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Evaluation of the combined glucose-insulin and intravenous glucose tolerance tests for insulin dysregulation diagnosis in donkeys

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Abstract

Background: Insulin dysregulation (ID) and donkey metabolic syndrome (DMS) are common in this species. Contrary to horses, diagnostic guidelines compiling insulin cut-offs values and dynamic testing interpretations have not been reported for this species.

Objectives: To evaluate resting serum insulin concentrations, the combined glucose-insulin test (CGIT) and the glucose intravenous tolerance test (IVGTT) for the diagnosis of DMS with ID suspicion.

Study design: Diagnostic test comparison.

Methods: Six of 80 mix-breed adult donkeys fulfilled the inclusion criteria for DMS based on history or clinical evidence of recurrent laminitis, body condition >6 and neck score >2 or baseline insulin and leptin concentrations >20 μIU/mL and >12 ng/mL respectively. CGIT and IVGTT were performed in all donkeys within a week and interpreted following guidelines reported for equine metabolic syndrome (EMS). Insulin and glucose curves were analysed, proxies calculated and correlations and multivariate analysis assessed.

Results: Following EMS guidelines, CGIT classified 2 (using glucose-positive phase duration) or 3 (using insulin concentration) and IVGTT classified 5 donkeys as ID. ID donkeys showed a lower glucose/insulin ratio, QUICKI and RISQI, and a higher insulin/glucose ratio, MIRG and HOMA-B%.

Main limitations: Comparison of these tests with additional dynamic testing including a larger number of ID donkeys is necessary.

Conclusions: This is the first study evaluating dynamic tests to assess ID/DMS in DMS-suspected donkeys. IVGTT detected more ID donkeys than CGIT. EMS recommendations could also be used for DMS diagnosis, although a baseline insulin cut-off value is needed.

KEYWORDS

donkeys, glucose, insulin, metabolic syndrome, obesity

1 | INTRODUCTION

Interest in donkeys has been increasing in recent years in developed countries, in particular related to assisted therapies for people with psychomotor and mental disabilities, as an alternative source of milk for people with cow's milk intolerance, in certain sport competitions and as companion animals.[1] Donkeys are afflicted by similar pathologies as horses; however, due to physiological and pharmacological differences between donkeys and horses,[2-4] a species-specific approach should be considered when dealing with diagnostics and therapies in donkeys.

While metabolic and endocrine disturbances are common in donkeys,[5-8] most studies in this area have been on equine metabolic syndrome (EMS),[9,10] with donkey-specific knowledge being sparse. As a result, clinicians often extrapolate from horses, which in some instances apply while in others could lead to misdiagnosis, inappropriate treatments and associated risks.

Donkey/Asinine metabolic syndrome (DMS or AMS) is a frequent endocrinopathy in this species. Factors contributing to DMS include genetic predisposition to obesity due to calm behaviour, energy efficiency (thrifty species), reduced physical activity and poor nutritional management.[11,12]

Most research on insulin dysregulation (ID) has been on the diagnosis of EMS, with guidelines being updated frequently.[13-15] The body condition score (BCS) and baseline insulin, triglyceride, leptin and/or adiponectin concentrations are used in the diagnosis and management of EMS.[16,17] However, the value of these clinical, metabolic and endocrine parameters in the diagnosis of DMS remains unclear. Dynamic tests are useful for EMS diagnosis,[16] but to date only the frequently sampled intravenous glucose-insulin tolerance test (FSIGT), which is tedious and expensive, has been evaluated in donkeys with ID.[18] Other dynamic tests have not been characterised in donkeys (eg intravenous insulin test [IVITT] and oral glucose tolerance test [OGTT]) or have only been assessed in healthy donkeys (eg combined glucose-insulin test [CGIT] and intravenous glucose tolerance test [IVGTT]).[19]

We hypothesised that donkeys have specific glucose and insulin disposal particularities that could misdiagnose the DMS diagnosis if equine reference values are used. Therefore, the objective of this study was to evaluate two of the commonly used dynamic tests for the diagnosis of EMS (CGIT and IVGTT) in DMS-suspected donkeys.

