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Intratumoural tigilanol tiglate in the multicentre treatment of equine sarcoids and cutaneous melanomas

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Abstract

Background: Intralesional chemotherapeutic administration represents an important treatment option for equine cutaneous neoplasia. Tigilanol-tiglate (TT), a novel molecule extracted from *Fontainea picrosperma*, an Australian rainforest plant, is registered for intratumoural treatment of canine MCT, leading to rapid oncosis and tumour slough. Evidence from horses is limited but suggests that efficacy may be similar.

Objectives: To evaluate the response to intratumoural TT treatment in horses with sarcoids (fibroblastic/nodular) and cutaneous melanomas.

Study Design: Two noncontrolled prospective multicentre clinical trials, one for each of sarcoids and melanomas.

Methods: Cases were enrolled across multiple sites and treated by the same site-specific clinician with intralesional TT (sarcoids: 0.35 mg/cm^3; melanomas: 0.2 mg/cm^3 of tumour volume – T_{vol}; max dose 2 mg). Quantitative (T_{vol} regression) and qualitative outcomes (likely tumour free (LTF) per expert opinion) were recorded, and potential determinants of efficacy were assessed using random effects logistic models. A full clinical response was complete T_{vol} regression and a LTF treatment site.

Results: Forty-one sarcoids and 97 melanomas were enrolled and treated. 73/74% of treated sarcoids/melanomas showed complete T_{vol} regression. 64/61% (sarcoids/melanomas) showed a full clinical response at medians of 546/247 days post final treatment. For both tumour types, this response was dependent on initial tumour volume ($P_{sarcoids} = 0.006$; $P_{melanomas} < 0.001$). The predicted probability of a full clinical response was 6 times greater for initially small sarcoids ($T_{vol} = 1$ cm^3) than for the maximum study volume ($T_{vol} = 6$ cm^3). For melanomas in the perineal region, this was 11 times greater for $T_{vol} \leq 0.3$ cm^3 than for tumours ≥2.0 cm^3. For melanomas, tumour location further affected treatment efficacy $= 0.005$). In total, 5 adverse events were reported.

Main Limitations: Lack of treatment control and histologic/biomolecular follow-up data.

Conclusions: The observed therapeutic efficacy of TT supports clinical use as well as early interventions in horses. Successful use necessitates knowledge of the drug's mode of action and management of associated local site responses.

KEYWORDS
horse, neoplasia, skin, stelfonta

1 | INTRODUCTION

Sarcoids and melanomas are the most prevalent tumours encountered in equine veterinary practice.[1,2] Recent advances[3–5] have resulted in tumour-specific, systemic and efficacious treatment options; however, these are yet to be commercially available, such that currently available treatment strategies remain nonspecific at targeting individual growths.[6,7] Complete regression rates with intratumoural chemotherapeutics exceeding 90% have been reported, and these may set the reference standard when assessing novel intratumoural drugs, but lower rates are not uncommon.[8,9] An effective and impactful treatment should also be convenient and safe to use, and patients should require minimal aftercare to promote early interventions and client uptake. These latter attributes would be expected to result in earlier interventions and acceptability to clients. In the case of conventional chemotherapeutics, associated safety protocols and the potential for delayed wound healing render use in general veterinary practice more complex.[10,11] Given the latter concerns along with more common tumour resistance, there is a need for novel compounds to serve as primary or adjunctive therapeutics.[12]

Tigilanol tiglate (TT; also known as EBC-46 and registered as Stelfonta®) is a small epoxytigliane molecule derived from the native Australian rainforest plant *Fontainea picrosperma* (Euphorbiaceae). It has been approved as a veterinary pharmaceutical for the treatment of nonmetastatic mast cell tumours in dogs in the United Kingdom,[13] European Union, Switzerland,[14] United States,[15] and Australia,[16] and is registered as Stelfonta®. Its tumour-agnostic effects are attributed to the disruption of the local vasculature, oxidative stress, direct tumour oncosis, and activation of the innate immune system. The tissue deficit developing from successful tumour ablation heals with a good cosmetic outcome.[17–22] There is limited reported evidence for efficacy against tumours in horses, with outcomes after the treatment of only six cases reported: one sarcoid,[23] three squamous cell carcinomas,[23,24] and two mast cell tumours.[25]

Our primary objective was to describe outcomes after TT treatment in much larger populations of horses with sarcoids and cutaneous melanomas by conducting two separate noncontrolled clinical trials. A secondary aim was to report the safety and any adverse events encountered during the trials. We hypothesised that up to three intratumoural injections with TT would result in tumour regression rates in line with comparable clinical standards and that reported adverse events or observed comorbidities would be minimal. We also assessed potential determinants of tumour outcomes within each of sarcoids and melanomas.

2 | MATERIALS AND METHODS

Two noncontrolled prospective multicentre clinical trials were conducted, one for sarcoids and one for melanomas. In the equine sarcoid study, the first to be designed and initiated, cases were enrolled from March 2021 to January 2023 at eight study sites (six university and two private veterinary hospitals) across six countries. Enrolments and treatments were performed by 11 clinical investigators who, for each site, remained the same throughout the study. Early learnings from the sarcoid trial were incorporated into the melanoma study design, and cases were enrolled from July 2022 to October 2023 at four study sites (three university and one private veterinary hospital) across three countries and were supported by five clinical investigators remaining the same throughout. All treatments were approved by relevant institutional animal welfare and regulatory bodies (Table S1). Tumours selected and subsequently treated by clinical investigators were retrospectively verified (at the time of final data collation) to have met inclusion criteria. For sarcoids, single or up to five discrete fibroblastic or nodular type sarcoids per horse that were confirmed by histology and/or molecular techniques (bovine papilloma virus PCR detection) at an accredited veterinary diagnostic laboratory were eligible for enrolment. For pigmented cutaneous tumours to be included as melanomas, they had to occur in grey horses and be of typical clinical presentation regarding site and appearance. Up to 3 discrete melanomas were eligible for enrolment per treatment round, and masses of atypical presentation required histologic confirmation. Melanocytic tumours could not have received prior chemo, radio, or immunotherapy. Noncutaneous melanocytic tumour masses (e.g., within the parotid salivary gland) or cutaneous tumours with extensive deep tissue involvement (e.g., deep pararectal tumours) were not eligible for enrolment. (Full eligibility criteria shown in Table 1). Treatment doses were determined based on tumour volume[26,27] and administered by intratumoural injection as outlined (Treatment protocol; Table 1). Different dose rates (0.35 mg TT/cm^3 in sarcoids vs. 0.2 mg TT/cm^3 in melanomas) were set, guided by the manufacturer's exploratory work which suggested that despite the lower dose rate, responses in melanomas are on par with those in sarcoids, while at the same time resulting in smaller tissue deficits. Details on permitted patient restraint,

TABLE 1 Tigilanol tiglate (TT) dose determination and administration as per study protocol.

