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ORIGINAL PAPER

Distribution of endocannabinoid system receptors in the equine hoof: dysregulation as a potential therapeutic target for laminitis

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Abstract

A growing body of evidence indicates that the endocannabinoid system (ECS) is essential for controlling the homeostasis of the skin and that the ECS is modified in the presence of skin disease. It is plausible to expect that the lamellar junction of the hoof expresses cannabinoid receptors and that their expression could be affected by lamellar disease. The goal of this study was to characterise the cannabinoid receptor type 1 (CB1R) and type 2 (CB2R) and the G protein-coupled receptor 55 (GPR55) within the dermo-epidermal junction of the hooves of healthy and laminitic horses. The expression of the CB1R, CB2R, and GPR55 within the dermo-epidermal lamellar junction of six healthy and 12 laminitic hooves was studied using polymerase chain reaction (PCR) and immunofluorescence. Both the mRNA and protein expression of the CB1R, CB2R, and GPR55 were found in the dermo-epidermal lamellar junction of horse hooves. The immunolabelling was expressed by the epithelial cells of the primary and secondary laminae of healthy hooves (CB2R > GPR55 > CB1R). The presence of CB1R, CB2R, and GPR55 immunoreactivity in the healthy laminar epithelial cells, coupled with increased protein expression in pathological epithelial cells, provided strong motivation for future investigation. These findings suggest that cannabinoid compounds which interact with these receptors may influence lamellar healing and mitigate inflammation in hoof diseases, particularly laminitis.

Keywords Cannabinoid receptors · *Cannabis* sp. · Lamellae · Epidermal lamellae · Horse

Introduction

The hoof is a complex structure crucial for weight-bearing, locomotion, and the overall health of horses. Hoof diseases, such as laminitis, cause significant morbidity and mortality, impacting animal welfare and the economy of the equine industry. The laminar junction, connecting the distal phalanx to the inner hoof wall, supports the entire weight of the horse.

Laminitis is a severe disease characterised by inflammation, degeneration, and damage to the hoof lamellae (Pollitt 2010). Its pathogenesis is complex, involving metabolic, endocrine, gastrointestinal, and systemic inflammatory factors. Despite advances in understanding laminitis, treatment remains challenging, highlighting the need for new therapeutic targets. The hoof lamellae share anatomical components with mammalian skin, including the epidermis, dermis, and basement membrane (BM) (Leise 2018).

Investigating the endocannabinoid system (ECS) as a potential therapeutic target for laminitis is promising, given its role in skin physiology and pathology (Ligresti et al. 2016). The ECS includes endogenous ligands (anandamide and 2-arachidonoyl glycerol), cannabinoid receptors (CB1R and CB2R), and enzymes for ligand degradation and recycling (Silver 2019). Cannabinoid-related receptors, such as GPR55 (Iannotti et al. 2016; Morales et al. 2017), and endocannabinoid-like molecules such as palmitoylethanolamide also play a role in ECS functioning (Gabrielsson et al. 2016; Petrosino and Di Marzo 2017). These receptors can be activated by exogenous phytocannabinoids, such as Δ-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Ligresti et al. 2016). A recent study has shown that the horse epidermis expresses the messenger RNA (mRNA)

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of CB1R and CB2R and cannabinoid receptor immunoreactivity (Kupczyk et al. 2022). The ECS has been investigated in equine sensory neurons (Chiocchetti et al. 2021; Galiazzo et al. 2022; Zamith Cunha et al. 2023a), the intestine (Galiazzo et al. 2021), and synoviocytes (Zamith Cunha et al. 2023c). Its role in chronic skin diseases suggests that it could influence hoof lamellae (Blázquez et al. 2006; Kupczyk et al. 2009; Scheau et al. 2020; Chiocchetti et al. 2022).

The aim of this study was to describe the distribution and expression patterns of CB1R, CB2R, and GPR55 in healthy and laminitic hooves. Particular attention was given to the epidermal cells of the dermo-epidermal junction, as this interface plays a central role in the structural integrity of the hoof and is one of the primary sites affected during the development and progression of laminitis, focusing on the basal cells and supra-basal cells of the epidermal component of the lamellar junction. We hypothesised that these receptors would be expressed in the specific lamellar cell types and that modulating their activity could be a therapeutic target for laminitis.

Material and methods

Animals

Inclusion criteria

In the group having healthy lamellae (HL), the horses were selected by age (range 1–5 years of age), with no signs of lameness in the thoracic or pelvic limbs, no history of laminitis, and, based on a clinical summary visit prior to slaughter, normal results of a complete blood count (CBC) and routine serum biochemical analyses. Sanitary history was evaluated by tracking health data. No macroscopic lesions or defects were present in the hooves.

The group having laminitic lamellae (LL) included horses with a clinical history/diagnosis of acute (ALL) or chronic (CLL) laminitis. Acute laminitis was defined as a clinical presentation occurring within a maximum of 72 h from the onset of clinical signs, with no prior history of laminitis and no evidence of structural failure. Chronic laminitis was defined as a condition persisting for more than 72 h after the onset of primary clinical signs, with structural failure and/or a history of recurrent laminitic episodes. The hooves were selected immediately post-mortem with or without the presence of macroscopic lesions and defects (coronary band deformation, sinking of the third phalanx, third phalanx rotation, excessive growth of the heels), with histopathological findings of laminitis, such as separation of the BM, marked irregular hyperplasia of the epidermal lamellae, irregular shape of the secondary lamellae, and inflammatory infiltrate (Kawasako et al. 2009).