2 | MATERIALS AND METHODS

2.1 | Animals and inclusion criteria

Eighty donkeys from two donkey sanctuaries were initially evaluated. Inclusion criteria for DMS phenotypically suspected were as follows: body condition score (BCS) higher than 6, a neck score (NS) higher than 2 and evidence of recurrent laminitis.[20] Blood samples were drawn from animals fulfilling the inclusion criteria (n = 12) and serum insulin (human insulin RIA kit [Millipore Corporation]) and leptin (Multi-species Leptin RIA kit [Millipore Corporation]) concentrations measured at the Animal Health Diagnostic Center from Cornell University using immunoradiometric assays. Animals with baseline serum insulin and/or leptin concentrations higher than 20 μIU/mL and 12 ng/mL, respectively, were further considered suspects of having DMS according to the guidelines for EMS diagnosis.[15-17,21]

Donkeys meeting clinical and endocrine inclusion criteria (n = 6) were considered DMS suspected and selected for dynamic tests. Animals were housed in a paddock (Brazos Valley Equine Hospital), where studies were carried out. Donkeys had free access to water and pasture. Donkeys were considered healthy based on physical examination and were under a regular deworming and vaccinations programmes.

This study received approval from both local and regional welfare committees, and animals were handled according to guidelines for research animals.

2.2 | Body morphometric measurements

Morphometric variables included: calculated bodyweight (BW), body mass index (BMI), body condition score (BCS), neck score (NS), neck circumference (NC) and neck circumference-to-height ratio (NCHR) following formulas previously described for donkeys and horses.[22,23] Three independent evaluators graded BCS and NS. The BCS ranged from 1 (very thin) to 9 (obese) and the NS from 0 to 4.[3,22]

2.3 | Testing protocols and interpretation

Donkeys were housed overnight (22:00 pm–08:00 am) with a flake of coastal Bermuda grass hay (1.5 kg) and free access to water.[13,14,15,17] The CGIT and IVGTT were performed to assess ID as previously described for donkeys.[11,19]

Briefly, for the CGIT, dextrose (150 mg/kg bwt; 50% dextrose solution [VetOne, MWI Animal Health]) was administered intravenously as a bolus, followed by recombinant human insulin (0.1 IU/kg bwt; Novolin® N [Novo Nordisk]) as an intravenous bolus. For the IVGTT, dextrose (300 mg/kg bwt; 50% dextrose solution [VetOne, MWI Animal Health]) was administered intravenously as a bolus. Blood samples in both tests were collected at the following time points: baseline, 5, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240 and 300 minutes. Test were carried out in all animals with 1 week washout period between tests. All tests were performed in summer time.

Based on criteria for EMS dynamic testing interpretation,[16,17,24-26] donkeys were considered ID positive either when the glucose-positive phase was longer than 45 minutes or plasma insulin concentrations were higher than 100 μIU/mL by 45 minutes in the CGIT, or when glucose and insulin concentrations did not return to baseline values after 2 hours for the IVGTT.

2.4 | Sample handling and measurement

A catheter was placed in the left jugular vein the day before of every study in all donkeys. Blood samples were collected into heparin-containing tubes for insulin and leptin and into oxalate tubes for glucose measurement. After collection, blood samples were chilled immediately on ice, subsequently centrifuged at 1500 g for 10 minutes at 4°C, aliquoted and stored at −20°C until analysis.

Every parameter was determined using tests and protocols previously validated for donkeys.[7,11] Plasma insulin (sensitivity limit 2.7 μIU/mL and intra-assay CV <4.4%) concentration was measured with a human-specific immunoradiometric assay (DIASource ImmunoAssays SA) and glucose by spectrophotometry (Heska Dry-Chem 7000 [Fujifilm, Minato-Ku]).

2.5 | Proxies and curves parameters calculation

Fasting baseline glucose and insulin concentrations were used to calculate the following proxies according to formulas previously used in donkeys and horses[27,28]: glucose/insulin ratio, insulin/glucose ratio, modified insulin-to-glucose ratio (MIRG), reciprocal of the square root of insulin (RISQI), quantitative insulin-sensitivity check index (QUICKI), homeostasis model assessment for insulin resistant (HOMA-IR) and homeostasis model assessment of percentage β-cell function (HOMA-B%).

Parameters obtained from dynamic tests curves have been previously described in donkeys and horses[11,19,29,30]: positive phase duration (PPD), positive glucose clearance rate (PGCR), negative phase duration, start to nadir interval, nadir concentration, valley duration (when applicable), negative glucose clearance rate and valley to baseline interval. The areas under the curve for glucose (AUCg) and insulin (AUCi) were calculated using the trapezoidal method.[11,19,30]

2.6 | Data analysis

Normality was assessed with the Shapiro-Wilk test. Data were normally distributed. Results are expressed as mean and standard deviation. Correlations were calculated using Pearson test. Binary logistic regression was used to determine the odds of ID based on the variables (predictors) studied. Statistical analysis was performed using a commercial statistical software (IBM SPSS Statistics 24 [IBM]).