Protocol component	Description/comments	
	Sarcoid	**Melanoma**

Inclusion criteria	• Signed owner consent form.
	• Have 'Not intended for human consumption' signed in the EU/UK horse passport.
	• ≥12 months of age.
	• At least one sarcoid with the following characteristics:
	  ○ Nodular or fibroblastic,
	  ○ Tumour edges are defined and distinct,
	  ○ Calculated tumour volume up to 6 cm^3,
	  ○ Total dose at time of treatment does not exceed 2 mg/horse,
	  ○ >8 weeks since previous treatment modalities including BCG, injectable chemotherapy, radiotherapy, surgery, or any topical therapies,
	  • Where previous treatment modalities have occurred, all inflammation from the treatment should be resolved prior to Treatment Day 0.
	  • Where a wound resulted due to previous treatment modalities, the treatment site should be fully healed.
	• For horses with multiple sarcoids that are:
	  ○ Nodular or fibroblastic,
	  ○ Tumour edges are defined and distinct,
	  ○ Calculated tumour volume up to 6 cm^3, and
	  ○ Total dose at time of treatment does not exceed 2 mg/horse,
	  ○ >8 weeks since previous treatment modalities including BCG, injectable chemotherapy, radiotherapy, surgery, or any topical therapies,
	  • Where previous treatment modalities have occurred, all inflammation from the treatment should be resolved prior to Treatment Day 0.
	  • Where a wound resulted due to previous treatment modalities, the treatment site should be fully healed.
	  ○ Up to 5 sarcoids on the same horse may be treated simultaneously provided that:
	  • the combined volume of all sarcoids treated at that timepoint does not exceed 6 cm^3,
	  • the total dose at that timepoint does not exceed 2 mg/horse, and
	• Horses are manageable, cooperative, and available for the entire study period.
	• Horses may have a concurrent medical condition other than the presenting tumour (e.g., lameness) but should be considered physically healthy and not have any known condition that may cause the patient to be immunocompromised.
	• Owners agree to remain in the study for at least 24 months which includes long term follow-up visits.
		• Signed owner consent form and that they have received information on and declined all other treatment options.
		• Physical examination within normal limits and free of severe underlying disease.
		• Horse is deemed fit for sedation.
		• Single or multiple melanomas (up to 3 per treatment per horse).
		  ○ Maximum total calculated volume of each treated melanoma must be ≤10 cm^3 (this equates to a maximum dose per lesion of 2 mL of 1 mg/mL tigilanol tiglate as Stelfonta).
		• Target lesions must have an intact surface (i.e., no unhealed biopsy site or ulcerated) to minimise leakage of TT during administration.
		• Each patient is eligible to receive treatment of up to 3 melanomas per treatment cycle for a maximum of 3 treatment cycles.
		• Each melanoma must be at least 2 cm apart to ensure that on lesion slough areas do not converge causing a large single tissue deficit.
		• Horses that have previously received Oncept vaccine ≥6 months prior to treatment in this study may be treated but will be analysed as a separate cohort and additional horses must be enrolled to ensure adequate numbers to meet statistical requirements of the study.
		• At least 6 months since previous treatment modalities including injectable chemotherapy, radiotherapy, surgery, or any topical therapies.
		  ○ Where previous treatment modalities have occurred, all inflammation from the treatment should be resolved prior to Treatment Day 0.
		  ○ Where a wound resulted due to previous treatment modalities, the treatment site should be fully healed.
		• Horses are manageable, cooperative, and available for the entire study period.
		• Horses may have a concurrent medical condition including other melanoma lesions (e.g., lameness) but should be considered physically healthy and not have any known condition that may cause the patient to be immunocompromised.
		• Owners agree to remain in the study for at least 24 months which includes long term follow-up visits.

Exclusion criteria	• Occult, verrucous or mixed sarcoids. • Sarcoid volume >6 cm^3. • Surface of sarcoid is likely to cause leakage of intratumoural tigilanol tiglate including ulcerated surfaces or unhealed biopsy sites. • Healing wound is still present following previous sarcoid treatment modalities. • Horse has received immunosuppressive therapy including chemotherapy or radiotherapy or any other form of sarcoid treatment <8 weeks prior to Treatment day (Day 0).	• Lesions larger than 10 cm^3 • Parotid melanoma or complicated melanoma cases such as deep para-rectal melanomas. • The horse has received systemic chemotherapy within 14 days prior to enrolment. • Horse has a previous history of gastric ulceration and/or another condition where the use of NSAIDs is contra-indicated. • The horse has received immunosuppressive therapy such as corticosteroids or long-acting corticosteroids ≤4 weeks prior to treatment.

(Continues)

TABLE 1 (Continued)

Protocol component	Description/comments	
	Sarcoid	**Melanoma**
	• Horse has a previous history of gastric ulceration and/or another condition where the use of NSAIDs is contra-indicated. • Horse has an ongoing condition or is undergoing treatment (including corticosteroid use) that could result in any form of immunocompromise up to 8 weeks prior to Treatment day (Day 0). • Horse has a concurrent medical disorder likely to result in death or euthanasia within six (6) months. • Ocular sarcoids that have conjunctival involvement. • The horse is pregnant or with foal. Where a general anaesthetic is required to achieve effective intratumoural injection.	• Treatment with Oncept vaccine <6 months. • Horse has an ongoing condition or is undergoing treatment (including corticosteroid use) that could result in any form of immunocompromise up to 4 weeks prior to Treatment day (Day 0). • Horse has a concurrent medical disorder likely to result in death or euthanasia within six (6) months. • The horse is considered unsuitable for study enrolment by the investigator. • The horse is pregnant or lactating with foal at foot.
Estimation of tumour volume	• Tumour volume estimated using calliper measurements and the following formula: T_{vol} (cm^3) = ½ (lesion length × lesion width × lesion thickness)	
Dose protocol	• Dose: 0.35 mg TT (1 mg/mL) per cm^3 of estimated tumour volume.[23] • Maximum cumulative dose: 2 mg TT per horse and treatment. • When treating multiple sarcoids at the same time (up to three per treatment cycle) masses were required to be at least 50 cm apart (or 2.5 cm apart for Australian and US sites). NB: When tumours enlarged to greater than 6 cm^3 post treatment, they were excluded from further trial participation as this would have resulted in permitted cumulative doses to be exceeded.	• Dose: 0.20 mg TT (1 mg/mL) per cm^3 of estimated tumour volume (dose guided by early experiences). • Maximum cumulative dose: 2 mg TT per horse and treatment. • When treating multiple melanomas at the same time (up to three per treatment cycle) tumours were required to be at least 2 cm apart.
Administration protocol	• Injected intratumoural using 'fanning' technique from a single needle entry site to reduce potential leakage and achieve optimal distribution. • Delivered using 1 mL Luer lock syringe.	
		• Treatment site 20 also used Quatron needles (a 34G 4 mm long multi-needle device) for injection of small lesions without fanning (Quatron®, ASTI corporation, Shizuoka, Japan; https://www.asti-med.com/quatron/).

Note: Details specific to the sarcoid trial are shaded green; those applicable to the melanoma trial are in yellow.

obligatory prophylactic, and continued management of inflammatory tissue responses are listed in a supplemental file (Table S2).

Post-treatment follow-up occurred using a combination of digital photography, in-person re-evaluations, and a reporting diary maintained by caretakers. In-person reassessments were guided by photographic updates, owner, and veterinary availability. Tissue deficits were expected to form after successful tumour destruction and slough, and these were left untreated to heal by second intention. In the event of an inadequate response (<100% T_{vol} regression) as judged by the treating veterinarian, protocols permitted retreatment from 14 days after the last treatment until a maximum of three treatments was provided (Tx1; Tx2; Tx3). Events identified as requiring retreatment included no response, tumour slough or change in treatment tumour volume, partial response with residual tumour present, or evidence of proliferative growth in the case of sarcoids.

Outcomes assessed were tumour volume changes from immediately before Tx1 and whether a full clinical response occurred, the latter determined by volume change considered jointly with expert assessment of photographs (Table 2). For each tumour that achieved an outcome of complete regression (CR; i.e., 100% reduction in tumour volume from before Tx1 to after last treatment), two experts reviewed available photographs documenting tumour appearance at critical time points (prior to last treatment, 7 and 28 days after last treatment and last available images) and reached consensus as to whether the treatment site was likely or not likely to be tumour-free as of the date that the last available images were recorded ('expert consensus'). Examples of these assessments are illustrated in Figure 1.

Five sarcoids became ineligible during the study either because treatments other than TT were used some time after their final TT treatment (Tx2 for 1 sarcoid; Tx3 for 2 sarcoids) or because the tumour volume increased to greater than 6 cm^3 (2 sarcoids). For the former tumours, post-treatment volumes after their final treatment were not available, but those tumours were known to be not

TABLE 2 Definition of treatment outcomes.

Outcomes	
Quantitative	$T_{vol\ regression}$ was calculated using repeat tumour volume measurements, using the following formula: $T_{vol\ regression} = \left(\frac{T_{vol\ end} - T_{vol\ start}}{T_{vol\ start}}\right) \times 100$ where: a. $T_{vol\ start}$ = Initial tumour volume. b. $T_{vol\ end}$ = Tumour volume after treatment. T_{vol} regression values were categorised as follows: a. 100% T_{vol} regression = Complete regression (CR). b. >30%; <100% T_{vol} regression = Partial regression (PR). c. >0%; <30% T_{vol} regression = No change in tumour volume – stable disease (SD). d. <0% T_{vol} regression = Progressive disease (PD).
Qualitative	*Tumour tissue presence*—expert consensus between two veterinary specialists (YE, blinded to trial details; RL unblinded) based on assessment of photographic images that treatment sites were likely or not likely to be tumour free on date of last available images (only performed for tumours with volume reduction 100% after last treatment).
Full clinical response	CR (T_{vol} regression 100%) *and* treatment site considered by expert consensus as likely to be tumour free at the time of last available images.