Samples were collected from horses slaughtered for human consumption at the Zerbini Giorgio and Ragazzi Maria Grazia Slaughterhouse (Correggio, Reggio Emilia, Italy) and from horses being treated at the Equine Hospital of the University of Bologna that were euthanised for animal welfare reasons.

Sample collection and processing

Hooves were collected from the thoracic limbs of six healthy horses included in the HL group (control), with ages ranging from 15 to 24 months (mean: 18 months; SD ±3 months), which were slaughtered for consumption. The horse breeds included two half-blood trotters, two trotters, and two mixed breeds, in which all the hooves collected were macroscopically intact.

Hooves were collected from the thoracic limbs of 12 laminitic horses included in the LL (laminitic lamellae) group, which were euthanised for animal welfare reasons, with ages ranging from 3 to 12 years (mean: 7 years; SD ±4 years). The horses included four mixed breeds, two warmbloods, four trotters, and two Haflingers. The horses were subdivided into two subgroups: the acute laminitic (ALL) horses (4/12), and the chronic laminitic (CLL) horses (8/12).

According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 regarding the protection of animals used for scientific purposes, Italian legislation (D. Lgs. n. 26/2014) did not require any approval by competent authorities or ethics committees, as this study did not influence any therapeutic decisions.

The digits were disarticulated at the metacarpophalangeal joint; lamellar tissues, including epidermal and dermal lamellae, were obtained by sectioning the hooves with a band saw according to the protocol described by Pollit (1996) and Douglas and Thomason (2000). Tissue processing started a maximum of 2 h after death. The tissues were then fixed and processed as follows in order to obtain cryosections. Lamellar tissues were fixed for 48 h at 4 °C in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2). The tissues were subsequently rinsed overnight in phosphate-buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and stored at 4 °C in PBS containing 30% sucrose and sodium azide (0.1%). The following day, tissues were transferred to a mixture of PBS/30% sucrose/azide and optimal cutting temperature (OCT) compound (Sakura Finetek Europe, Alphen aan den Rijn, Netherlands) at a ratio of 1:1 for an additional 24 h before being embedded in 100% OCT in Cryomold® (Sakura Finetek Europe). The sections were prepared by freezing the tissues in isopentane cooled in liquid nitrogen. The cryosections (14 µm thick) of the lamellar tissues were cut on a cryostat and mounted on polylysinated slides; 10 slides ($n = 10$) were made for each single staining receptor, and an additional three slides

($n = 3$) were made for each co-localisation for each horse. The cryosections were oriented transversely to the major axis of the horse digit and included the primary (PEL) and secondary epidermal lamellae (SEL) (Fig. 1), a portion of the innermost layer of the hoof capsule, and a portion of the sub-lamellar dermal tissue, the last being necessary for identifying the receptors studied in the fibroblasts, the inflammatory cells/immunocytes, and the blood vessels. Three cryosections (20 µm thick) for each sample were washed in PBS and stored at −80 °C for gene expression analysis. Each round of molecular reactions was carried out with all subjects and receptor by receptor (i.e., 16 tissues stained for CB1r at once; 16 tissues stained for CB2r at once).

Histology

Tissue cryosections from each horse were stained with haematoxylin and eosin for histopathological examination to assess the lamellar damage. Histopathological examinations included separation of the BM, marked irregular hyperplasia of the epidermal lamellae in the SEL, the irregular shape of the secondary lamellae, and the presence of inflammatory infiltrate (Kawasako et al. 2009).

Immunohistochemistry on cryosections

Cryosections from 18 horses (6 HL and 12 LL) were hydrated in PBS. Endogenous peroxidase was blocked by immersion in 3% H_2O_2 in methanol for 30 min at room temperature (RT) (22–25 °C), followed by rehydration. The blocking of non-specific antigenic sites was achieved by incubating the slides in a 3% solution of bovine serum albumin (Sigma-Aldrich, Milan, Italy) and 0.25% Tween 20 (Sigma-Aldrich, Milan, Italy) for 30 min at RT and were then incubated overnight in a humid chamber at 4 °C

Table 1 Primary antibodies used in the study

Primary antibodies	Host	Code	Dilution	Source
CB1R	Rabbit	ab23703	IF 1:100 IHC 1:300	Abcam
CB2R	Mouse	sc-293188	IF 1:50	Santa Cruz
CB2R	Rabbit	PA1-744	IF 1:250 IHC 1:400	Thermo Fisher
GPR55	Rabbit	NB110-55498	IF 1:200 IHC 1:400	Novus Biol

Primary antibody suppliers: Abcam, Cambridge, UK; Novus Biologicals, Littleton, CO, USA; Santa Cruz Biotechnology, Dallas, TX, USA; Thermo Fisher Scientific, Waltham, MA, USA. *IF* immunofluorescence; *IHC* immunohistochemistry