3 | RESULTS

3.1 | Morphometric variables, basal concentrations and proxies results

Six mix-breed adult donkeys (1 jack and 5 nonpregnant jennies, 35 ± 12.1 years old), weighting 218.5 ± 52.9 kg, fulfilled the inclusion criteria. All donkeys were obese with a BCS >6 and NS >2 (Table 1). All donkeys were normoglycaemic, with insulin and leptin concentrations (Table 2) higher than reference values reported for healthy donkeys and horses,[3,31,32] except for donkey 4, which was phenotypically suspected and fulfilled most criteria but had a baseline insulin concentration lower than 20 μIU/mL. Baseline insulin and leptin concentrations from donkeys excluded from the study (6/12) are shown in Table S1. Ratios and proxies are listed in Table 2.

Glucose and insulin concentrations were negatively correlated (Table 3) with bodyweight ($r = -0.89$, $P = .01$; $r = -0.81$, $P = .04$, respectively) and NS ($r = -0.99$, $P < .01$; $r = -0.77$, $P = .05$, respectively). Pearson correlations among baseline glucose, insulin and leptin concentrations and proxies are shown in Table 3.

3.2 | Combined glucose-insulin test

No donkey had adverse effects from the tests. Glucose concentrations peaked (approximately 200% of basal glucose concentrations) between 1 and 10 minutes (blood sample collected at 5 minutes) in all donkeys. Based on the EMS guidelines for CGIT,[16,17,33] only 2 donkeys (2/6) were classified as ID positive using PPD. When insulin cut-off values were used, 3/6 animals were ID positive (Figure 1; Table 4A). Those diagnosed as ID positive based on PPD have a PGCR lower than 1.5 mg/dL/min. In contrast, when insulin concentration was used, donkeys with ID had an AUCi higher than 25×10^3 mg/dL/min (Table 5).

Baseline insulin concentration was positively correlated (Table S2) with PPD ($r = 0.79$, $P < .05$), AUCg ($r = 0.95$, $P < .01$) and AUCi ($r = 0.81$, $P < .05$). The binary logistic regression analysis did not identify any variable as a predictor for ID using the CGIT.

3.3 | Intravenous glucose tolerance test

No adverse effects were observed with this test. Glucose administration induced an increase in plasma glucose above 250% of basal concentration, peaking by 5 minutes. Five of 6 donkeys (5/6) were ID positive with the IVGTT (Figure 2; Table 4A) following the protocol interpretation previously reported for horses.[24,25] Donkeys showed an AUCg higher than 32×10^3 mg/dL/min, and a PGCL lower than 1.5 mg/dL/min (Table 5).

Baseline glucose, insulin and leptin concentrations were positively correlated (Table S3) with PPD ($r = 0.79$, $r = 0.82$ and $r = 0.80$, $P < .05$; respectively). Also insulin and leptin concentrations were negatively correlated with PGCR ($r = -0.84$ and $r = -0.91$, $P < .05$; respectively). The binary logistic regression analysis did not identify any variable as a predictor for ID using IVGTT.

4 | DISCUSSION

Contrary to horses, there are no validated protocols for DMS diagnosis in donkeys. ID criteria described for horses were used to

TABLE 1 Morphometric measurements for each donkey (n = 6)

Parameter	Donkey 1	Donkey 2	Donkey 3	Donkey 4	Donkey 5	Donkey 6	Mean ± SD
BW (kg)	131.9	223.6	215.9	252.2	269.0	160.7	218.5 ± 52.9
BCS	6	8	7	7	9	7	7.4 ± 1.1
BMI (kg/m^2)	125.3	204.2	169.8	142.4	204.1	150.0	169.1 ± 35.7
NC (cm)	64.1	71.1	72.1	82.2	83.3	65	74.6 ± 8.1
NS	2	3	3	3	3	2	2.8 ± 0.5
NCHR (cm/m)	62.5	67.9	63.9	61.8	72.6	62.8	65.7 ± 4.5

Abbreviations: BCS, body condition score; BMI, body mass index; BW, bodyweight; NC, neck circumference; NCHR, neck circumference-to-height ratio; NS, neck score; SD, standard deviation.