CR. For these 3 tumours, T_{vol} reductions were estimated assuming the volume was unchanged from before to after their final treatment. For the sarcoids whose volumes increased to greater than 6 cm^3 after treatments 1 and 2, respectively, those tumours were ineligible for further treatments.

2.1 | Adverse events

Reported adverse events (AE) defined as any observations that were unfavourable and unintended were reviewed (TDR; RL) to ascertain if they were likely related to treatment, and if so, whether they were associated with protocol aberrations or consistent with expected or known effects of TT administration. Expected known effects include mild to moderate inflammation, diffuse tissue swelling followed by tissue necrosis and slough of the target site and margins, regional lymphadenopathy, mild to moderate pain response on site palpation, and lethargy and reduced food intake lasting no longer than 3 days. Functional deficits arising from treatment of tumours on eyelid margins were not considered an AE given that tumour slough had been intended. Serious AEs were those that resulted in significant disability/incapacity compared with the initial presenting condition, those that required hospitalisation, were life threatening, or resulted in the death of the animal.

2.2 | Sample size calculations

Sarcoid sample size calculations were based on achieving the desired precision of estimated proportions of tumours that achieve CR with TT treatment. Assuming this would be either 50% or 75% and disregarding clustering of outcomes when treating multiple sarcoids in the same horse, for precision at the 95% confidence level of 20%, 19–25 sarcoids would suffice. In the presence of considerable clustering (intra-cluster coefficient 0.5) and assuming a mean of 2 tumours enrolled per horse, 27–36 sarcoids would be required. Consequently, 40 sarcoids were chosen as an appropriate sample size. These sample size calculations were performed using WinPepi Version 11.65 (Abramson, J.H. WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiologic Perspectives & Innovations 2011, 8:1).

Melanoma sample size determination was approached using a Simon's two-stage design as a proposed alternative to the sarcoid sample size method, given uncertainties of tumour responses at the lower dose rate (http://cancer.unc.edu/biostatistics/program/ivanova/simonstwostagedesign.aspx). In this design, the final number relied on the outcome of an initial trial, utilising 23 melanomas and a dichotomous outcome variable (volume reduction shortly after first treatment: yes/no). For this, the null hypothesis was that 75% of tumours would show volume reduction shortly after first treatment, the one-sided Type-1 error was 0.05, and 80% power was used for assessing the alternative hypotheses that 87.5% of tumours would show volume reduction shortly after first treatment. The full trial was to be abandoned if, in the initial phase, less than 18 tumours showed volume reduction shortly after first treatment. If fully executed, the trial was to include a total of 76 tumours. These calculations did not account for clustering of tumour within horse.

2.3 | Data analysis

All statistical analyses were performed using Stata (version 18, StataCorp). Five binary tumour-level outcomes were assessed to describe treatment efficacy: CR/Not CR after Tx1, Tx2, and Tx3; CR/Not CR after last treatment; full clinical response (i.e., CR and likely tumour free) after last treatment. For each outcome, 95% confidence intervals (CIs) for proportions of tumours that were CR were calculated using Stata's—proportion—command. Logit-transformed CIs were calculated using the robust standard error (i.e., the standard error derived from the Huber-White or sandwich estimator of variance) that accounted for clustering of tumour within horse.

Associations between possible determinants (for sarcoids: tumour type [nodular vs. fibroblastic], prior treatment, initial tumour volume and tumour location; for melanomas: initial tumour volume and tumour location) and each of three outcome variables (CR/Not CR after first treatment, CR/Not CR after final treatment; full clinical response) were assessed using univariable multilevel logistic models, fitted using Stata's—melogit—command. For full clinical response for melanomas, a multivariable model with initial tumour volume and

FIGURE 1 Example of photographic data reviewed by experts to ascertain if a sarcoid or melanoma achieving complete regression was likely tumour free (LTF; 1—Sarcoid A–D; 3—Melanoma I–L or not likely tumour free (NLTF; 2—Sarcoid E and F; 4—Melanoma M–P).

tumour location simultaneously fitted as fixed effects was also implemented. For all models, horse was fitted as a random effect, and maximum likelihood estimation was used. For sarcoids, due to near complete clustering of the outcome variables (in horses that contributed more than one sarcoid), mixed effects logistic models with horse set as a random effect did not converge. Consequently, for analyses of possible determinants of outcomes for sarcoids, only one sarcoid per horse was used. In each horse with more than one study sarcoid, one was chosen using a random number generator. To assess whether short times from treatment to assessment affected our conclusions about risk factors, we also assessed strengths of association between potential determinants and both CR after first treatment and CR after final treatment after excluding cases that did not have a minimum of 28 days between treatment and volume assessment. Results were similar and conclusions the same when cases that did not have a minimum of 28 days between treatment and volume assessment were excluded.

Predicted probabilities and 95% confidence intervals for the outcome event were calculated using the linear prediction from estimated fixed effects (coefficients) in the model with the random effect of horse set to the mean value of 0. Linearity in the logit for relationships between initial tumour volume and each outcome variable was first assessed using fractional polynomial regression with multilevel logistic models and from results of locally weighted regression of each outcome variable on initial tumour volume; results were plotted after transforming the dependent variable to logits; initial tumour volume was modelled either as a continuous or categorical variable depending on the presence of a linear relationship. Data were too sparse to meaningfully assess interaction between the possible determinants.

Analyses were performed as 'intention to treat', thus all initially eligible tumours were included regardless of whether or not retreatment was implemented for eligible tumours. For initially eligible tumours that became ineligible during the study period, treatment volumes after their last treatment before becoming ineligible were used as their final volumes.

3 | RESULTS

3.1 | Sarcoid trial population

Forty-one sarcoids in 30 horses were enrolled, met study inclusion criteria, were treated at least once, and remained compliant with the trial protocol at least until T_{vol} regression after Tx1 was assessed. 16/41 tumours received two, and 7/41 were administered three treatments while adhering to study protocols. Further detail on case enrolment, progression, and losses due to noncompliance are illustrated in Figure 2.

The 30 horses consisted of 13 geldings, 15 mares, and 2 stallions, with an average (min/max) age of 9.3 (2/25) years. Predominant breeds were Warmbloods and their crosses (8/30), Australian stock horses (6/30), quarter horses (5/30) and Thoroughbred horses (4/30). Twenty-two horses contributed one, five horses each contributed two, and three horses each contributed three study sarcoids. Thirty-four of the 41 sarcoids were confirmed histologically, and for the remaining seven tumours, the PCR for bovine papilloma virus (BPV) was positive. The majority of enrolled sarcoids were nodular sarcoids (33/41; 80%) and 9/41 (22%) sarcoids had received previous treatments. The 41 sarcoids were predominantly located on the horses' head (29/41; 71%) with most of those located in the periocular region (17/29; 59%).

Median (IQR; range) initial T_{vol} at Tx1 was 1.4 (2.3; 0.3–6) cm^3 with 61% (25/41) of participants having a volume of less than 2 cm^3 (Figure S1).

3.2 | Sarcoid trial outcomes

T_{vol} regression: This outcome variable was first assessed at median (IQR; range) 62 (91; 15–262) days after first treatment. In 32/41 sarcoids, T_{vol} decreased following Tx1 and 23/41 sarcoids (56%; 95% CI 36%–74%) showed CR. After Tx2 ($n = 16$ tumours), CR was achieved in four (25%; 95% CI 9%–54%). After Tx3 ($n = 7$ tumours), CR occurred in three (43%; 95% CI 9%–85%). Representing the cumulative effect of all treatments, T_{vol} regression was also assessed for each sarcoid from before Tx1 to after the last treatment [the latter assessed at median (IQR; range) 54 (40; 19–262) days after last treatment]. Of the 41 tumours, 30 (73%; 95% CI 53%–87%) were classified as CR (Figure 2, Table 3). Four tumours partially responded with T_{vol} reductions of 90%, 81%, 83% and 34%, and 5 tumours increased in volume with proportional increases of 1%, 3%, 51%, 110% and 1567% (Figure 3).