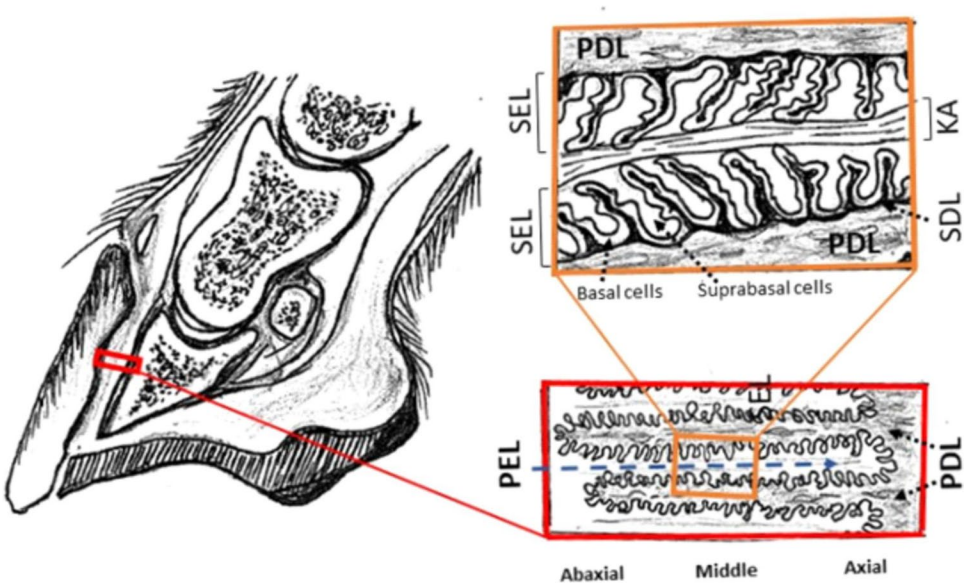

Fig. 1 Schematic representation of the anatomical structure of the lamellar tissue in the equine hoof. Left: a cross-sectional sagittal drawing of a horse's foot illustrating the location of the sampling, and the transverse section plane utilised for the tissue collection in relation to the hoof wall and the distal phalanx (DP). Bottom right: schematic sketch of the cross-sectional plane of the sample. The primary epidermal lamellae (PEL) and the primary dermal lamellae (PDL) are highlighted. Their positions in relation to the skeletal axis are indicated as axial (closest to the DP), middle, and abaxial (closest to the hoof wall). Top right: a drawing illustrating the organisation of the lamellar tissue at higher magnification. Dermal tissue, primary dermal lamellae (PDL) and secondary dermal lamellae (SDL) are observed

with the primary antibodies diluted in the blocking solution (Table 1). The slides were rinsed in Tris buffer and were then incubated with secondary biotinylated anti-rabbit immunoglobulin G (IgG) antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in the blocking solution. After two washes in Tris buffer, immunoreactions were detected with avidin–biotin immunoperoxidase (Colorado Serum Co., Denver, CO, USA) for 30 min and then, after two washes in Tris buffer, visualised with 3,3′-diaminobenzidine chromogen (Histo-Line Laboratories, Pantigliate, Milan, Italy). The slides were counterstained with Harris hematoxylin (Vector Laboratories, Burlingame, CA, USA), dehydrated, and permanently mounted using Bio Mount HM (Bio-Optica, Milan, Italy). The images were acquired using an optical microscope (Nikon, Shinjuku, Japan) equipped with an Imaging Source 33 Series USB 3.0 camera (cat. no. DFK 33UX264, Bremen, Germany). Neurons present in the trigeminal ganglion of a healthy horse were used as positive control (Zamith Cunha et al. 2023b). Each round of molecular reactions was carried out with subjects and receptor by receptor (i.e., 16 tissues stained for CB1r at once; 16 tissues stained for CB2r at once).

Immunofluorescence on cryosections

The cryosections from 18 horses were hydrated in PBS and processed for immunostaining. To block non-specific binding, the sections were incubated in a solution containing 20% normal donkey serum (Colorado Serum Co., Denver, CO, USA), 0.5% Triton X-100 (Sigma-Aldrich, Milan, Italy), and bovine serum albumin (1%) in PBS for 1 h at RT. The cryosections were incubated in a humid chamber overnight at RT with the antibodies directed against CB1R, CB2R, and GPR55 (single immunostaining) or with a cocktail of primary antibodies (double immunostaining) (Table 1) diluted in 1.8% NaCl in 0.01 M PBS containing 0.1% sodium azide. After washing in PBS (3 × 10 min), the sections were incubated for 1 h at RT in a humid chamber with the secondary antibodies (Table 2) diluted in PBS. The cryosections were then washed in PBS (3 × 10 min) and mounted in buffered glycerol at pH 8.6 with 4′,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each round of molecular reactions was carried out with subjects and receptor by receptor (i.e., 16 tissues stained for CB1r at once; 16 tissues stained for CB2r at once).