TABLE 2 Biochemical variables and proxies for each donkey (n = 6)

Variable	Donkey 1	Donkey 2	Donkey 3	Donkey 4	Donkey 5	Donkey 6	Mean ± SD
Glucose (mg/dL)	93.5	79.0	79.0	77.5	78.0	98.0	82.1 ± 6.2
Insulin (μIU/mL)	42.4	26.9	20.4	13.8	23.8	30.6	24.6 ± 11.9
Leptin (ng/mL)	24.0	44.9	41.1	22.9	28.2	38.6	32.2 ± 10.1
Glucose/insulin ratio	2.2	2.9	3.9	8.2	3.3	3.2	4.1 ± 2.4
Insulin/glucose ratio	0.45	0.34	0.26	0.12	0.30	0.31	0.30 ± 0.12
RISQI	0.15	0.19	0.22	0.33	0.21	0.18	0.22 ± 0.10
MIRG	12.3	13.1	10.9	6.4	12.4	10.1	11.1 ± 2.70
QUICKI	0.28	0.30	0.31	0.35	0.31	0.29	0.31 ± 0.02
HOMA-IR	175.9	94.7	71.6	32.4	82.4	133.4	91.4 ± 52.8
HOMA-B%	9.4	7.1	5.4	2.5	6.4	6.5	6.2 ± 2.5

Abbreviations: HOMA-B%, homeostasis model assessment of percentage β-cell function; HOMA-IR, homeostasis model assessment for IR; MIRG, modified insulin-to-glucose ratio; QUICKI, quantitative insulin-sensitivity check index; RISQI, reciprocal of the square root of insulin; SD, standard deviation.

assess the donkeys of this study[16,17,21]. Results showed differences between both dynamic tests, with the CGIT detecting a lower number of ID donkeys compared with IVGTT.

Based on the results of this study, when dynamic tests are indicated to confirm DMS/AMS, such as donkeys with a moderate increase in insulin concentrations (eg 20-50 μIU/mL), it would be preferable to use the IVGTT over the CGIT. Following horse endocrine interpretation,[16,26] CGIT identified 2/6 (using PPD) or 3/6 (using insulin concentration by 45 minutes) ID-positive donkeys, while IVGTT identified 5/6. Moreover, using a donkey-specific curve analysis based on reference values from healthy donkeys,[19] the number of donkeys identified as ID positive decreased to 2/6 with CGIT, using both PPD (median: 41.4 minutes) or insulin concentration by 45 minutes (Table 4B). Despite these findings, all donkeys included in this study had a higher AUCg and AUCi values than previously published for healthy ones (median: 15.9×10^3 mg/dL/min and 14×10^3 μIU/mL/min, respectively).[19] Therefore, additional factors likely influenced the response to the CGIT when used in donkeys with a moderate increase in insulin concentrations.

The IVGTT classified a higher number of donkeys as ID (5 of 6), both using the interpretation described for horses (PPD >120 minutes)[24,25] and a donkey-specific approach (PPD >150 minutes; Table 4). Donkeys with ID had IVGTT curve parameters different from those published for healthy donkeys,[19] with longer PPD (median healthy: 156.3 minutes) and higher AUCg (median healthy: 21.4 mg/dL/min) and AUCi (median healthy: 7.2 μIU/mL/min).[19]

Although the IVGTT classified a larger number of donkeys as ID, it has the disadvantage that it is more time-consuming.[19] In this sense, other criterion could be proposed for a faster interpretation; for instance, to the author's knowledge, an insulin cut-off at a certain time of the curve has not been established for donkeys or horses for this test, but could be validated as an earlier indicator of ID. For example, a cut-off of 100 μIU/mL at 45 minutes could be used, shortening the test duration as done during CGIT. Applying this new criterion to IVGTT, all donkeys were ID positive except donkey 4 (which did not fulfil the basal insulin criterion and was ID negative) and donkey 6. This last animal, despite fulfilling the inclusion criteria, had a baseline insulin concentration <20 μIU/mL on the day of the test,[25] which could have caused a lower AUCi despite being positive for ID (PPD >120-150 minutes; Table 5). Increasing the number of donkeys with ID doing this test is necessary to validate this criterion.

The CGIT evaluates tissue insulin sensitivity while IVGTT also assesses β-cell sensitivity and glucose disposal. A previous study in healthy donkeys showed that the IVGTT curve is right shifted and displayed a negative phase, indicating delayed insulin sensitivity, although glucose clearance rate was similar to horses.[19] Therefore, we

TABLE 3 Pearson correlation coefficients (*r*) among resting hormone concentrations, morphometric parameters and proxies from six adult donkeys