3.3 | Sarcoid tissue presence

Of the 30 sarcoids with CR after the last treatment, photographs of the treatment site after the last treatment were available for 25. For these 25 tumours, photographic images were recorded at a median (IQR; range) 546 (367; 84–1011) days after the final treatment. The second-shortest interval was 235 days after the final treatment. For 23 of these 25 tumours, the treatment site was classified by expert consensus as likely to be tumour free. For the remaining 2 tumours, a slightly hyperkeratotic treatment site raised concerns that the tumours had recurred or persisted. Thus, consequently, for 92% of tumours with the quantitative outcome of CR, subsequent expert consensus at least 84 days after the final treatment rated that the site was likely to be tumour free (23/25 CR with follow-up). A full clinical response occurred for 64% (23 of 36 sarcoids with follow up if CR; 95% CI 44%–80%) (Figure 2, Table 3).

3.4 | Determinants of sarcoid outcomes

Possible determinants of outcomes for sarcoids were assessed using univariable analyses. Sarcoids previously treated using other modalities prior to enrolment in this study were less likely than others not previously treated to be CR after final treatment (odds ratio 0.08; 95% CI 0.01–0.6; $p = 0.01$) and to have a full clinical response (odds ratio 0.04; 95% CI 0.00–0.45; $p = 0.009$).

There was some evidence that the odds of CR after Tx1 decrease as initial tumour volume increases (odds ratio for each 1cm^3 increase in initial volume 0.62; 95% CI 0.38–1.03; $p = 0.07$) and after final treatment (odds ratio 0.59; 95% CI 0.36–0.98; $p = 0.04$). Odds of a full clinical response also decreased as initial tumour volume increased (odds ratio 0.50; 95% CI 0.28–0.89; $p = 0.02$). Tumours that were initially 1 and 2 cm^3 had predicted probabilities of a full clinical response of 0.81 and 0.68, respectively, whereas tumours that were initially 4 and 5 cm^3 had predicted probabilities of a full clinical response of only 0.36 and 0.22, respectively (Table 4, additional analyses of determinants reported in Data S1 [Tables 1.1–1.3]).

3.5 | Melanoma trial population

Ninety-seven melanomas in 27 horses were enrolled, met study inclusion criteria, were treated at least once, and remained compliant with the trial protocol at least until T_{vol} after Tx1 was assessed. Twenty-two/97 received a second treatment, including 2/97 that received a third treatment while adhering to study protocols. Further details on case enrolment, progression, and losses are shown in Figure 4.

The population consisted of 15 geldings and 12 mares with an average (min/max) age of 14.6 (5/25) years. Predominant breeds were Thoroughbreds (8/27), Australian stock horses (4/27), and Arabians (4/27). The majority of horses (22/27) had a single melanoma treated.

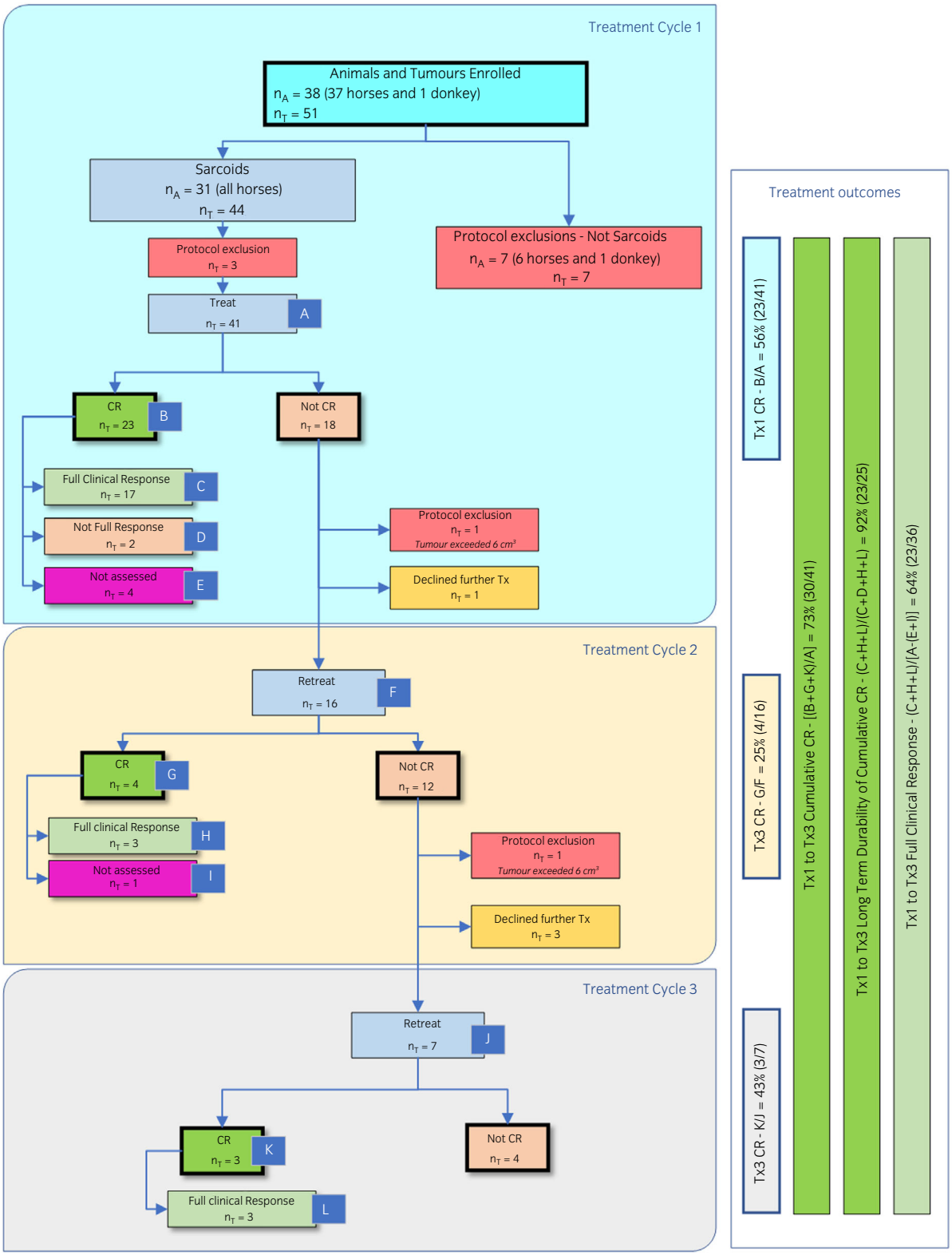

FIGURE 2 The progression of animals and fibronodular sarcoids treated in each treatment cycle of the study. n_A = number of horses and donkeys treated; n_T = number of tumours treated; CR = Complete regression, that is, 100% T_{vol} regression; Not CR = <100% T_{vol} regression. Full clinical response = CR *and* likely tumour free (LTF) by expert opinion; Not Full Response = CR but not likely tumour free (NLTF). Following Tx1, 23 tumours achieved CR (B); of these, 17 were classified as showing a full clinical response (C), and two were considered NLTF (D), 4 were not assessed due to being lost to follow-up or having no photographs (E). Following Tx2, four sarcoids achieved CR (G), with three showing a full clinical response (H), and the remaining case was not available for assessment (I). All three sarcoids that received a third treatment and were CR (K) also assessed as showing a full clinical response (L).

TABLE 3 Final outcomes after up to three intratumoural tigilanol tiglate administrations.

