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Evaluation of the appropriateness of using a continuous glucose monitoring system and a point-of-care glucometer for measuring blood glucose in horses

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Summary: Hand-held glucometers and continuous glucose monitoring systems are standard tools in diagnostics and the management of several diseases in humans, and are applied more frequently in veterinary medicine. The fast and accurate measurement of the glucose concentration plays a decisive role in equine medicine, both in the care of intensive care patients and the implementation of dynamic diagnostic tests for the diagnosis of endocrinological disorders. The objective of the study was to evaluate the accuracy and practicability of a point-of-care glucometer and a continuous glucose monitoring system in horses. The Accu-Chek® Guide (ACG) and FreeStyle Libre™ (FL) systems were tested on seven Icelandic horses subjected to oral glucose tests and insulin-response tests, resulting in transient and dynamic changes in blood glucose concentrations. The measurements obtained were compared with a standard colorimetric glucose assay and checked for accuracy and requirements in compliance with DIN EN ISO 15197 and US Food and Drug Administration standards. Both systems tested correlated well with the reference method. The ACG had a mean absolute relative difference of 6.4 % and a correlation coefficient of $\rho = 0.96$, and was, therefore, far more accurate than the FL, with a mean absolute relative difference of 35.4 % and a correlation coefficient of $\rho = 0.56$. While the ACG complied fully with the requirements of DIN EN ISO 15197 and the US Food and Drug Administration, the FL met the requirements of neither. The suitability for use in horses was shown for ACG and FL. Fast and large fluctuations in the glucose concentration could not be captured by FL, which makes this system rather unsuitable for use in dynamic diagnostic test procedures, such as insulin-response or oral glucose tests. Nevertheless, it could be a promising option for the long-term monitoring of intensive care inpatients. The ACG is a safe and fast alternative to the reference method, and could be a reliable tool for use in horses in various clinical situations.

Keywords: Glucose measurement, continuous glucose monitoring system, point-of-care glucometer, foreign body reaction, interstitial fluid glucose concentration

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Introduction and background

Intensive monitoring of glycaemia has been shown to notably enhance the results for patients in critical care, both in human and veterinary medicine[1–3]. Blood glucose concentrations (BGCs) have been established as dependable/reliable indicators for management and evaluating survival probabilities in different disease contexts, such as sepsis or acute abdominal pain in horses[4,5]. Minor variations in the measurement accuracy could potentially hinder decision-making regarding treatment plans for critically ill patients, with potentially fatal consequences.

In addition to the use in an intensive care context, reliable and valid glucose measurements are essential for endocrinologic diagnostic procedures, such as the insulin-response test (IRT)[6,7]. However, frequent blood sampling for the monitoring of intensive care patients or during diagnostic dynamic tests of metabolic disorders can be a source of stress, which affects glucose homeostasis by reducing the distribution of insulin through the action of somatostatin and catecholamines[6,8]. Moreover, frequent blood withdrawal could lead to iatrogenic anaemia in neonates. Finally, fast changes in the BGC may be missed even with frequent sampling.

There have been significant advancements in continuous glucose monitoring systems (CGMS) in the last decade. These systems continuously measure glucose levels in the interstitial fluid instead of blood, and have found successful application in both human and small animal medicine for managing patients with different forms of diabetes[9–15].

In addition to intensive validation and usage in humans[16–21], the FreeStyle Libre™[a] (FL) has been validated in dogs[22–27], cats[28–31] and horses[32–34].

The equine studies were conducted on neonatal foals, healthy and critically ill adult horses and reported the system's ease of use. Trials in humans have reported an effect of the body mass index on the accuracy of CGMS[19,20,35,36], which is of interest since obese horses or equids with endocrinological disorders may especially benefit from continuous glucose monitoring.

The point-of-care system (POC) Accu-Chek® Guide (ACG)[b] was used in this study. Although it is commonly used in human and veterinary medicine, it is, to date, not validated in horses.

The DIN EN ISO 15197 is a quality standard which allows one to objectively evaluate POCs based on their measurement accuracy. There are also accuracy criteria for interoperable CGMS published by the US Food and Drug Administration (FDA).

The aim of the study was to evaluate the accuracy of the FL and ACG in comparison to the reference method hexokinase assay in an experimental setting of induced hypo- and hyperglycaemia in horses.

Furthermore, factors potentially influencing the devices' accuracy, such as endocrinological status, Body Condition Score (BCS) and wearing time of the sensor, were investigated.

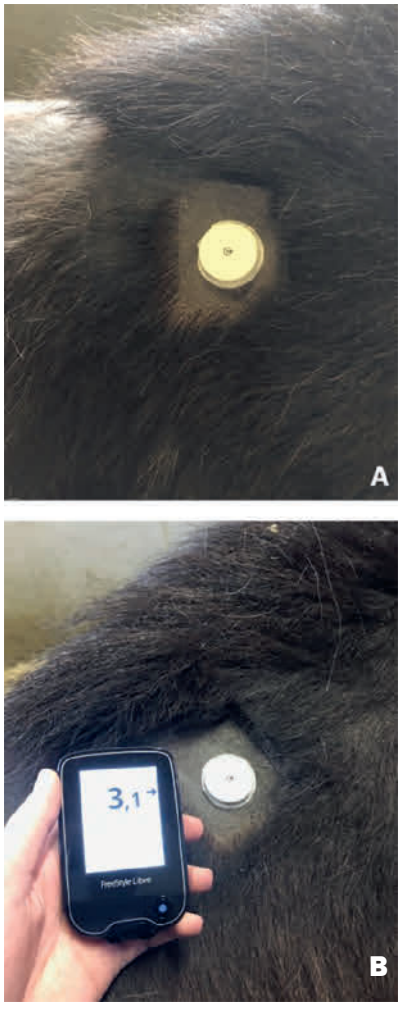

Fig. 1 Sensor applied at the transition from neck to withers after clipping and decreasing of the skin area (A). Sensor and reading device during read off (B). | *Nach Scheren und Entfetten der Haut am Übergang von Hals zu Wiederrist angebrachter Sensor (A). Sensor und Lesegerät während Auslesen des Sensors (B).*

Material and methods

Horses

Seven Icelandic horses owned by the university (1 stallion, 3 geldings, 3 mares) aged 18–29 years (Med.: 21 years) and weighing 230–417 kg (Med.:355 kg), were enrolled in this trial. The BCS was assessed according to *Henneke* et al.[37] and ranged between 3 and 7 (Med.: 5.5), while the cresty neck score[38] ranged between 0 and 3 (Med.: 2). Horses were selected based on BCS criteria to cover a broad range of body conditions. One horse, previously diagnosed with pituitary pars intermedia dysfunction, was treated with pergolide-mesylatec, according to the manufacturer's instructions, during the study period. Two of the seven horses had been previously diagnosed with insulin dysregulation based on oral (Oral Glucose Test and Oral glycaemic challenge with pelleted carbohydrate formulation) and intravenous (Insulin-Response Test) dynamic testing. The horses were fed hay ad libitum and were primarily group-housed on paddocks. Access to pasture was not permitted during the study. Full clinical examinations were performed daily to exclude any clinical infection. The horses were cared for according to accepted veterinary practices. The study was approved by the State Office for Consumer Protection and Food Safety (LAVES) in accordance with the German Animal Welfare Law (Ref: 33.19-42502-04-18/3006).