	BW	BCS	NS	Glucose	Insulin	Leptin	G:I	I:G	MIRG	RISQI	QUICKI	HOMA-IR	HOMA-B%
BW	—	0.77*	0.92**	−0.89**	−0.81*	−0.03	0.52	−0.68	−0.22	0.67	0.75	−0.88*	−0.67
BCS	—	–	0.62	−0.57	−0.33	0.22	−0.05	−0.18	0.30	0.10	0.21	−0.43	−0.17
NS	—	—	—	−0.99**	−0.77*	0.16	0.45	−0.60	−0.10	0.60	0.69	−0.87*	−0.59
Glucose	—	—	—	—	0.73	−0.02	−0.46	0.56	0.10	−0.60	−0.70	0.83*	0.55
Insulin	—	—	—	—	—	0.04	−0.86*	0.97**	0.69	−0.93**	−0.95**	0.98**	0.97**
Leptin	—	—	—	—	—	—	−0.40	0.13	0.41	−0.34	−0.31	−0.01	0.12
G:I	—	—	—	—	—	—	—	−0.92**	−0.92**	0.98**	0.95**	−0.79*	−0.92**
I:G	—	—	—	—	—	—	—	—	0.83*	−0.96**	−0.95**	0.92**	1**
MIRG	—	—	—	—	—	—	—	—	—	−0.85*	−0.77*	0.56	0.84*
RISQI	—	—	—	—	—	—	—	—	—	—	0.99**	−0.89*	−0.95**
QUICKI	—	—	—	—	—	—	—	—	—	—	—	−0.93*	−0.94*
HOMA-IR	—	—	—	—	—	—	—	—	—	—	—	—	0.91*
HOMA-B%	—	—	—	—	—	—	—	—	—	—	—	—	—

Abbreviations: BCS, body condition score; BW, bodyweight; G:I, glucose/insulin ratio; HOMA-B%, homeostasis model assessment of percentage β-cell function; HOMA-IR, homeostasis model assessment for insulin resistant; I:G, insulin/glucose ratio; MIRG, modified insulin-to-glucose ratio; NS, neck score; QUICKI, quantitative insulin-sensitivity check index; RISQI, reciprocal of the square root of insulin.
*$P < .05$; **$P < .01$.