Tumour	Trial outcome variables	No. of tumours	%	Median time (days) between first treatment and respective assessment (IQR; range)
Sarcoids	CR after final treatment[a]	30/41	73	56 (46; 19–262)
	Full clinical response[b]	23/36	64	546 (367; 84–1011)
Melanomas	CR after final treatment[a]	72/97	74	167.5 (162; 28–531)
	Full clinical response[b]	55/90	61	247 (95; 156–531)

[a]CR (Complete T_{vol} regression) = 100% T_{vol} regression.
[b]Full clinical response = CR *and* treatment site considered likely to be tumour-free by expert consensus. Five sarcoids and 7 melanomas with CR but no photographs available for expert assessment were excluded from analyses of full clinical response.

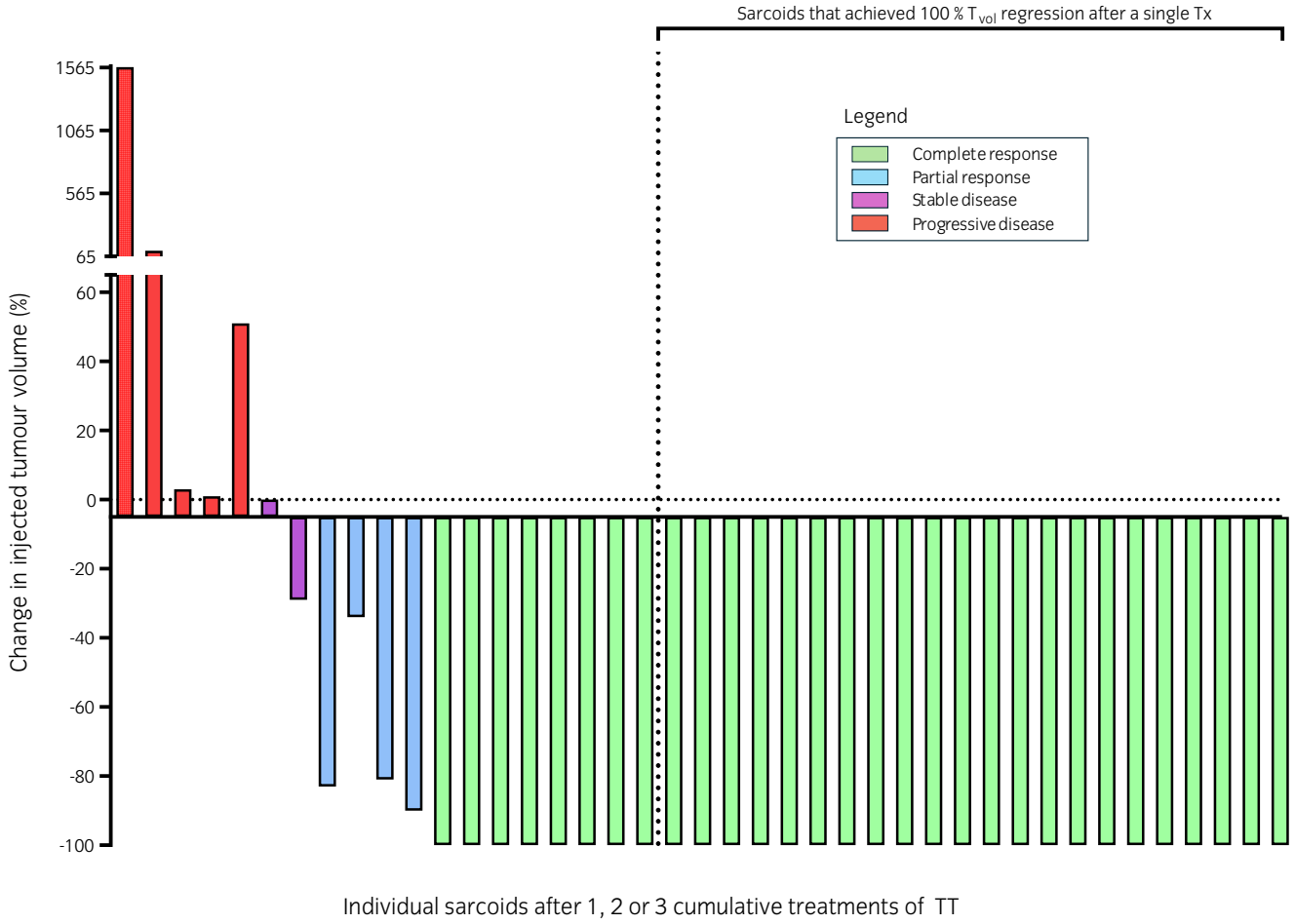

FIGURE 3 Waterfall plots showing the change in tumour volume (%) for 41 individual sarcoids following 1–3 treatments with tigilanol tiglate (TT). Efficacy after 1–3 TT treatments with all tumour volume regression calculations related to the initial tumour volume calculated prior to Tx1: 30/41 CR; 4/41 PR; 2/41 SD; 5/41 PD. Green = Complete Regression (CR; 100% T_{vol} regression). Blue = Partial regression (PR; <100 to 30% T_{vol} regression). Purple = Stable Disease (SD; 0 to <30% T_{vol} regression). Red = Progressive Disease (PD; <0% T_{vol} regression).

Nine horses had three tumours, four had four, and two had either two, six, or eight masses treated. Seven and nine tumours were enrolled in one horse each. Sixty percent (58/97) of all melanomas were located on the tail, 27% (26/97) were in the anal/perineal region, and 13% (13/97) were elsewhere on the body.

Median (IQR; range) T_{vol} at Tx1 was 0.7 (1.7; 0.01–10.4) cm^3 with 74% (72/97) less than 2 cm^3 (Figure S2).

3.6 | Melanoma trial outcomes

T_{vol} regression: Tumour volumes after Tx1 were assessed at a median (IQR; range) 60 (169; 14–531) days after that treatment (Figure 4, Table 3). In 89% (86/97) of melanomas, T_{vol} regressed following a single TT treatment, with 60/97 achieving CR (62%; 95% CI 51%–72%). Retreatment was not consistently implemented; only 22/37 (59%)

TABLE 4 Predicted probabilities and 95% confidence intervals of a full clinical response for various initial sarcoid volumes (A) and initial melanoma volume category by tumour location (B).

(A) Sarcoids	
Initial tumour volume (T_{vol}, cm^3)	**Predicted probability (95% CI) of a full clinical response**
1.0	0.81 (0.57–0.93)
2.0	0.68 (0.46–0.84)
3.0	0.52 (0.29–0.75)
4.0	0.36 (0.13–0.68)
5.0	0.22 (0.05–0.63)
6.0	0.13 (0.02–0.59)

(B) Melanomas				
	Predicted probability (95% CI) of a full clinical response by initial T_{vol} (cm^3) category			
Tumour location	**0.01 to <0.3**	**0.3 to <0.7**	**0.7 to <2.0**	**≥2.0**
Head/body	1.00 (0.85–1.00)	0.94 (0.41–1.00)	0.91 (0.34–0.99)	0.84 (0.31–0.98)
Perineal region	0.93 (0.41–1.00)	0.23 (0.04–0.70)	0.14 (0.01–0.68)	0.08 (0.01–0.50)
Tail	0.99 (0.71–1.00)	0.67 (0.26–0.92)	0.54 (0.16–0.87)	0.38 (0.09–0.79)

Note: Depending on the relationship with the outcome measure (linear or nonlinear on the logit scale), initial tumour volume was analysed as a continuous variable for sarcoids and a categorical variable for melanomas. For melanomas, treatment efficacy also varied by tumour location, so probabilities were predicted by initial T_{vol} category by location (assuming no interaction between location and T_{vol} category) using a multivariable model with both variables fitted as fixed effects.

tumours which did not achieve CR after Tx1 received a second treatment. Of these 22 tumours, CR occurred for 12 (55%; 95% CI 28%–79%). Only 2/10 tumours which did not show CR after Tx2 were treated a third time, with none achieving a CR outcome. Considering all three treatment cycles together, T_{vol} regression was also assessed for each melanoma from before Tx1 to after the last treatment [the latter assessed at median (IQR; range) 180 (182; 14–531) days after first treatment]. CR was observed for 72/97 melanomas (74%; 95% CI 62%–84%; Figure 4).

For the 17 melanomas with PR, T_{vol} reduced on average by 76% (median 84%; IQR 29%; range 34%–99.6%). Of the remaining eight melanomas, five were classified as having stable disease (SD) and three as having progressive disease (PD; Figure 5).