Sensor placement

Sensor placement was performed about 12 h prior to the first dynamic challenge. The sensors of the FL were applied on the left or right side at the transition from the neck to the withers, after clipping and degreasing the skin. Cyanoacrylate adhesive was used for better adhesion in addition to the sticky surface on the bottom of the sensors. The sensor placement was performed following the manufacturer's manual using the applicator designated (Fig. 1a and 1b).

Blood glucose fluctuation models

Up to three oral glucose tests were performed in three horses, each with 0.5 g/kg body weight glycaemic carbohydrates as stimuli, for the purpose of induction of hyperglycaemia[39]. An indwelling catheterd was placed in one of the jugular veins of each horse to collect blood samples for each test.

Blood sampling and an FL sensor reading started directly prior to the oral stimulation and 30, 60, 120, 180 and 240 min afterwards.

Two two-step IRTs[7] were performed in all seven horses for the induction of rapid changes in the BGC and interstitial fluid glucose concentration (ISFGC), with a wash-out period

of three days in between. A dose of 0.1 IE/kg body weight insulin[e f] was rapidly injected through the intravenous catheter. A 50 % dextrose solution[g] was administered 30 min after the insulin injection to prevent insulin-mediated and clinically relevant hypoglycaemia. Blood sampling and a FL sensor reading were performed prior to and 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150 and 180 min after the insulin injection. The blood samples obtained were divided among sodium fluoride and ethylenediaminetetraacetic acid tubes and centrifuged at 1000 g for 6 min within 3 h. The ethylenediaminetetraacetic acid plasma samples were then aliquoted and stored at -80 °C until further analysis.

Glucose measurement

Glucose measurements were performed using three different methods: two of them measuring the BGC, and the FL monitoring the ISFGC.

The BGC was measured at the laboratory of the Clinic for Small Animals, University of Veterinary Medicine Hannover, with the Gluc-3 test[h] with Cobas® C311/501 (CO)[i] from sodium fluoride tubes using the hexokinase method (40). This is considered a reference standard according to DIN EN ISO 15197. The lower detection limits for this system are 0.1 mmol/l, while the upper detection limit is 33.3 mmol/l, according to the manufacturer.

In addition, the BGC was measured with the POC ACG using electrochemical measurement principles. According to the manufacturer, the ACG measures the BGC in a range between 0.6 and 33.3 mmol/l within 4 s. All test strips used in this trial were from the same lot.

The FL, which was used to measure the ISFGC, consists of a round sensor (35 × 5 mm) with a small catheter (0.44 × 5 mm) which enables the glucose measurement.

The FL utilises wired-enzyme sensing technology, in which glucose oxidase converts glucose into glucuronic acid and hydrogen peroxide. This reaction generates an electric current that is directly proportional to the glucose level in the interstitial fluid. The current can be converted and displayed as glucose values in either mg/dl or mmol/l. The system measures the ISFGC every minute and stores the data for up to 8 h. Unlike previous systems, the data can be easily read out using a reading device, exported via a USB port and analysed using a computer programme provided by the manufacturer.

Insulin measurement

The plasma insulin concentration was also determined at the time points of the glucose measurement. An equine insulin enzyme-linked immunosorbent assay[j] was used for this purpose.

Skin examination

The skin area where the FL sensor was applied was examined daily for any indications of inflammation, discomfort or itching. Since the horses were euthanised promptly after this trial for reasons unrelated to this experiment, it was possible to obtain full thickness skin biopsies from both the application site and the corresponding area on the contralateral side in due course. Approximately 4 × 4 cm square skin samples were extracted with a scalpel. The skin samples were preserved in 4 % formalin for 24 h and then transferred to isopropanol. Paraffin cross-sections and longitudinal sections were prepared at the Institute of Pathology, University of Veterinary Medicine Hannover, prior to staining with haematoxylin-eosin and azan. The tissue sections were examined microscopically by a blinded investigator for signs of foreign body reaction [41].

Statistical analysis

Statistical analysis was performed with R 4.0.4[k] and GraphPad Prism[l]. Data were checked for normality using the Shapiro-Wilk normality test. Agreement between different methods for glucose measurement was assessed using the mean absolute difference (MAD) and the mean absolute relative difference (MARD). In addition, the proportion of overestimated and underestimated values were calculated. The correlation between measurements from different methods was described using Spearman's rank correlation coefficient, while their agreement was investigated by means of *Bland-Altman* plots [42]. The limits of agreement for the latter were estimated to evaluate compliance with DIN EN ISO 15197, which is applicable in human medicine. In addition, the accuracy of the FL was checked against the specifications for the measurement accuracy of interoperable CGMS published by the FDA.

In order to differentiate the measurement accuracy in different glycaemic ranges, the values measured were divided into hypoglycaemic (0–3 mmol/l), euglycaemic (3–6 mmol/l) and hyperglycaemic (> 6 mmol/l) measurement ranges based on the corresponding CO value. In addition, the samples from intravenous and oral stimulation procedures were isolated and analysed. The square root transformation was used to ensure approximately normally distributed residuals for MARD and MAD, which were analyzed depending on test type, glycaemic range and measurement method using mixed models fitted with the ´lme4´ R-package [43].

The *Clarke*-Error Grid [44] was used to visualise and evaluate hypothetical clinical consequences following any inaccurate measurements of the IFSG or BGC. The impact of the BCS of the horse, sensor age and plasma insulin concentration measured at the corresponding time points were investigated using mixed models [45] fitted using the afex R package and including 'horse' as a random factor besides the respective predictors. The time course of the BCG and ISFGC was assessed using generalised additive models fitted using the mgcv R package [46]. The glucose concentration was modelled as a function of time as a spline with one level per measurement method, also included as fixed effects, while horse was added as a random effect spline. Based on model residuals, a scaled t-distribution with an identity link function was chosen as the model family [47]. P-values were obtained by F-tests. Statistical significance was accepted at $P < 0.05$.