The preparations were examined using a Nikon Eclipse Ni microscope equipped with a filter system providing excitation light at a wavelength of 360 nm to reveal blue DAPI nuclear labeling (Set Filter EX361-389, DM415, BA430-490). The microscope was also equipped with filter cubes for distinguishing between fluorescein isothiocyanate (Set Filter FITC EX 465-495, DM505, BA512-558, 450–490 nm) and Alexa 594 (TRITC 530–585 nm excitation filter and 615 nm emission filter, DM570) fluorescence. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Correction of chromatic aberrations was performed with a described algorithm in the NIS software (Kozubek and Matula 2000). The figure panels were prepared using CorelDRAW (Corel Corporation/Alludo, Ottawa, ON, Canada). For the published version, slight brightness and contrast adjustments were made using Corel Photo-Paint.

Specificity of the primary antibodies

The specificity of the primary antibody anti-CB1R utilised in the current study had been validated on rat (Chiocchetti et al. 2019) and human (Galiazzo et al. 2021) tissues, tested on horse tissue using western blot (WB) analysis (Chiocchetti et al. 2021), and by immunohistochemistry in the trigeminal ganglion of the horse (Zamith Cunha et al. 2023b).

The rabbit anti-CB2R antibody (PA1-744) utilised in the current study for immunohistochemistry and some of the immunofluorescence reactions (Table 1) had previously been tested on horse tissue using WB analysis (Kupczyk et al. 2022). For double immunofluorescence, another anti-CB2R antibody, raised in mice (sc-293188), was also used, the specificity of which had not been tested on horse tissue; however, both the mouse and rabbit anti-CB2R antibodies were tested using a double-staining protocol and were co-localised in horse tissue (Supplementary item 1).

The rabbit anti-GPR55 antibody (NB110-55498) utilised in the current study had recently been used on horse tissue (Zamith Cunha et al. 2023d). The immunogen used to obtain the anti-GPR55 antibody was a synthetic 20-amino-acid peptide from the third cytoplasmic domain of human GPR55 in

Table 2 Secondary antibodies used in the study

Secondary antibodies	Host	Code	Dilution	Source
Anti-mouse IgG Alexa-594	Donkey	A-21203	1:500	Thermo Fisher
Anti-rabbit 488	Donkey	A-21206	1:1000	Thermo Fisher
Anti-rabbit biotinylated	Goat	BA-1000	1:200	Vector laboratories

Secondary antibody suppliers: Thermo Fisher Scientific, Waltham, MA USA; Vector laboratories, Newark, CA, USA

amino acids 200–250. The homology between the full amino acid sequences of the horse and human GPR55 was 80%, and the correspondence with the specific sequence of the immunogen was 78% (https://www.uniprot.org/, accessed 7 January 2022). This antibody, which was recently used on horse sensory neurons of the ileum by immunofluorescence (Galiazzo et al. 2021) and in the trigeminal ganglion of the horse by immunohistochemistry (Zamith Cunha et al. 2023b), had previously been tested on rat and dog dorsal root ganglia (DRG) using immunofluorescence (Chiocchetti et al. 2019) and on mice tissue using WB analysis (Galiazzo et al. 2018).

Specificity of the secondary antibodies

The specificity of the secondary antibodies was tested by applying them on the sections after the omission of the primary antibodies. No stained cells were detected after omitting the primary antibodies.

Qualitative and quantitative analysis: real-time polymerase chain reaction (RT-PCR) for *Cn1r*, *Cn2r*, and GPR55, immunofluorescence and immunohistochemistry for CB1R, CB2R, and GPR55

For gene expression analysis, total mRNA extraction was carried out using an RNeasy FFPE [formalin-fixed/paraffin-embedded] Kit (Qiagen, Hilden, Germany), with a few modifications. Three cryosections of 14 µm for each sample ($n=4$ HL group; $n=4$ ALL group; $n=8$ CLL group) were defrosted and briefly centrifuged in 240 µl of Buffer PKD [Proteinase K Digestion Buffer], followed by the addition of Proteinase k (10 µl). The samples were incubated at 56 °C for 3 h; every 30 min, the samples were mixed/homogenised using a micro-pestle (Eppendorf, Hamburg, Germany). The samples were then incubated at 80 °C for 15 min, and the manufacturer's protocol was followed up to elution (20 µl). After spectrophotometric quantification, the total RNA (1000 ng) was reverse-transcribed to complementary DNA (cDNA) using 5× iScript RT Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a final volume of 20 µl. To evaluate the gene expression profiles, RT-PCR was carried out in a CFX96 thermal cycler (Bio-Rad Laboratories, Inc.) using SYBR green detection to target the genes. Specific primers for horses (Zamith Cunha et al. 2023d) were used to evaluate the gene expression for cannabinoid receptors 1 and 2 (*Cn1r* and *Cn2r*), and G protein-coupled receptor 55 (*GPR55*). The reference genes, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *HPRT* (hypoxanthine phosphoribosyltransferase 1), and *ACTB* (beta actin) were based on horse sequences as reported previously (Zannoni et al. 2014). All the amplification reactions were carried out in 20 µl aliquots and analysed in duplicate; the reaction contained 10 µl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc.), 0.8 µl of the forward and reverse primers (5 mM each) of each target gene, 2 µl cDNA, and 7.2 µl of water. The real-time procedure included an initial denaturation period of 3 min at 95 °C, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s, followed by a melting step with ramping from 55 °C to 95 °C at a rate of 0.5 °C/10 s. The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis. The relative expression of the genes of interest (IGs) were normalised based on the geometric mean of the three reference genes (RGs). The relative mRNA expression of the genes tested was evaluated using the ΔC_t method with $\Delta C_t = (C_t$ geometric mean reference gene $- C_t$ gene of interest), which correlated directly with the expression level (ΔC_t values very negative, lower expression; ΔC_t values less negative higher expression).