3.7 | Melanoma tissue presence

Expert consensus was available for 65 of the 72 melanomas that were CR after the last treatment. For these 65 tumours, photographic images were recorded at median (IQR; range) 247 (95; 156–531) days after the final treatment. Of these 65 tumours, 55 were judged to be likely tumour-free. Thus, for 85% of tumours with the quantitative outcome of CR, subsequent expert consensus at least 156 days after the last treatment was that the site was likely to be tumour free (55/65 CR with follow-up). A full clinical response occurred for 61% (55 of the 90 melanomas with photographic follow-up; 95% CI 52%–73%) (Table 3). All of the 10 tumours with CR but not considered a full clinical response (i.e., classified by expert consensus as not likely to be tumour-free) were located on the tail or in the perineal/anal area compared with 81% (45) of the 55 tumours with CR and a full clinical response.

3.8 | Determinants of melanoma outcomes

From univariable analyses, there was evidence that the odds of CR after Tx1 varied by initial tumour volume in Tx1, with tumours ≥2 cm^3 being less likely to completely regress after Tx1 than those <0.3cm^3 (crude odds ratio 0.17; 95% CI 0.04–0.63; $p = 0.01$). A full clinical response was strongly linked to both initial tumour volume and tumour location, with lower odds and probabilities of full clinical response for initially large tumours and for tumours in the perineal region relative to those on the head or body (Tables 4B and 5, further analyses conducted on determinants are reported in Data S1 [Tables 2.1–2.4]).

3.9 | Reported adverse events (AE)

Sarcoid trial: Four AEs were reported in three of 30 horses treated. All were likely attributable to TT administration, and all exclusively arose during the treatment of periocular sarcoids. Two events were considered serious AEs; in each, a dose greater than 1.5 mg of TT had been administered. Of the four AEs, two were attributable to protocol aberrations, representing inadequate use of nonsteroidal anti-inflammatory drugs (NSAIDs) and the injection of a TT dose

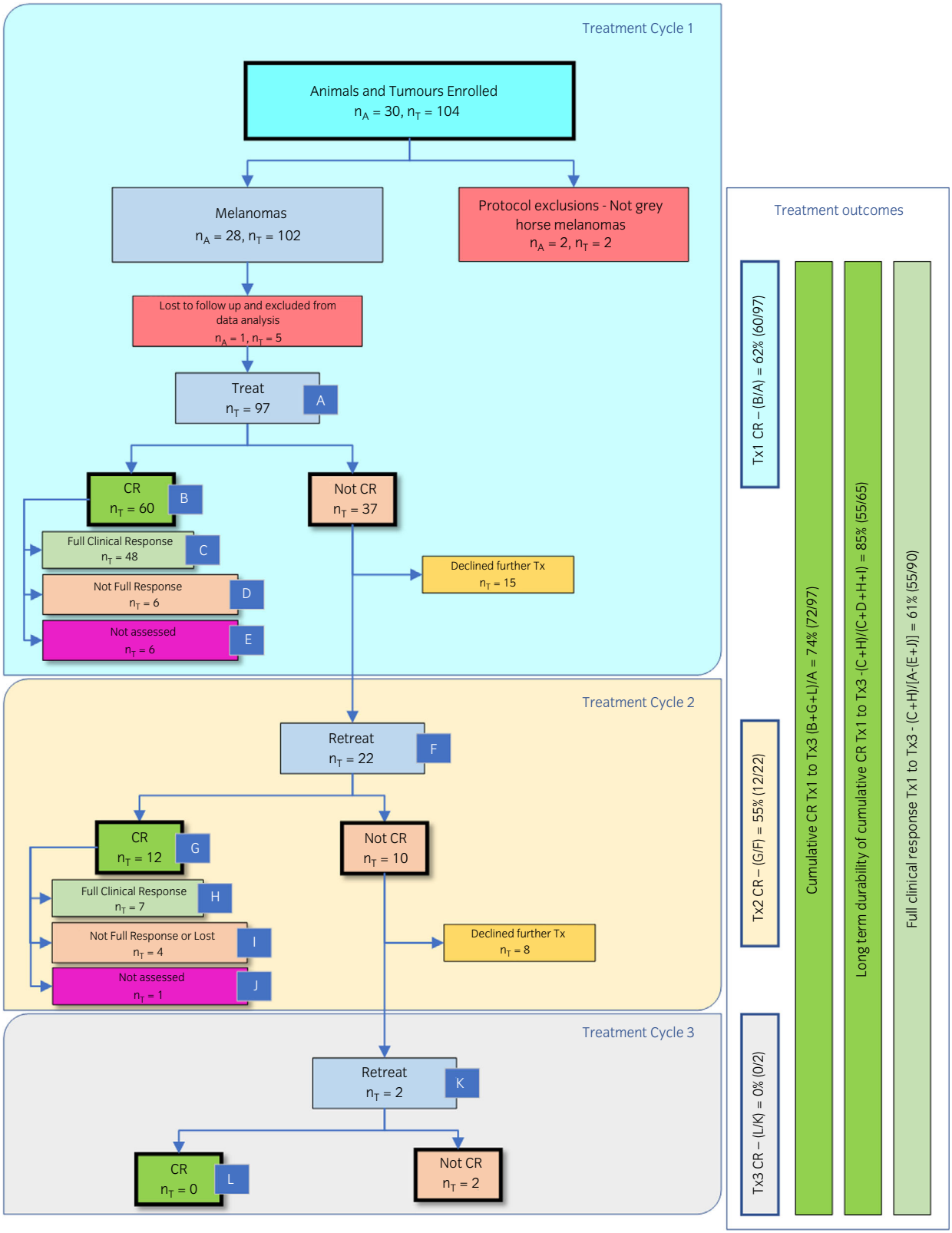

FIGURE 4 Progression of animals and melanomas treated in each treatment cycle. n_A = number of horses treated, n_T = number of tumours treated. CR = Complete regression, that is, 100% T_{vol} regression; Not CR = <100% T_{vol} regression. Full clinical response = CR *and* likely tumour-free (LTF) by expert opinion; NLTF = not likely tumour-free. Expert review post Tx1 determined that of 60 tumours recording CR (B), 48 were LTF (C), six were NLTF (D) and a further six were not able to be assessed, being lost to follow-up (E). Following Tx2, 12 of 22 melanomas achieved CR (G), seven were assessed as LTF (H), four as NLTF (I) and one melanoma was not reviewed, being lost to follow-up (J). No melanomas achieved CR post Tx3 (L).