Results

Usability of FL

Sensor insertion was safe, easy and well-tolerated by all horses, with no adverse reactions or pain observed. The ISFGC could be read after the 60 min launching period in six out of seven horses. However, the sensor in one horse had to be replaced twice due to initiation failure during this period. The sensor remained operational for the intended duration of 14 days in use for six out of seven horses. Only one device required replacement due to a sensor defect, not associated with sensor detachment, which did not occur in any horse during the study. No signs of discomfort, such as scratching or biting the sensor attachment area, were observed. The ISFGC measures could be captured easily at any time, except for some cases of sensor errors in very low ambient temperatures (-5 °C). These errors did not occur during dynamic testing but in the nights between test days. After warming the sensor and reader, glucose estimation was possible without any problems or the need for sensor replacement.

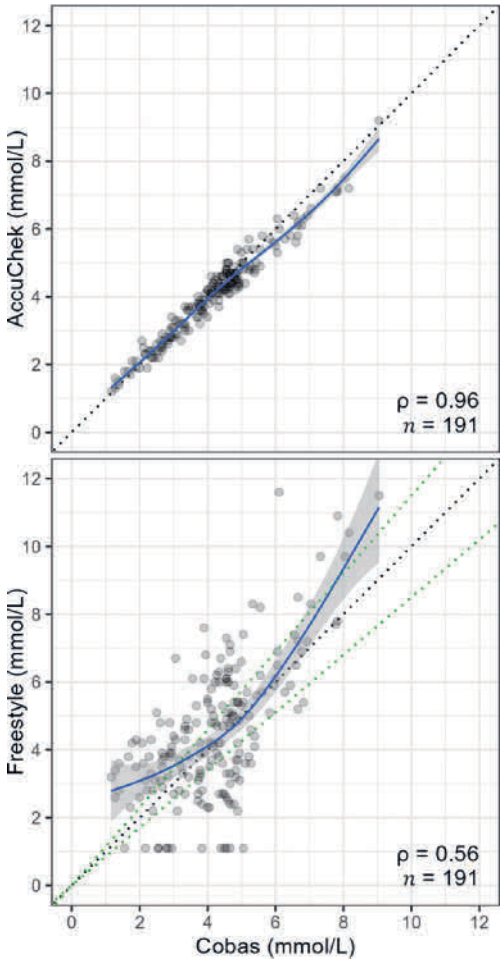

Fig. 2 Correlation of the Accu-Chek® Guide and FreeStyle libre™ glucose measurements with the reference method (Cobas). Green dotted line markes ± 15% tolerance range. | *Korrelation der mit dem Accu-Chek® Guide bzw. FreeStyle libre™ und der Referenzmethode (Cobas) gemessenen Glukosewerten. Die grün gestrichelte Linie markiert den ± 15% Toleranzbereich.*

Accuracy of the FL and ACG

A total of 191 sampling time points were taken and analysed with the different measurement methods described above, of which 75% (143/191) were obtained after intravenous stimulation and 25% (48/191) following oral stimulation. The BGC ranged between 1.2 and 9.1 mmol/l with a mean of 4.2 mmol/l measured with the reference method, and between 1.2 and 9.2 mmol/l with a mean of 4.1 mmol/l measured with the ACG. The ISFGC ranged between 1.1 and 11.6 mmol/l with a mean of 4.5 mmol/l.

Using CO as the gold standard, the BGC was classified as follows: 44 samples were in the hypoglycaemic range (BGC 0–3 mmol/l), 126 in the euglycaemic range (BGC 3–6 mmol/l) and 21 in the hyperglycaemic range (BGC > 6 mmol/l).

The deviation from the BGC measured provided by the reference method ranged from 0 to 5.6 mmol/l, resulting in a MAD of 1.22 mmol/l for the ISFGC measurement with the FL, and from 0 to 1 mmol/l with an MAD of 0.28 mmol/l for the BGC measurement with the ACG. The ACG system demonstrated greater accuracy compared to the FL system, with a MARD of 6.4% for the ACG compared to one of 35.4% for the FL, relative to CO. This difference in accuracy is further supported by the higher correlation coefficient observed between the ACG and CO (ρ = 0.96) compared to the FL and CO (ρ = 0.56) (Fig. 2).

Table 1 Proportion of over- and under-estimated and correctly classified glycaemic range values, mean absolute difference (MAD; mmol/l) and mean absolute relative difference (MARD; %) of the FreeStyle Libre™ (FL) and ACCU-CHEK® Guide (ACG) compared to the gold standard in hypoglycaemic, euglycaemic and hyperglycaemic ranges with corresponding correlation coefficients and confidence intervals. | *Anteil der über-, unterschätzten und bezüglich des glykämischen Bereichs korrekt klassifizierten Werte, MAD (mmol/l) und MARD (%) von FL und ACG im Vergleich zum Goldstandard in hypo-, eu- und hyper-glykämischen Bereichen mit entsprechenden Korrelationskoeffizienten und Konfidenzintervallen.*

	FreeStyle Libre™ (FL)			Accu-Chek® Guide (ACG)		
	Hypoglycaemia	Euglycaemia	Hyperglycaemia	Hypoglycaemia	Euglycaemia	Hyperglycaemie
Overestimated Values	35/44 (79.5 %)	69/126 (54.8 %)	14/21 (66.7 %)	25/44 (56.8 %)	47/126 (37.3 %)	2/21 (9.5 %)
Underestimated Values	9/44 (20.4 %)	57/126 (45.2 %)	7/21 (33.3 %)	19/44 (43.2 %)	79/126 (62.7 %)	19/21 (90.4 %)
Correctly classified	16/44 (36.4 %)	81/126 (64.3 %)	17/21 (81 %)	41/44 (93.2 %)	121/126 (96 %)	15/21 (71.4 %)
MAD (mmol/l)	1.26	1.15	1.19	0.16	0.26	0.54
MARD (%)	62.69	28.57	18.73	7.42	5.86	7.75
ρ	0.0531	0.2450	0.599	0.9208	0.8984	0.9233
95 % confidence interval	-0.2477–0.3446	0.07319–0.4026	0.2271–0.8194	0.8587–0.9563	0.8584–0.9276	0.8175–0.9688

When compared to the reference method CO, 38% (73/191) of the values measured by the FL were underestimated and 62% (118/191) were overestimated, while 61% (117/191) of the values measured by the ACG were underestimated and 39% (74/191) were overestimated. No consistent pattern or proportional error of the BCG and ISFGC could be detected for either system.