For immunofluorescence investigations, the intensity of the expression of CB1R, CB2R, and GPR55 in the primary (PEL) and secondary epidermal lamellae (SEL) was quantitatively analysed. Five random photomicrographs of the PEL at the abaxial portion and five random sections of the PEL at the axial portion of each horse were acquired at high magnification (400×) using the same exposure time for all the images and analysed blindly by two independent observers for positive cellular immunoreactive staining. ImageJ software (version 1.52t, National Institutes of Health, Bethesda, MD, USA) was used to analyse the signal intensity of each image using the colour histogram (gMEAN) tool from the software.

The immunohistochemical reactivity of the antibodies was evaluated, and the cellular localisation of the immunohistochemical positivity for each antibody was assessed as membranous and/or cytoplasmic and/or nuclear. The intensity of the expression was assessed as faint, moderate, or strong when more than 70% of the lamellae showed this type of positivity based on microscopic observation.

Statistical methods

For gene expression data (ΔC_t values), the normal distributions were evaluated using Shapiro–Wilk tests and Kolmogorov–Smirnov tests, and depending on the results, a parametric statistical test was performed. Comparisons between the groups were carried out using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. A p-value ≤ 0.05 was considered significant (GraphPad Prism software, version 8.3, La Jolla, CA, USA).

For each cannabinoid receptor investigated using immunofluorescence, five photos were obtained from each region of the PEL (abaxial and axial) on five different slides per horse. The normality of the data was assessed using both

the Shapiro–Wilk and Kolmogorov–Smirnov tests, with p-values > 0.05 considered indicative of a normal distribution (Table 3). Initially, comparisons between the regions for each receptor in the HL group were carried out using a paired t-test, with a 95% confidence interval (CI) and a significance threshold of $p < 0.05$. If differences were observed within the control group, subsequent comparisons were carried out with the diseased group by comparing each region with its respective region. In the absence of differences within the control group, comparisons were carried out considering the entire PEL as one. After confirming normality, the data were analysed for comparisons between control and diseased groups, using an ordinary one-way ANOVA followed by Tukey's multiple comparisons test, with a 95% CI and a significance level of $p < 0.05$.

Results

Histology

Based on the histological examination, it was possible to confirm the healthy and the pathological status of the two groups (HL and LL) by evaluating the presence of the histomorphological features, including separation of the BM, marked irregular hyperplasia of the epidermal lamellae, the irregular shape of secondary lamellae, and the inflammatory infiltrate (Fig. 2). In addition, two horses were excluded from the control group due to the presence of multiple focal lesions on the laminar junction, even though they did not present clinical signs or a history of laminitis; therefore, only four horses were considered for the control group .

RT-PCR for *Cn1r*, *Cn2r*, and *GPR55*

Quantitative PCR data demonstrated that *Cn2r* was detected in all samples of the epidermal–dermal lamellar junction in the HL (4/4) and the ALL (4/4) groups, whereas in the CLL group, *Cn2r* was detected in only five samples (5/8). The *GPR55* transcripts were detected in almost all the samples in the HL group (3/4) and in all the samples in the ALL group (4/4), whereas in the CLL group, GPR55 was detected in only three samples (3/8). The transcript of *Cn1r* was detected in only a few samples (2/4 CLL; 1/4 ALL; 1/8 LL). As reported in Fig. 3, gene expression for both *Cn2r* and *GPR55* in the acute laminitic lamellae group (ALL) was significantly lower than in the CLL (chronic laminitic lamellae) and HL (control) groups (Table 4).

Immunohistochemistry on cryosections

Cannabinoid receptor 1

In the HL group, CB1R-IR was expressed from faintly to moderately by the cytoplasm of the SEL epidermal cells. More specifically, 50% (2/4) of the healthy lamellae showed faint CB1R-IR, and the other 50% (2/4) showed moderate CB1R-IR (Figs. 4a, 5). In the LL group, 33% (4/12) showed faint CB1R-IR, 58% (7/12) showed moderate CB1R-IR, and 9% (1/12) showed strong CB1R-IR in the epidermal cells (Figs. 4b, 5).

Cannabinoid receptor 2

In the HL group, CB2R-IR was moderately to strongly expressed by the cytoplasm of the epidermal cells of the PEL and the SEL (Figs. 4c, 5). More specifically, in 50% (2/4) of the HL group, CB2R-IR was moderate, and in the other 50% (2/4) it was strong. In the LL group, 56% (7/12) of CB2R-IR was moderate, and in the remaining 44% (5/12) it was strong (Figs. 4d, 5).