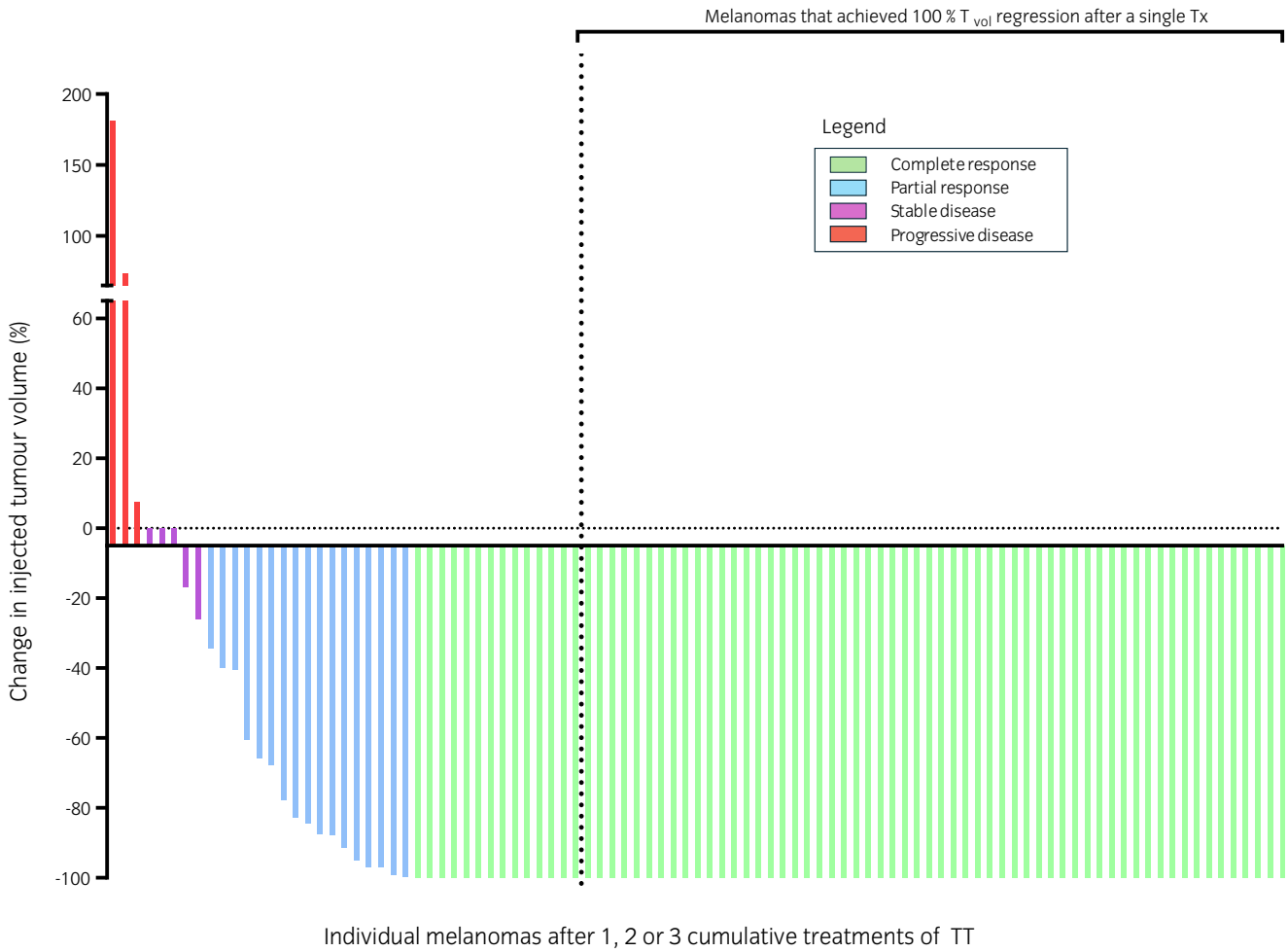

FIGURE 5 Waterfall plots showing the change in tumour volume (%) for 97 individual melanomas following 1–3 treatments with tigilanol tiglate (TT). Efficacy after 1–3 TT treatments with all tumour volume regression calculations related to the initial tumour volume calculated prior to Tx1: 72/97 CR; 17/97 PR; 5/97 SD; 3/97 PD. Green = Complete Regression (CR; 100% T_{vol} regression). Blue = Partial regression (PR; <100 to 30% T_{vol} regression). Purple = Stable Disease (SD; 0 to <30% T_{vol} regression). Red = Progressive Disease (PD; <0% T_{vol} regression).

exceeding the study limit. The two remaining AEs included regional lymph node abscessation and concurrent ipsilateral thrombophlebitis and a deep corneal ulcer developing subsequent to marked conjunctivitis and periocular swelling.

Melanoma trial: One AE was reported in the 97 melanomas treated. The AE occurred in a 13-year-old gelding that received treatment for an 8 cm^3 tumour between the tail base and anus. The subsequent swelling that occurred in the 2 days after treatment was more excessive than expected and resulted in delayed defecation and rectal impaction. It was successfully managed in hospital with the additional use of anti-inflammatory therapy and manual emptying of the rectum, including the use of rectal enemas.

4 | DISCUSSION

Given complete regression rates (73%–74% of tumours) and minimal adverse events observed (5/190 treatment interventions), it can be concluded that intratumoural tigilanol tiglate (TT) facilitates clinical outcomes in line with comparable treatment standards for equine sarcoids and melanomas.[6–9,28,29] Consequently, our hypothesis was accepted. Incorporating stringent quantitative and qualitative long-term outcome measures, this approach achieved a full clinical response rate of 61%–64% of tumours when followed for an average of eight (sarcoids) or 18 months (melanomas) post treatment. Although higher success rates have been reported using combination therapies, study differences, critical bias, and lack of power limit comparative analyses of treatment efficacy across published studies. With that in mind, radiotherapy, cryotherapy, and intralesional cisplatin or electrochemotherapy are considered the most effective published sarcoid treatments to date.[8] For melanomas, surgical excisions outperformed any medical treatment modality.[9] While this study provides relevant data on treatment outcomes, it does not permit within-study comparison of treatment efficacy, that is, between sarcoids and melanomas, as trials were not conducted in parallel and methodologies differed. The sarcoid study was the first to be designed, commencing a year earlier, thereby guiding the final design of the melanoma trial. Consequently, differences in treatment

TABLE 5 Adjusted odds ratios for full clinical response for melanomas by tumour location and tumour volume immediately prior to first treatment from a multivariable model with both variables fitted as fixed effects.

Exposure variable and level	No. tumours	% (no.) with full clinical response	Adjusted odds ratio[a]	95% CI	*P*-value[b]
Tumour location					**0.005**
Head/body	13	77% (10)	Reference category		
Perineal region	24	46% (11)	0.02	0.00–0.51	0.02
Tail	53	68% (36)	0.12	0.01–1.86	0.1
Initial tumour volume (cm^3)					**<0.001**
0.01 to <0.3	21	90% (19)	Reference category		
0.3 to <0.7	26	58% (15)	0.02	0.00–0.55	0.02
0.7 to <2.0	19	58% (11)	0.01	0.00–0.79	0.04
≥2.0	24	50% (12)	0.01	0.00–0.36	0.01

Note: Given its nonlinear relationship with the outcome measure, tumour volume was analysed as a categorical variable. The model includes all melanomas for which final T_{vol} information and expert follow-up were available ($n = 90$).

[a]Odds ratio estimates for each of location and initial tumour volume are adjusted for the other variable; estimated with horse fitted as a random effect.

[b]Bolded values are overall likelihood ratio test *p*-values for variables; unbolded values are Wald *p*-values for levels relative to the reference category.

protocol (in sarcoids dosing at 0.35 mg/cm^3 vs. in melanomas at 0.2 mg/cm^3), length of follow-up (for sarcoids a median more than twice as long as for melanomas) and in initial tumour volumes (for sarcoids a median three times larger than for melanomas) are expected to affect outcomes.

Recognition of residual or recurrent melanocytic tissue is straightforward due to the presence of indicative black pigment. In the absence of histopathology and biomolecular test results, recognition of residual or recurrent sarcoid tissue is more complex but generally appropriate, provided adequate clinician experience.[30] Irrespectively, given the potential absence of pathohistological findings and the presence of bovine papilloma virus genome in unaffected tissues, the validity of follow-up tests could be questioned.[31,32] In this investigation, a full clinical response was recorded when T_{vol} regression was 100%, and experts agreed that target sites were likely tumour free, thereby counterbalancing the absence of follow-up testing and the potential for measurement error. This approach also meant that tumours scoring CR but missing photographs for expert review could not achieve a full clinical response score. Consequently, the reported treatment efficacy is likely a conservative estimate. Nonetheless, the lack of an objective measure should be considered when interpreting the presented data.