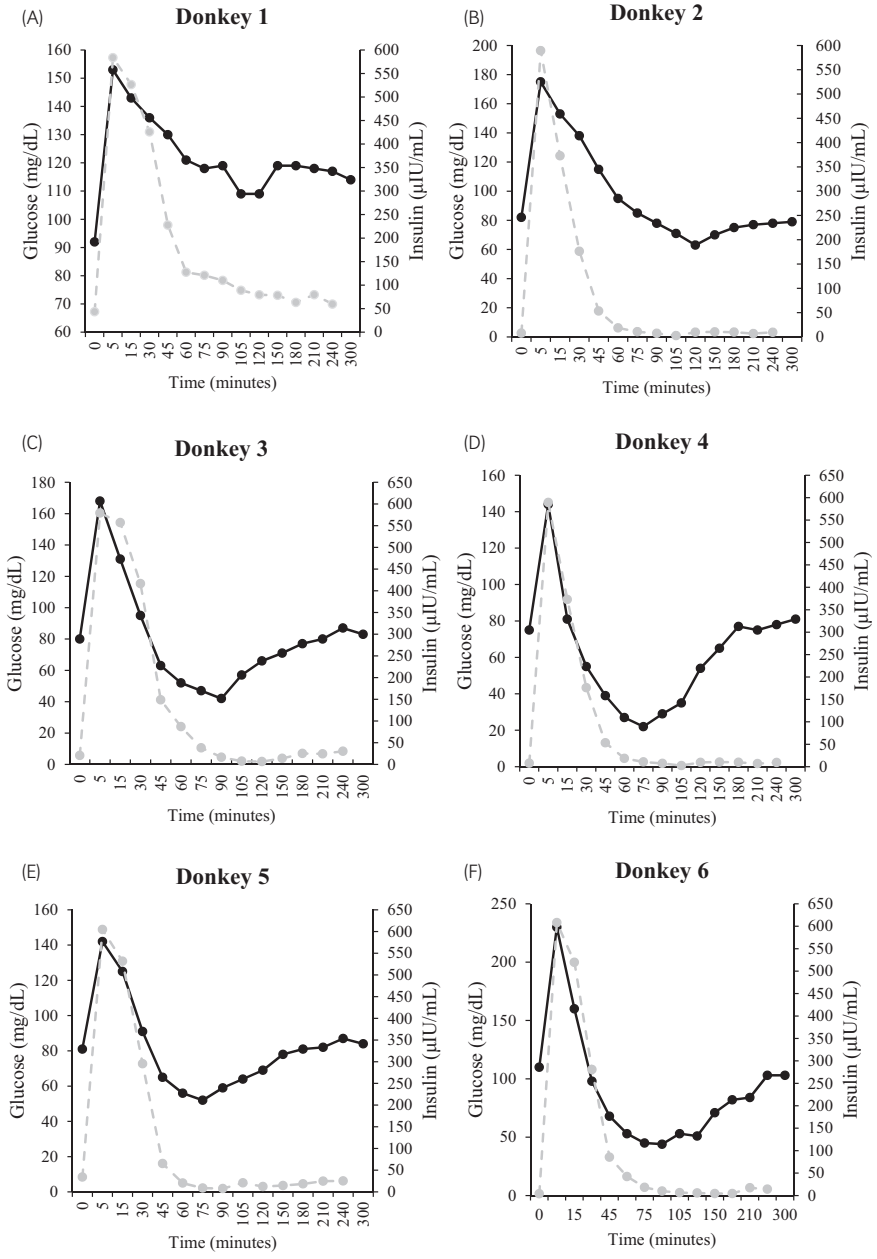

FIGURE 1 Glucose (continuous line) and insulin (dashed line) concentrations for combined glucose-insulin test (CGIT) in each donkey. From A to F correspond to Donkey 1 to Donkey 6, respectively.

TABLE 4 Insulin dysregulation diagnosis (n = 6) following previously reported guidelines for (A) equine metabolic syndrome[16,17,24,25,26] or (B) according to donkey-specific dynamic testing interpretation[19]

Test	Donkey 1	Donkey 2	Donkey 3	Donkey 4	Donkey 5	Donkey 6	
Inclusion criteria	+	+	+	+	+	+	**Interpretation**
(A)							
CGIT	+	+	−	−	−	−	PPD >45 min
CGIT	+	+	+	−	−	−	Insulin >100 µIU/mL by 45 min
IVGTT	+	+	+	−	+	+	PPD >120 min
(B)							
CGIT	+	+	−	−	−	−	PPD >60 min
CGIT	+	+	−	−	−	−	Insulin >100 µIU/mL by 60 min
IVGTT	+	+	+	−	+	+	PPD >150 min

Abbreviations: CGIT, combine glucose-insulin test; IVGTT, intravenous glucose tolerance test; PPD, positive phase duration.

can speculate that discrepancies between CGIT and IVGTT could be attributed to differences in ID severity in the donkeys included and/or to the sensitivity of each dynamic challenge. Thus, based on the results of this study, the IVGTT should be considered for donkeys with suspected mild ID and the CGIT for more severe ID. Moreover, evaluation of the enteroinsular axis in donkeys could add valuable information on how intestinal factors influence insulin secretion in this species.[34] In addition to low sensitivity, the CGIT also has poor repeatability in horses,[35] which has not been evaluated in donkeys but could have influenced our results.

Although proxies offer minimal advantages in EMS diagnosis,[14,15] they could be a complementary tool for the diagnosis of DMS/AMS and enhance our understanding on energy dynamics in this species. In fact, correlations between proxies and an ID-positive diagnosis using IVGTT (but not CGIT) were observed, and every ID-positive donkey showed proxies outside the reference ranges for this species.[19] A glucose/insulin ratio <4 was observed in all ID-positive donkeys in this study, which is lower than the value proposed for horses (<10) regardless of the severity of ID.[36] According to our results, this cut-off for this proxy could be proposed as a satisfactory tool for ID diagnosis in this species. Similarly, MIRG >10 or RISQI <0.22 values could also be proposed for the diagnosis of ID in donkeys, being different from those proposed for horses (<5.6 and <0.32 respectively).[17,36] The greatest difference with previously described donkeys reference ranges was for HOMA-IR, where ID-positive donkeys have a value >70 versus >30 in healthy donkeys,[19] although donkeys in our study were older. Since cut-off values for proxies were generated using a low number of donkeys, it would be advisable to repeat the study in a larger population of donkeys with different degrees of ID to confirm their clinical utility.

Although donkey 4 had a baseline insulin concentration lower than 20 μIU/mL, it was included in the study based on the rest of the inclusion criteria. However, based on both dynamic tests, it was later classified as not having ID. While this was observed in one animal, it suggests that insulin is the main laboratorial parameter to consider when facing an ID diagnosis. Future studies in larger donkey populations with different ID degrees using other laboratorial parameters to assess EMS such as adiponectin or protein C concentrations[15] deserve investigation.