Table 3 Normality test results for immunoreactivity (IR) and RT-PCR values

Receptor	Test	Statistic	p-value	Passed the normality test (alpha = 0.05)?
CB1R–IR	Shapiro–Wilk	W = 0.9324	0.2960	Yes
	Kolmogorov–Smirnov	KS = 0.1601	> 0.1000	Yes
CB2R–IR	Shapiro–Wilk	W = 0.9259	0.2371	Yes
	Kolmogorov–Smirnov	KS = 0.1793	> 0.1000	Yes
GPR55–IR	Shapiro–Wilk	W = 0.9406	0.0946	Yes
	Kolmogorov–Smirnov	KS = 0.1187	> 0.1000	Yes
GPR55 (RNAm)	Shapiro–Wilk	W = 0.8557	0.0680	Yes
	Kolmogorov–Smirnov	KS = 0.2313	> 0.1000	Yes
CB2R (RNAm)	Shapiro–Wilk	W = 0.8858	0.0856	Yes
	Kolmogorov–Smirnov	KS = 0.1921	> 0.1000	Yes

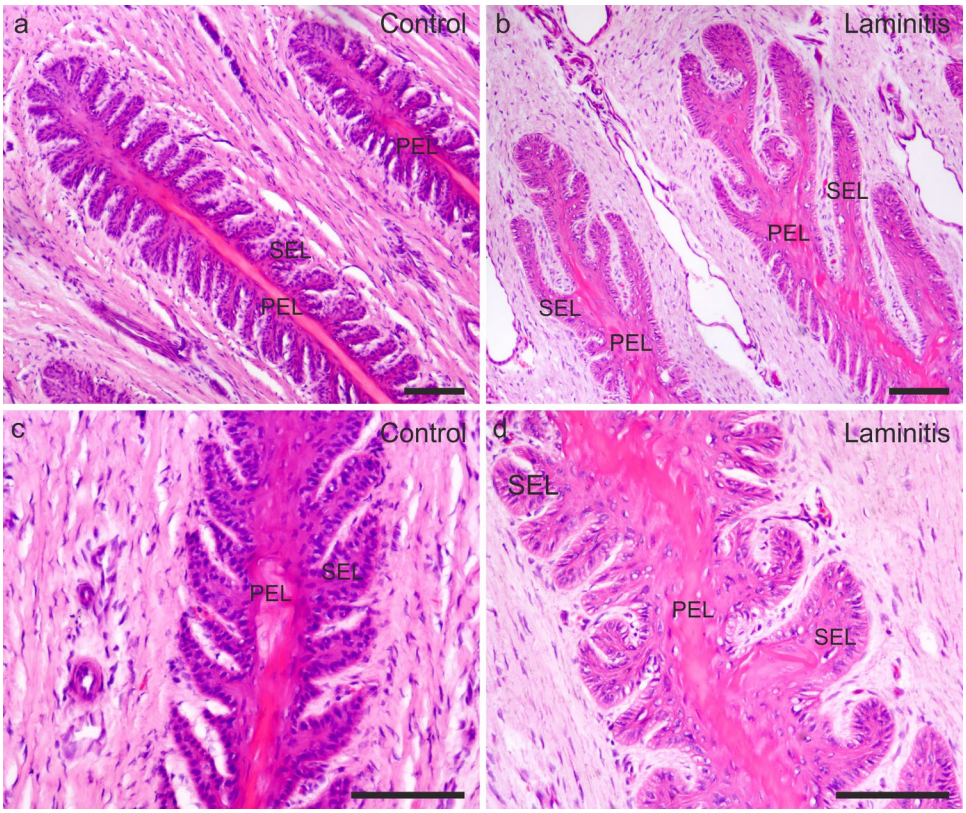

Fig. 2 Representative haematoxylin and eosin images of healthy lamellae (a, c) (control) and laminitic lamellae (b, d) (laminitis) in which irregular hyperplasia of the primary epidermal lamellae (PEL) and the irregular shape of the secondary epidermal lamellae (SEL) are evident. Scale bar: 100 µm

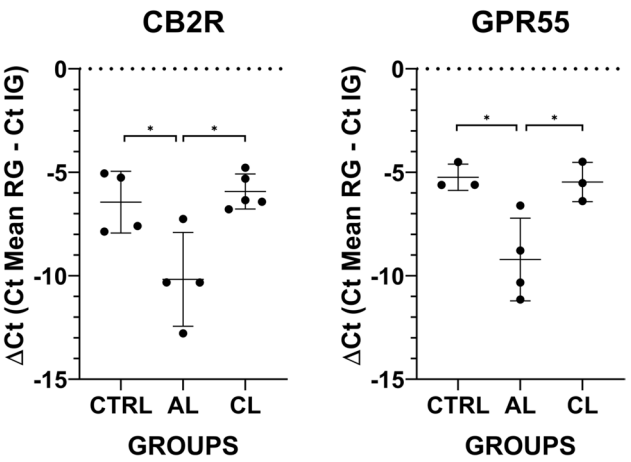

Fig. 3 Gene expression of *Cn2r* and *GPR55* in the epidermal–dermal lamellar junction of the horse's hoof. The results are presented as $\Delta C_t = (C_{t\ \text{mean reference gene}} - C_{t\ \text{gene of interest}})$. The symbols indicate individual animals. For each gene, mean ± SD are indicated by horizontal bars. The asterisks indicate statistically significant differences ($p < 0.05$, Kruskal–Wallis test, Tukey's multiple comparisons post hoc test). *CTRL* healthy lamellae, *ALL* acute laminitic lamellae, *CLL* chronic laminitic lamellae