A strong correlation with the reference method could be observed in all three glycaemic ranges for the ACG.

The MARD of the ACG did not differ significantly across hypo- and euglycaemic ranges ($p = 0.7152$), eu- and hyperglycaemic ranges ($p = 0.7883$) or hypo- and hyperglycaemic ranges ($p = 0.9966$). The ACG was able to correctly classify 93% of the hypoglycaemic, 96% of euglycaemic and 71% of the hyperglycaemic values.

However, the MARD for the FL varied significantly across hypo- and euglycaemic ranges ($p < 0.0001$) as well as hypo- and hyperglycaemic ranges ($p = 0.0001$). There was no significant difference of MARD between hyper- and euglycaemic ranges ($p = 0.9395$). Specifically, the FL showed a lower accuracy in hypoglycaemic conditions compared to euglycaemic and hyperglycaemic conditions. In fact, there was no correlation found between the values of FL measured and those obtained with CO in the hypoglycaemic range. The FL accurately classified only 36% of the hypoglycaemic samples and 64% of the euglycaemic samples, while correctly classifying 81% of the hyperglycaemic samples. For a more detailed presentation of measurement accuracy across different blood glucose ranges, please refer to Table 1.

When assessing the glucose curve with experimental models, the general difference between the ACG and FL compared to the reference method could be determined during oral stimulation. This could not be narrowed down to specific time points since the time course did not differ significantly.

The FL differed significantly from the reference method at most time points during intravenous stimulation, with mea-

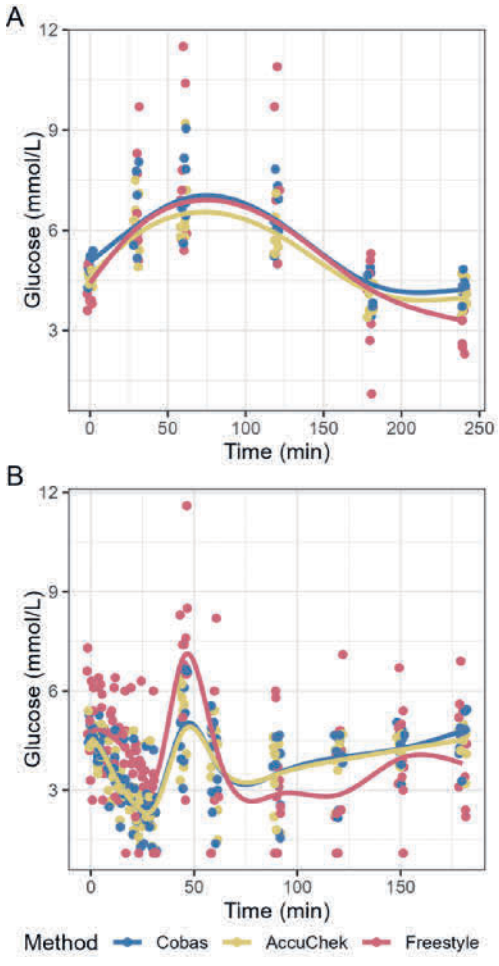

Fig. 3 Time course of glucose concentrations for each measurement method during oral dynamic testing (A), and during intravenous dynamic testing (B). | *Zeitlicher Verlauf der Glukosekonzentration in Abhängigkeit von der Messmethode bei oralen dynamischen Tests (A), und bei intravenösen dynamischen Tests (B).*

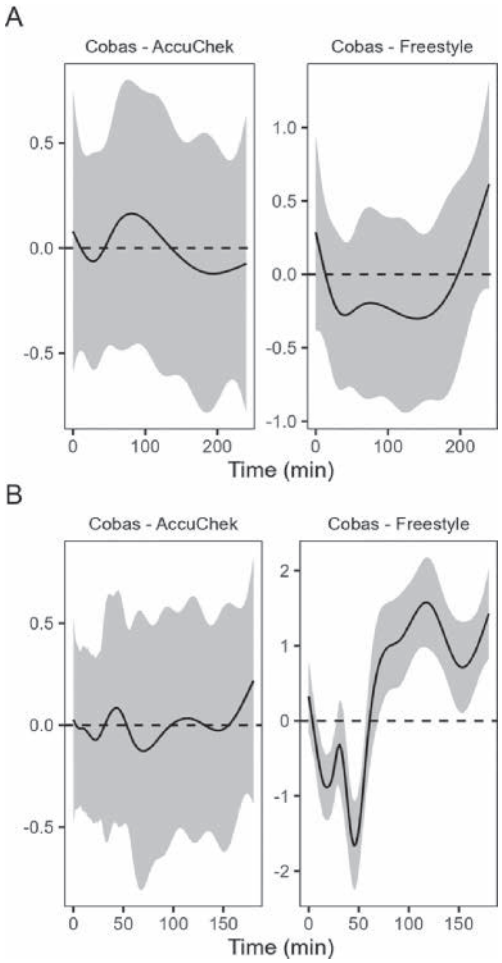

Fig. 4 Difference between splines used to model the time course of glucose for each method during the oral dynamic test (A) and the intravenous dynamic test (B). The difference between splines is significant when 0 (dashed line) is outside the confidence interval of the spline difference (grey area). Only the difference between Cobas and FreeStyle libre™ during the intravenous test reveals significant differences. | *Vergleich zwischen den als Splines modellierten Zeitverläufen der Blutglukosekonzentration mit den unterschiedlichen Messmethoden für den oralen dynamischen Test (A) und dem intravenösen dynamischen Test (B). Die Differenz zwischen den Splines ist zu den Zeitpunkten signifikant, an denen 0 (gestrichelte Linie) sich außerhalb des grauen Konfindenzintervalls befindet. Signifikante Unterschiede bestehen ausschließlich bei dem FreeStyle libre™-Cobas Vergleich, während des intravenösen Test.*

surements being higher during the first third of the time course and lower at later time points. By contrast, there were no major differences between the ACG and CO during intravenous stimulation (Fig. 3 + 4).

A consistent time lag of glucose dynamics was not observed between the BGC and ISFGC.