It has been shown that baseline insulin concentration had a poor sensitivity to diagnose ID,[10,37] which could explain why some of donkeys in this study have contradictory results when using dynamic tests. Although species-specific insulin cut-off value cannot be accurately established with our results, it seems reasonable to propose that baseline insulin concentrations higher than 20 μIU/mL in fed donkeys make the animal suspicious of DMS/AMS, as described in the EMS guidelines.[15] Future studies with a larger donkey population with different ID degrees could evaluate this value. Olley et al (2019)

TABLE 5 Analysis of the CGIT and IVGTT curves for each donkey (n = 6)

	Parameter	Donkey 1	Donkey 2	Donkey 3	Donkey 4	Donkey 5	Donkey 6
CGIT	Positive phase duration (min)	>300	81.4	37.1	18.5	35.8	27.1
	Positive glucose clearance rate (mg/dL/min)	0.2	1.1	2.4	3.7	1.7	4.4
	Negative phase duration (min)	—	218.6	172.9	156.5	144.2	272.9
	Start-to-nadir interval (min)	—	120	90	75	75	90
	Nadir (mg/dL)	—	63	42	22	52	44
	Valley duration (min)	—	45	60	60	60	60
	Negative glucose clearance rate (mg/dL/min)	—	−0.5	−0.7	−0.9	−0.7	−1.1
	Valley-to-baseline interval (min)	—	180	120	100	105	210
	AUC glucose ($\times 10^3$ mg/dL/min)	35.9	26.1	23.2	19.1	23.8	25.4
	AUC insulin ($\times 10^3$ mg/dL/min)	41.7	31.1	25.7	14.7	21.1	19.5
IVGTT	Positive phase duration (min)	300	198.7	255	90	161.5	300
	Positive glucose clearance rate (mg/dL/min)	0.4	0.9	0.7	2.1	1.5	0.6
	Negative phase duration (min)	—	101.2	45	210	42.5	—
	Start-to-nadir interval (min)	—	240	300	150	180	—
	Nadir (mg/dL)	—	60	75	69	67	—
	Valley duration (min)	—	—	—	60	30	—
	Negative glucose clearance rate (mg/dL/min)	—	−0.4	−0.1	−0.2	−0.4	—
	Valley-to-baseline interval (min)	—	—	—	150	24	—
	AUC glucose ($\times 10^3$ mg/dL/min)	40.1	35.1	37.9	29.6	32.3	48.3
	AUC insulin ($\times 10^3$ mg/dL/min)	27.9	35.5	29.1	4.8	21.9	8.6

Note: Positive phase duration: time glucose returned to baseline; positive glucose clearance rate: ratio between the difference in the highest glucose concentration minus glucose concentration returned to baseline and time glucose returned to baseline minus time of baseline glucose; Start-to-nadir interval: time glucose returned to baseline.