G protein-coupled receptor 55

In the HL group, faint GPR55-IR was found in 25% (1/4) of the horses, whereas in 75% (3/4) it was moderate (Figs. 4e, 5). In the LL group, GPR55-IR was faint in 33% (4/12), moderate in 22% (3/12), and strong in 44% (5/12) of the horses (Figs. 4f, 5).

No differences were observed between the acute and the chronic laminitic horses for all of the receptors above.

Immunofluorescence on cryosections

Cannabinoid receptor 1

In the HL group, CB1R-IR was faintly expressed by the cytoplasm of the epidermal cells of the hoof lamellae of all the studied horses (4/4; 100%) (Fig. 6a, e, i). There was no significant difference in CB1R immunolabelling between the axial and the abaxial regions of the PEL in the HL control group ($p = 0.48$).

In horses with acute laminitis (LL group), CB1R-IR was brightly expressed by the basal and supra-basal cells of the SEL with BM detachment (Fig. 6b–c, f, g, j–l). Random basal cells showed brighter cytoplasmic CB1R-IR than the other cells. The epidermal cells of the axial portion of the PEL undergoing a remodelling process expressed a brighter and granular pattern of CB1R-IR.

In horses with chronic laminitis (LL group), CB1R-IR was moderately to faintly expressed by keratinocytes of the middle axis of the PEL, and brightly expressed by the

Table 4 mRNA statistical comparison results

Comparison	Mean diff	95% CI of diff	Significant	Summary	Adjusted *p*-value
Cn2r					
HL vs. ALL	3.731	0.6713 to 6.790	Yes	*	0.0186
HL vs. CLL	−0.5132	−3.416 to 2.389	No	ns	0.8800
ALL vs. CLL	−4.244	−7.146 to −1.342	Yes	*	0.0064
GPR55					
HL vs. ALL	4.308	1.068 to 7.548	Yes	*	0.0140
HL vs. CLL	0.5606	−2.903 to 4.024	No	ns	0.8843
ALL vs. CLL	−3.748	−6.988 to −0.5076	Yes	*	0.0269

ns not significant

*Significant difference

epidermal cells at the axial portion of the PEL with loss of shape and orientation deviation (Fig. 6d, h, l).

In the horses with acute and chronic laminitis, CB1R immunolabelling showed no statistical difference between the axial and the abaxial regions of the PEL ($p=0.19$ and $p=0.32$, respectively) (Supplementary item 3).

The comparison of CB1R immunolabelling between the HL and the LL groups revealed a statistically significant difference for acute laminitis ($p=0.04$) but not for chronic laminitis ($p=0.81$) (Supplementary item 2), indicating higher expression of CB1R in acute laminitis. There was no significant difference between acute and chronic laminitic horses ($p=0.06$). Notably, within the chronic group, two distinct patterns emerged: three horses showed very faint epidermal CB1R expression, which was significantly lower than in the HL group ($p=0.003$), while five horses exhibited moderate to bright expression, which was significantly higher than in the HL group ($p=0.001$).

Cannabinoid receptor 2

Cannabinoid receptor 2 immunoreactivity (CB2R-IR) was brightly expressed by the cytoplasm of the epidermal cells of the hoof lamellae of all the horses studied (16/16; 100%).

In the HL group, CB2R-IR was expressed by both the basal and the supra-basal cells of the SEL, and with less intensity by the keratinocytes of the PEL (middle axis) (Fig. 7a, e, i). There was no significant difference in the CB2R immunolabelling between the axial and the abaxial regions of the PEL ($p=0.23$).

In the horses with acute (Fig. 7b–c, f, g, j–l) and chronic (Fig. 7d, h, l) laminitis (LL group), CB2R-IR was brightly expressed by the supra-basal cells of the SEL and by the keratinocytes of the middle axis of the PEL undergoing stretching and hyperplasia/dysplasia.

In the horses with acute and chronic laminitis, no statistical difference in CB2R-IR was observed between the axial and the abaxial regions of the PEL (0.16 and $p=0.26$, respectively).

The comparison between the HL group and the LL group revealed a statistically significant difference in the acute group ($p=0.0005$) and a non-statistically significant difference in the chronic group ($p=0.08$) (Supplementary item 2). In contrast, p was statistically different between the acute and the chronic laminitic horses ($p=0.007$). Notably, within the chronic group, two distinct patterns emerged: four horses showed moderate epidermal CB2R expression, not statistically different from the HL group ($p=0.56$), while four horses exhibited much brighter expression, significantly higher than the HL group ($p=0.01$) (Supplementary item 3).