The type of stimulation, intravenous or oral, had a significant impact on the measurement accuracy of the FL. While the MAD was 0.91 mmol/l and MARD was calculated at 16% for oral stimulation, significantly poorer precision was found during intravenous stimulation, with a MAD of 1.32 mmol/l ($p = 0.0003$) and a MARD of 42% ($p < 0.0001$). Similarly, the correlation with the reference method is much higher during oral ($p = 0.9591$) than with intravenous stimulation ($p = 0.0558$). Such differences were not observed for the ACG. The MARD ($p = 0.2191$) did not differ significantly across different stimulation types, but the MAD was significantly higher during oral stimulation ($p = 0.0009$) (Tab. 2).

Comparing the ACG measurements with CO using the *Clarke*-Error Grid, it appears that 100% of the data points fall into zone A. This means that the ACG has a less than 20% deviation from the reference method, and the ACG measurements would not have led to incorrect treatment decisions, according to the definition of this grid derived from human medicine. Regarding the FL, merely 56% of the data points were located in zone A and 31% in zone B. Consequently, 87% of the measurements would not result in treatment failure. However, 13% were located in zone D, indicating potentially fatal treatment errors due to unrecognised severe hypo- or hyperglycaemia (Fig. 5).

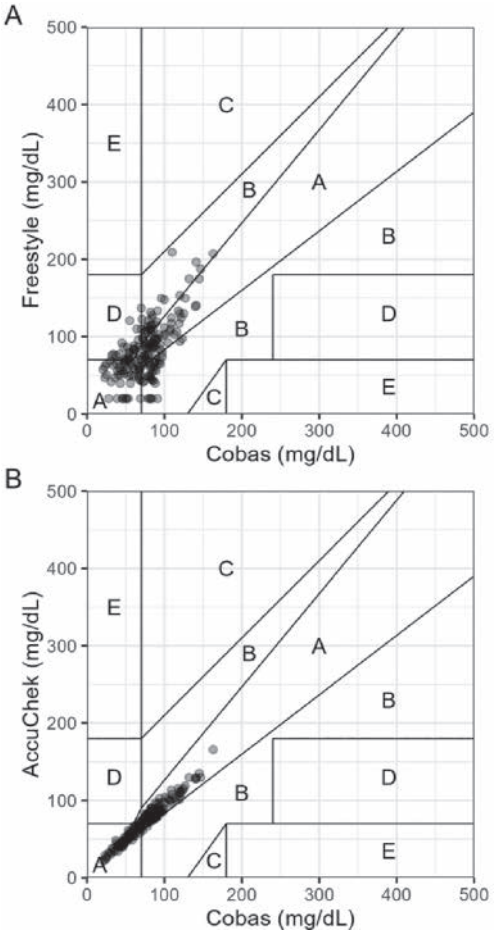

Fig. 5 Scatterplot of blood glucose measurements performed with the Accu-Chek® Guide compared to Cobas (A) and Freestyle libre™ compared to Cobas (B) superimposed with the Clarke Error Grid to quantify the methods' clinical accuracy based on the clinical consequences implied by different levels of disparity. Region A: Values within 20% of reference method, Region B: Values outside of the 20% not leading to inappropriate treatment; Region C: Values leading to unnecessary treatment, Region D: Values indicating a potentially dangerous failure to detect hypo- or hyperglycaemia, Region E: Values leading to confuse treatment of hypo- or hyperglycaemia. | *(A) Streudiagramm der Blutglukosemessungen mit dem Accu–Chek® Guide im Vergleich zum Cobas (A) und Freestyle libre™ im Vergleich zum Cobas (B) mit dem Clarke Error Grid zur Quantifizierung der klinischen Genauigkeit der Methoden basierend auf der klinischen Auswirkung potenzieller Messabweichungen. Region A: Werte innerhalb von 20% der Referenzmethode, Region B: Werte außerhalb der 20%, die nicht zu einer unangemessenen Behandlung führen, Region C: Werte, die zu einer unnötigen Behandlung führen, Region D: Werte, die auf ein potenziell gefährliches Versagen bei der Erkennung von Hypo- oder Hyperglykämie hinweisen, Region E: Werte, die zu einer falschen Behandlung der Hypo- oder Hyperglykämie führen.*

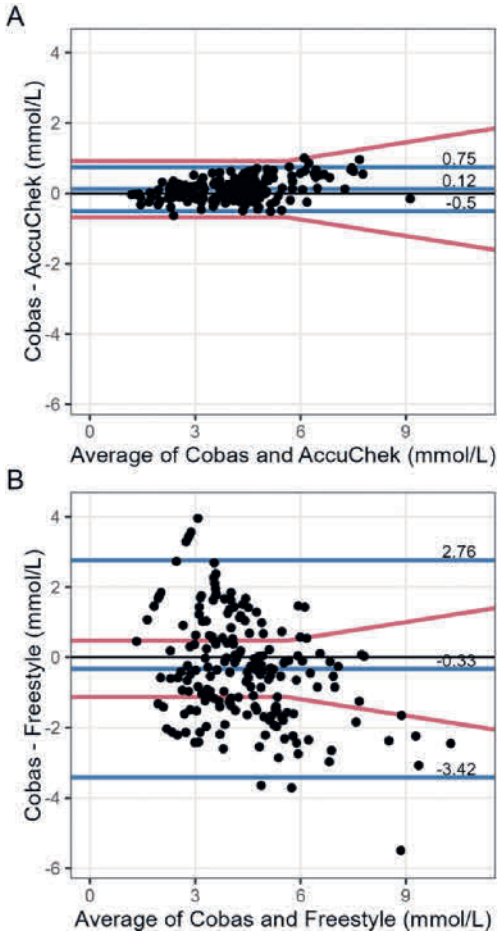

Fig. 6 *Bland-Altman* plots of the Accu-Chek® Guide against Cobas (A) and FreeStyle libre™ against Cobas (B) with red lines representing the tolerable deviation as defined in the guideline DIN EN ISO 15197 and blue lines representing the limits of agreement determined using a mixed model. | *Bland-Altman-Plot des Accu-Chek® Guide im Vergleich zu Cobas (A) und des Freestyle libre™ im Vergleich zum Cobas (B). Die roten Linien stellen die zulässige Abweichung gemäß den Richtlinien der DIN EN ISO 15197 und die blauen Linien die mittels des gemischten Modells ermittelten Grenzen der Übereinstimmung dar.*

The DIN EN ISO 15197 demands a maximal deviation of 15 mg/dl or 0.5 mmol/l for the values measured lower than 100 mg/dl or 5.6 mmol/l, respectively, compared to the reference method, which is the hexokinase technique. Furthermore, there is a maximal permissible deviation of 15% for values higher than 100 mg/dl or 5.6 mmol/l, respectively. Finally, 95% of the values measured should be within these limits.