Tigilanol tiglate, a PKC/C1 domain activator, causes vascular disruption and oncolysis by binding to a cell's endoplasmatic reticulum (ER) and initiating a stress response. This ER resident signalling pathway culminates in the generation of reactive oxygen species (ROS), the release of damage associated molecular patterns (DAMP), the loss of mitochondrial membrane potential, caspase activation, upregulation of pro-inflammatory cytokines and chemokines and tumour specific T-cell and innate immune cell recruitment.[18,33–35] This mode of action is not considered tumour or species specific as positive treatment effects have been observed in various applications[17,20,23,25,36] with CR rates being identical[21] and the potential for abscopal treatment effects.[36] Intratumoural use of immunogenic cancer therapeutics is an established treatment principle in horses. Administration of live or attenuated *Mycobacterium bovis* strain bacille Calmette-Guérin (BCG) is, depending on tumour location and type associated with relevant treatment efficacy.[28,37,38] Treatments are typically repeated three to four times, associated with inflammatory tissue responses and rarely anaphylactic responses are observed. However, depending on geographic location, BCG is not always readily available. More recently the efficacy of a commercial *Mycobacterium phlei* cell wall extract for use in equine sarcoids has been reported. With fewer cases enrolled and shorter and less structured follow-up, treatment efficacy was lower than reported here (53% vs. 64% of cases).[39] A similar approach but using complete Freund's adjuvant in equine melanomas, was associated with a modest clinical response (2/11 horses treated showing complete regression).[40]

Equine sarcoids and grey horse melanomas represent two of the most common tumour types in equine cutaneous neoplasia.[1,2] Despite their different aetiologies the treatment paradigm should be considered the same, that is, 'treat masses when they are small'. An argument for this treatment approach can be readily made: Successful outcomes are generally more likely with treatment of smaller sarcoids, an observation supported by this and work by others.[37,41] For melanocytic tumours progression follows an age dependent pattern with small masses expected to enlarge by both growth and confluence.[42] Consequently, a requirement to treat is likely and while radical resections can be successful, large tumour interventions are associated with increased treatment costs and greater risk of complications including recurrence.[43,44] Furthermore, results suggest that when masses are located within the tail/perineal region, an often widely affected area in study horses, outcomes are less likely to be successful. Irrespective of the logic of early tumour interventions, small masses often go unnoticed when dedicated skin checks are not performed or are deliberately ignored when stakeholders are deterred by the prospect of surgical interventions. Therefore, a change in both owner and veterinary behaviour is needed to enable regular

examinations of at-risk horses and subsequently early treatment interventions of small masses. Use of a 'chemical scalpel' in the form of an easily administered intratumoural therapeutic may facilitate this process. In the case of TT, the tissues' typical treatment response provides further benefit as a developing eschar and semi-occlusive wound cover protects healing deeper tissues and requires no specific wound care (Figure 1). The drug's mode of action is associated with a wide safety profile in comparison to standard cytotoxic agents in clinical practice, which adds to the practicality of its use in early and repeated interventions.[17,18,20,33,34,36] Adverse events nevertheless occurred and were by and large attributable to administration of excessive doses, inadequate prevention of ensuing inflammatory responses or an unexpected level of inflammation post treatment. The observed abscessation of a locoregional lymph node and ipsilateral thrombophlebitis were however unexpected especially since the affected vein had not been used for intravenous administrations. It could be speculated that lymphadenopathy occurred due to TT gaining access to relevant lymphatics or conversely the lymph node being overburdened by the level of target tissue oncosis. Thrombophlebitis on the other hand may have been caused by an inflammation dependent procoagulative disease process referred to as immunothrombosis aiming to limit pathogen spread.[45] Adverse events certainly emphasise the need for judicious use of anti-inflammatory medications. Initial experiences from sarcoid trials informed later protocol developments for melanoma treatment. Consequently, use of dexamethasone (0.04 mg/kg bwt/IV/SID) prior and as needed to after treatment was recommended in addition to a standard 3–5-day nonsteroidal anti-inflammatory regimen (Table S2). This adjustment proved well supported and better controlled ensuing reactions. Regardless, administrations around the eye, in the perineal/anal region or adjacent to a relevant vascular structure require diligent after care and monitoring to prevent more serious comorbidities. Likewise, alternatives to an undoubtedly imperfect administration method (i.e., fanning through tumour tissue) are expected to further efficacy by establishing truly equal tissue distribution. Currently ongoing developments focusing on dose reductions and precision in dose administration are expected to further the utility of intratumoural TT administration by better harnessing required tissue responses.

It is acknowledged that follow-up assessment times were not tightly controlled, a reality of clinical trials in client-owned animals. However, for those tumours that were classified as achieving a full clinical response (i.e., complete tumour volume regression (CR) and likely tumour free) the latter expert assessment was conducted a median of 490 (sarcoids) and 80 (melanomas) days after concluding that their tumour had been successfully ablated. This ensured that, if tumour tissue was still present at the site but had not caused a mass to reform, there was considerable time for disease to progress and to be recognised by experts. The difference in sarcoid and melanoma follow-up times can be justified and explained by the ease with which residual melanocytic tissue is identified and treatment success determined. This means that tumours showing a full clinical response were unlikely to be misclassified and outcomes were not expected to be affected by inherent variability in follow-up time.

Lastly, acknowledging additional limitations, this work did not include a placebo group. In oncology, the provision of this level of control is often unattainable due to the negative impact of withholding treatment or treating in an ineffective manner, particularly when considering sarcoid behaviour. Consequently, efficacy is compared against current clinical outcomes which, in light of the aforementioned shortfalls, are not without their own limitations.[41,46,47] It should further be acknowledged that our analyses were based on the intention to treat and that eligible tumours not presenting for retreatment were classified as treatment failures. Considering that this more typically occurred in the melanoma trial, one could speculate that the proportion of tumours with positive outcomes is likely higher under 'per protocol' management (i.e., where all non-CR tumours receive a further treatment to a maximum of 3 treatment) than those reported in the current study. Finally, readers are reminded that reported results should be interpreted in consideration of inclusion criteria, and uniform validity to all sarcoids or melanomas cannot be inferred by this work.

In summary, intratumoural tigilanol tiglate administration results in a good clinical response rate, especially in initially smaller tumours. When complete resolution occurred for sarcoids and melanomas, respectively, this typically persisted for at least 18 and 8 months after the first treatment. Repeat treatment cycles, either of the same or different growths but in the same horse, were well tolerated. Taken together, with the greater efficacy in smaller masses, this treatment approach would lend itself well to early and ongoing interventions.

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CONFLICT OF INTEREST STATEMENT

R. Labens serves as a scientific consultant for QBiotics Group Ltd., for which a salary is received. T. De Ridder and C. McGee are employees of QBiotics Group Ltd. P. Reddell is an Executive Director and Chief Scientific Officer of QBiotics Group Ltd. The remaining authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

R. Labens: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; supervision; resources; data curation; project administration. **C. Saba:** Investigation; writing – review and editing; validation; supervision; resources. **J. Williams:** Supervision; resources; investigation; writing – review and editing; validation. **A. Hollis:** Investigation; writing – review

and editing; validation; supervision; resources. **J. Ensink:** Investigation; writing – review and editing; validation; resources; supervision. **E. L. V. José-Cunilleras:** Investigation; writing – review and editing; validation; supervision; resources. **M. Jordana-Garcia:** Investigation. **K. Bergvall:** Investigation; writing – review and editing; validation; supervision; resources. **M. Ruppin:** Investigation; writing – review and editing; supervision; resources; validation. **F. Condon:** Supervision; resources; validation; investigation; writing – review and editing. **C. Spelta:** Investigation; validation; writing – review and editing; supervision; resources. **Y. Elce:** Validation; writing – review and editing. **T. De Ridder:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; project administration; data curation; supervision; resources. **J. Morton:** Formal analysis; writing – review and editing; data curation; software; methodology; validation; visualization. **C. McGee:** Investigation; writing – review and editing; project administration. **P. Reddell:** Writing – review and editing; conceptualization; funding acquisition.

DATA INTEGRITY STATEMENT

R. Labens had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

ETHICAL ANIMAL RESEARCH

This study was approved by the Animal Care and Ethics Committee, Charles Sturt University, Australia (A21014 and A21394); the Clinical Research Committee and Hospital Board, University of Georgia, USA (07012021); Department of Agriculture and Fishers (DAF) Community Access Animal Ethics Committee, Malanda Tableland Veterinary Service and Clermont Veterinary Clinic, Australia (SA 2021/03/778); University of Cambridge Ethics and Welfare Committee, UK (CR521); Jordbruksverket, Sveriges lantbruksuniversitet (SLU)—Uppsala Veterinary School, Sweden (5.8.18-10515/2021); Comisión de Ética en la Experimentación Animal y Humana (CEEAH), Universitat Autònoma de Barcelona, Spain (CEEAH 5594); and the Animal Welfare Body, Utrecht University, The Netherlands (5204-3-2).

INFORMED CONSENT

Signed informed consent was obtained from owners of each animal at study entry.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14502.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by editor for this clinical report.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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