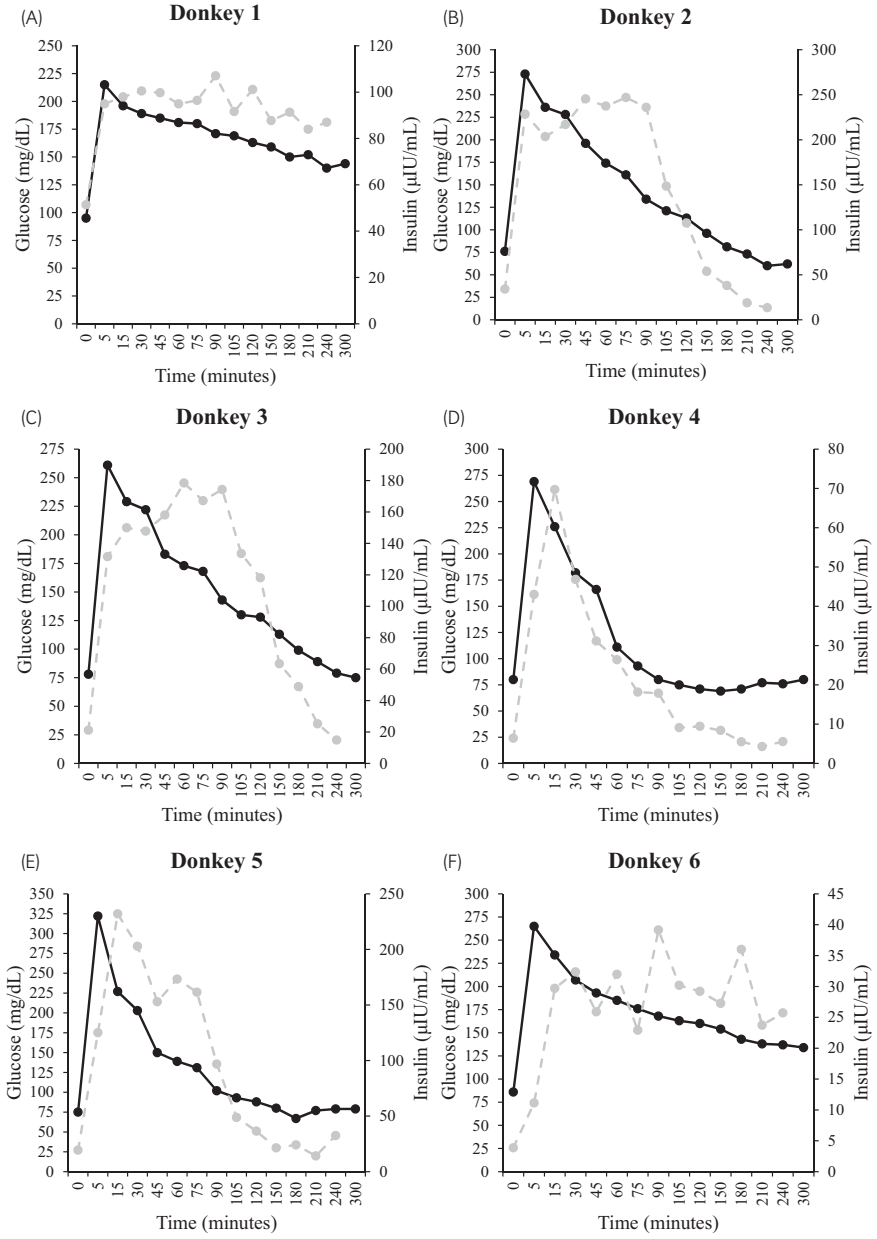

FIGURE 2 Glucose (continuous line) and insulin (dashed line) concentrations for intravenous glucose tolerance test (IVGTT) in each donkey. From A to F correspond to Donkey 1 to Donkey 6, respectively.

reported that a lower insulin cut-off (5.2 μIU/mL) would have better sensitivity, although worse specificity, compared to 20 μIU/mL.[38] However, this study was carried out in animals fasted for 8-12 hours, using chemiluminescent assay and the cut-off value obtained included horses and ponies. A recent study in fasted healthy donkeys showed that feed deprivation for 12 hours induced changes in insulin homeostasis[39]; thus, fasting and duration of fasting alter the response to dynamic tests and should be considered in data interpretation in donkeys.

A major factor to take into account in data interpretation and clinical classification is the method used for insulin measurement. A number of validated immunoassays are used to measure insulin concentrations in horses and results are not equivalent among assays.[15] This issue has not been investigated in donkeys. Thus, in addition to species differences, cut-off values should be assay specific as extrapolation may result in misclassification; in particular, when insulin concentrations are close to cut-off values. This is important as both false-positive and false-negative results have clinical and economic implications. Further studies are necessary to evaluate if the proposed insulin cut-off for immunoradiometric assays is valid when ELISA or chemiluminescent assays are used.

It has been shown in horses that season (β-cell sensitivity), circadian rhythm, diet (structural carbohydrate content), fasting, stress, α_2-adrenergic agonists and pregnancy could influence endocrine variable and response to dynamic tests.[28,30,38,40-42] Since all baseline samples for the inclusion criteria were collected in the morning in early July, all dynamic tests were performed in the last 2 weeks of July, all animals were kept in the same facility under a feeding protocol similar to the farm of origin, stress was reduced and no treatments were administered; these factors could be discarded.

It is important to highlight some shortcomings from this study, in particular that a small number of donkeys with moderate ID (insulin concentration <50 μIU/mL) were included. Further studies with a larger population of donkeys with varying degrees of ID would yield valuable information. In addition, since age affects insulin sensitivity in absence of ID, comparing these results with a case-control group of healthy young and old donkeys would be compelling.

In conclusion, guidelines for EMS diagnosis could also be useful for DMS/AMS diagnosis. Based on our results, the IVGTT identified more donkeys with ID than the CGIT. Additional dynamic studies in a larger population of healthy and ID donkeys will provide valuable insight on the pathogenesis of DMS as well as practical diagnostic methods and an insulin cut-off value.

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CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

S. Mejia-Moreira contributed to study design and study execution. A. Perez-Ecija, R. Toribio and F. Mendoza contributed to study design, data analysis and interpretation and preparation of the manuscript. B. Buchanan contributed to study design, data analysis and interpretation and preparation of the manuscript. All authors gave their final approval of the manuscript.

ETHICAL ANIMAL RESEARCH

This study received approval from Welfare Committees (2015PI/04 and 19-03-15-214).

INFORMED CONSENT

Representatives of the donkey sanctuaries caring for these animals gave consent for their inclusion.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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