Similarly, all the ACG data was in full compliance with the DIN EN ISO 15197, while only 52% of the FL data was within the acceptable range in the *Bland-Altmann* plot described previously (Fig. 6).

According to the FDA's specifications regarding the measurement accuracy of interoperable CGMS, in a measurement range of less than 3.9 mmol/l (70 mg/dl), between 3.9 and 10 mmol/l (70–180 mg/dl) and above 10 mmol/l (> 180 mg/dl), 85, 70 and 80%, respectively, must lie within a tolerance range of ± 15%. In our study, these requirements were only met by 23% of the values in the low range and 69% in the middle range. The tolerance range of ± 15% is shown as a dotted green line in Figure 2.

There was no effect of the sensors' dwell time ($F(1,56.93) = 3.88$, $p = 0.054$), the horses' BCS ($F(1,1) = 0.90$, $p = 0.516$) or the plasma insulin concentration at the time of the sensor readout ($F(1,57.85) = 1.95$, $p = 0.168$) on the FL accuracy.

A statistical evaluation of the influence of the endocrinological status was not possible due to the small sample size and the unbalanced distribution within the classes studied.

Skin examination and histological findings

Skin sections were available from six out of seven horses. Histological changes were observed in four out of six haematoxylin-eosin-stained sections and two out of six azan-stained sections collected from the sensor areas. None of the tissue sections from contralateral control areas showed any histological abnormalities. The puncture site enclosing the glucose sensor exhibited a low to moderate level of inflammatory cell infiltration and concurrent fibroblast proliferation. Additionally, there was a segmental increase in the eosinophilic extracellular matrix in the cross-section. Some animals also showed early signs of capsule formation, characterised by young collagen fibres without birefringence. However, the thickness and expansion of these fibrotic capsules could not be accurately measured due to sample processing artifacts. Small vessels were observed in close proximity to the sensor in certain sections (Fig. 7).

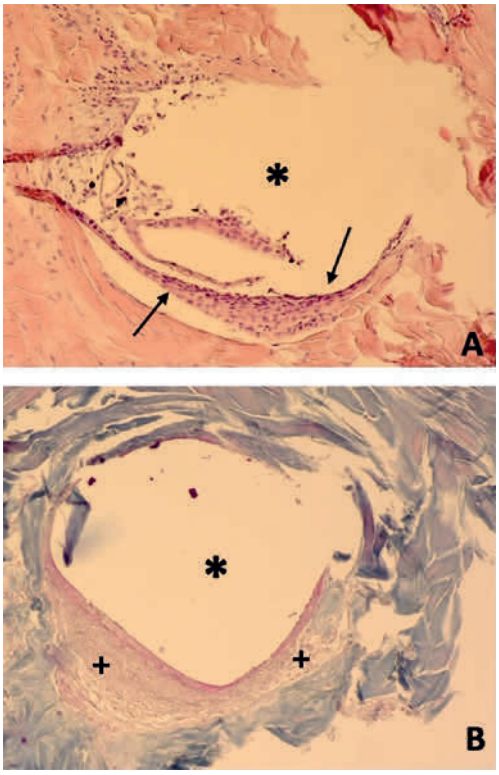

Fig. 7 Haematoxylin-eosin-stained cross-section of the skin area of the sensor attachment with branch canal (*) and fibroblast proliferation with increased eosinophilic extracellular matrix (↑); ×100 (A) Azan-stained cross-section of the skin area of the sensor attachment with branch canal (*) and incipient capsule formation consisting of mainly young collagen fibres (+) (B); ×100. | *Hämatoxylin-Eosin-gefärbter Querschnitt der Haut im Bereich des Sensors mit Stichkanal (*) und Fibroblasten Proliferation mit vermehrt eosinophiler extrazellulärer Matrix (↑); ×100 (A) Azan-gefärbter Querschnitt der Haut im Bereich des Sensors mit Stichkanal (*) und beginnender Kapselbildung, hauptsächlich bestehend aus jungen Kollagenfasern (+) (B); ×100.*

Table 2 Proportion of over- and under-estimated and correctly classified glycaemic range values, MAD (mmol/l) and MARD (%) of FL and ACG compared to the gold standard during oral and intravenous dynamic tests with corresponding correlation coefficients and confidence intervals. | *Anteil der über-, unterschätzten und bezüglich des glykämischen Bereichs korrekt klassifizierten Werte, MAD (mmol/l) und MARD (%) von FL und ACG im Vergleich zum Goldstandard bei oralen und intravenösen dynamischen Tests mit entsprechenden Korrelationskoeffizienten und Konfidenzintervallen.*

	FreeStyle Libre™ (FL)		Accu-Chek®Guide (POC)	
	PO	IV	PO	IV
Overestimated Values	26/48 (54.2 %)	98/143 (68.5 %)	10/48 (20.8 %)	64/143 (44.8 %)
Underestimated Values	20/48 (41.7 %)	43/143 (30.1 %)	38/48 (79.2 %)	79/143 (56.4 %)
Correctly classified	39/48 (81.3 %)	76/143 (53.1 %)	45/48 (93.8 %)	131/143 (91.6 %)
MAD (mmol/l)	0.9125	1.324	0.4016	0.2230
MARD (%)	16	42	7.137	6.185
ρ	0.9029	0.4305	0.9667	0.9591
Confidence Interval	0.8296–0.9456	0.2821–0.5588	0.9400–0.9816	0.9430–0.9708

Discussion

The aim of the study was to assess the applicability, practicality and accuracy of a CGMS and a commonly used POC glucometer in horses, specifically in an experimental setting that simulated clinically relevant scenarios including hypo- and hyperglycaemia. Additionally, the study aimed to investigate any potential factors that could influence the measurement accuracy of the CGMS.

As previously reported in humans and other species, the handling of both systems was simple[22–25,27,28,31–34]. The ACG and FL delivered quick results for the assessment of the BGC and ISFGC, and FL could be applied to the horse without any complications.

In contrast to other trials investigating the application of FL in horses[32–34], the horses were kept in a herd, whereby generally more movement and a strongly pronounced social behavior, in terms of playing behavior and grooming, can be assumed[48]. The horses spent most of the study period outside under partly unfavourable weather conditions. Despite these aggravated conditions, most sensors stayed in place and could be used for the period of 14 days intended without any signs of discomfort or inflammation. This contrasts with other studies performed in the field of veterinary medicine investigating the applicability and usability of the FL, for example, in cats and dogs. Early sensor dysfunction occurred in up to 60% in dogs and up to 70% in cats. The sensors could be used for an average of 8.3 days in cats and 5.5 days in dogs[22,23,26,28,29,31]. No signs of skin irritation were observed in our study despite the additional use of cyanoacrylate adhesives. This contrasts with other veterinary trials where additional securing, such as adhesive foils, bandages or skin staples, resulted in mild erythema and stress during sensor manipulation, particularly in cats[28,31].

Foreign body reaction, such as inflammation, formation of a hypo-permeable capsule and vascular regression, have been shown to affect the accuracy of glucose measurements in the interstitial fluid of subcutaneous tissue[41,49]. Even though no macroscopic signs of inflammation could be observed after the sensor placement in horses[32,33] and only mild skin issues occurred in human patients[36,50], cats[28] and dogs[22,26], one cannot exclude that a reaction on the cellular level may distort the results.

The reaction caused by the sensor on the histological level is comparable to a mild foreign body reaction. The formation of a solid capsule, which alters the tissue environment around the sensor and prevents the effective diffusion of glucose[41,51], is only expected to occur after three to four weeks in rodents[52], i.e. far beyond the anticipated period of use in humans, cats, dogs and horses. The impact of the histological reaction on the nature and conductivity of the sensor environment and, consequently, on the measurement accuracy, remains uncertain. Our study did not reveal any significant influence of the wearing time on the accuracy of the FL sensor measurements. This suggests that the accuracy is not or only minimally affected, which contradicts earlier findings that showed improved measurement accuracy over time[22].

Equine trials investigating the accuracy of the FL noted a good to excellent agreement between the FL and BGC under induced hyper- and hypoglycaemia with a correlation coefficient between 0.82 and 0.83[32–34]. Our results do not corroborate their observation, as we found the correlation between the FL and the gold standard to be weaker, with a correlation coefficient of 0.56. This finding also differs from previous results where the correlation between the FL and a BGC measurement system is reported to be good, with correlation coefficients ranging between 0.88 and 0.84 in dogs[22,23,25], 0.69 and 0.93 in cats [28–31], and 0.92 and 0.96 in humans[18,20]. In addition, the MARD in our study was 35.35%, which is much higher than in other studies, where the MARD ranged from 17–25.2% in dogs[23,25], 14.9% in cats[31], and 11.8–16.7% in humans[18–21,36,53]. However, it should be noted that the MARD is normally determined under conditions of a constant glycaemia. A constant glucose concentration over a longer period was present at almost no time in our study, as we focused primarily on induced fluctuations and margins of the normal ranges, which could probably explain the higher MARD.

Up to now, the MARD has been used as a parameter for the measurement accuracy of CGMS, which should not exceed 10% if the CGMS is used as a basis for therapy decisions. This requirement was not met in our study, but the MARD determined in clinical studies can be significantly influenced by the study protocol used and the selection of patients studied[54]. A direct comparison of two CGMS measured simultaneously in the same patient, also called precision absolute relative difference, provides more reliable information on the measurement quality of a CGMS. Using two CGMS in each horse was not possible in our study.

Despite the overall good correlation reported for glucose concentrations measured in interstitial fluid and blood[10,55,56], it can be assumed that there is a delay between the BGC variations and the glucose concentrations measured via GCMS in the interstitial fluid owing to the equilibration of glucose among these two compartments[55]. Previous studies have reported a delay of 4–15 min, depending on the species investigated and CGMS used[36,57–63]. *Cunneen* et al.[32] reported a delay from 10–60 min, depending on glucose rise, nadir or rest in healthy horses, while other trials with the FL and older CGMS reported a delay of 15 min in horses[34,62].

Several studies attempting to evaluate the accuracy of FL have found that the degree of deviation between the BCG and glucose concentration measured by CGMS depends on the glycaemic range and the dynamics of the glucose concentration. The best agreement was found in hyperglycaemic ranges, ahead of euglycaemic and hypoglycaemic ranges[32]. Deviation was shown to be smaller during glucose- than insulin-induced hyperglycaemia[17,22], which is in accordance with our results, although the insulin-induced hypoglycaemic ranges were associated with rapid fluctuations in the glucose concentration, impeding the accuracy of the FL. Even though we could not detect a manifest delay in the measurement, it can be assumed that the redistribution of glucose between the compartments requires some time, due to the diffusion of glucose from the capillaries through the capillary wall and into the tissue surrounding the sensor. This could distort the measurement accuracy in phases with rapid glucose fluctuations. It is likely that the ISFGC is dependent on both the glucose concentration in blood and insulin-related clearance from tissues[55,56,61]. In

our study, we intentionally manipulated both factors to a degree that may surpass the variations caused by regular physiological processes. This may explain the higher MARD and weaker correlation with intravenous stimulation, which has a rapid impact, compared to oral stimulation that takes longer due to the gastrointestinal glucose absorption and the activation of the entero-insular axis and, thus, multifactorial mitigation. Additionally, our study resulted in a poorer correlation and measurement accuracy compared to other studies conducted in more realistic clinical conditions without intentional, pronounced fluctuations in endocrinological conditions[22,23,28].

Since this study is used as a stress test for the reliability of the CGMS, these rapid fluctuations were present in our study for almost the entire experimental period. The resulting lack of phases with a constant glucose trend can be seen as a possible reason for the FL not meeting the DIN EN ISO 15197.

The DIN EN ISO 15197 is a guideline for determining the accuracy of POC glucometers in human medicine and not specifically designed for the equine species or the evaluation of CGMS. In previous veterinary and human medical studies that used the DIN EN ISO 15197 as a benchmark for evaluation of CGMS, such as the FL, these systems fell short of meeting the requirements to the same extent[17,22,23,28,30,31]. The FDA has also issued accuracy criteria for interoperable CGMS. The requirements of this guideline were not met by the FL in this study.

The fact that 13 % of the values measured with the FL fall into zone D of the Clarke-Error Grid indicates that the measurements could result in fatal treatment errors, according to the definition of this grid. Caution should be advised when evaluating these values measured and, in case of doubt, it is advisable to check the BGC, especially in situations in which the patient is in critical hypo- or hyperglycaemia. Regarding the use of the system for research purposes, the FL does not appear to be the appropriate instrument for detecting rapid fluctuations in the BGC. On the other hand, the values reported by the FL may reflect the actual supply of glucose in the interstitial tissue fluid, which could also be interesting depending on the question of the study.

Another reason for the unfavourable correlation between the FL and CO could be the fact that the system is only approved for use in humans and, therefore, calibrated based on human data. Since sensor accuracy is dependent on its localisation in humans, which is why the upper arm is preferred to the abdomen as an attachment site, it can be assumed that both the fat distribution and vascularisation in horses cause a slight difference in the milieu of the sensor[17,64]. The sensors in horses are usually attached to the cranial aspect of the neck[32–34], while sensor attachment in small animals is performed at the neck or the lateral thorax wall[22–25,27,28,31–34]. Studies investigating the differences in accuracy at different locations in these species do not exist.

Calibration with capillary blood is not necessary for the FL. This lack of reliance on an external calibration could potentially lead to CGMS errors, especially when using the system on a species other than humans. The previous GCMS systems, such as the Freestyle Navigator™ by Abbott or the MiniMed™ by Medtronic, required calibration with capillary blood at least twice a day. Additionally, the measurements could only be evaluated retrospectively after disconnecting the sensor from the patient. These limitations have resulted in these older systems not being commonly used in clinical practice, despite their satisfactory performance reported in various veterinary trials[11,65–69].

The ACG demonstrated a convincing performance with its user-friendly interface, reliable and quick measurement results, and excellent correlation with the gold standard. Despite being a human medicinal product, it was able to fully meet the requirements of DIN EN ISO 15197 when used in horses. One trial investigating the practicability and accuracy of the ACG in dogs revealed no statistically significant differences compared to the reference method[70]. However, there is a lack of other veterinary studies investigating the usability and performance of the ACG in multiple species in veterinary settings, which makes it difficult to compare the performance described in this study with data from other experiments or species.

However, other POC devices have already undergone evaluation according to DIN EN ISO 15197 standards when being used in clinical settings in horses. *Hackett* and *McCue*[71] reported a good clinical usability of the AlphaTrak system, which is specifically designed for veterinary purposes and validated for use in multiple species[71–74]. A correlation of 0.67–0.92 was found for the use of the human POC systems ACCU-CHEK® Aviva, ACCU-CHEK® Advantage and Accutrend® Plus in horses. These results apply to the use of whole blood as the sample material, as was also used in our study. Significantly higher correlations could be obtained with the use of plasma as the sample fluid[75–77].

It should be noted that the ACG has a financial advantage over laboratory glucose measurement, with costs per sample being approximately 20 times lower. In addition, there are logistical and practical advantages, including the small blood volume required, which should be noted. These are a major advantage especially in cases where frequent measurements and quick results are required. In these cases, it could also pay off to use the FL, which is comparatively cost-intensive, but enables the stress-free and frequent elicitation of glucose concentrations.

We did not investigate the influence of the sample type on the measurement accuracy of the ACG, which is a limitation of this study. Moreover, we were unable to investigate the influence of the endocrinological status on the measurement accuracy due to the small sample size and low number of insulin-dysregulated horses in the study population. Finally, few data were collected under resting circumstances without induced large oscillations in glycaemia, which would have been indispensable for a better basic assessment of the measurement accuracy under normal clinical conditions.

Conclusion

Although the FL proves to be an easy-to-use and practical system in terms of handling, the performance regarding the measurement accuracy did not meet the expectations and requirements. Since we did not evaluate the accuracy under

physiological, non-stimulated conditions, it is possible that the FL could be a useful tool for evaluating glucose dynamics in clinical or research settings where no significant fluctuations are expected. The lack of accuracy in states of (iatrogenic) hypo- or hyperglycaemia significantly limits the potential usefulness for research and dynamic testing methods, as rapid glucose and insulin dynamics are often required not only in study models to gain sufficient insight but also in clinical settings where severe impairments of glucose homeostasis could easily be missed or recognised too late. More research is needed to evaluate the FL under clinical conditions. The ACG was able to meet all requirements in the best possible way and, thus, represents a valid, reliable, simple and fast method for BGC determination in horses. The ACG has great potential for monitoring intensive care patients and for usage during dynamic tests for the diagnosis of endocrine disorders in which the BGC is the key parameter for diagnosis.

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Abbreviations

ACG	Accu-Chek® Guide
BCS	Body condition score
BGC	Blood glucose concentration
CGMS	Continuous glucose monitoring system
CO	Cobas®
FL	FreeStyle Libre™
FDA	United States Food and Drug Administration
ID	Insulin dysregulation
IRT	Insulin response test
ISFGC	Interstitial fluid glucose concentration
MAD	Mean absolute difference
MARD	Mean absolute relative difference
POC	Point-of-care

Manufacturer's addresses

[a] FreeStyle Libre™, Abbott laboratories, Illinois, USA
[b] Accu-Chek® Guide, Roche Diabetes Care Deutschland GmbH, Mannheim, Deutschland
[c] Prascend®, Böhringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany
[d] Intraflon 2 IV Canulla 12 g × 80 mm, Vygon, Aachen, Deutschland
[e] Insuman® Rapid 40 I.E./ml, Sanofi - Aventis Deutschland GmbH, Frankfurt, Germany
[f] Caninsulin® 40 I.E./ml, Intervet Deutschland GmbH, Unterschleißheim, Germany
[g] Glucose 50 % B. Braun Konzentrat zur Herstellung einer Infusionslösung, B. Braun Melsungen AG, Melsungen, Germany
[h] GLUC3, Cobas®, Roche Diagnostics GmbH, Mannheim, Germany
[i] Cobas® C311 Analyzer, Roche Holding, Basel, Schweiz
[j] Mercodia Equine Insulin ELISA, Mercodia, Uppsala, Sweden
[k] R 4.0.4, R Core Team, R Foundation for Statistical Computing, Vienna, Austria
[l] Graph Pad Prism Version 9.4.1, Graph Pad Software, Boston, U.S.

Conflict of interest statement

Tobias Warnken is now employed by Boehringer Ingelheim Vetmedica GmbH. All other authors have declared that no competing interests exist.

Animal welfare statement

The study was approved by the State Office for Consumer Protection and Food Safety (LAVES) in accordance with the German Animal Welfare Law (case number: 3.19-42502-04-18/3006).

Authors' contributions

TW and KF designed the experiments. AJG and TW performed the experiments. AJG measured the insulin concentrations. JD, AJG and TW analysed the data and prepared the figures. AJG, JD and TW wrote the manuscript. All authors contributed to the interpretation of the results, reviewed drafts of the manuscript and accepted its final version.

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