G protein-coupled receptor 55

Moderate to bright GPR55-IR was observed in the cytoplasm of the epidermal cells of the lamellae in all the horses (16/16; 100%).

In the HL group, bright GPR55-IR was expressed by both the basal and the supra-basal cells of the SEL. The keratinocytes of the PEL (middle axis) did not show GPR55-IR (Fig. 8a, e, i).

A significant difference ($p=0.02$) in GPR55-IR was observed between the axial and the abaxial regions of the PEL (Supplementary item 4).

In horses with acute laminitis (ALL group), bright GPR55-IR was observed across all the epidermal cells in the axial portion undergoing remodelling, accompanied by a loss of anatomic shape and orientation (Fig. 8b, c, f, g, j–l). At the abaxial portion, the SEL with BM detachment and loss of shape showed a brighter GPR55-IR at the basal cells, with random basal cells of the tips of the SEL having brighter GPR55 cytoplasmic labelling.

In horses with acute laminitis, there was no significant difference between the different regions of the PEL ($p=0.13$) (Supplementary item 3). In the horses with chronic laminitis (Fig. 8d, h, m), there was a significant p-value ($p=0.01$) between the regions of the PEL (Supplementary item 3).

Comparisons between the HL and the LL groups showed significant differences between the axial and the abaxial

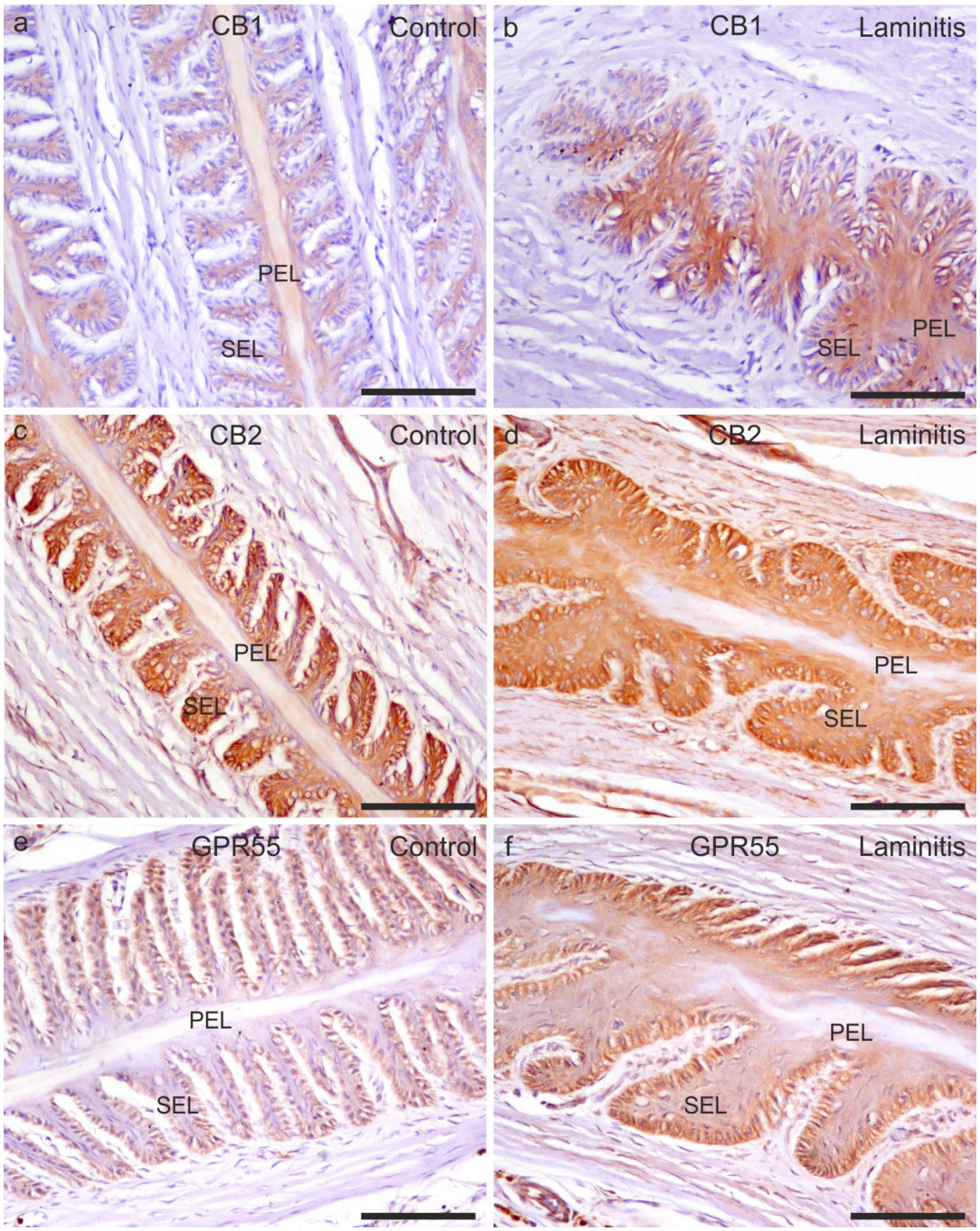

Fig. 4 Immunohistochemical staining for CB1R (**a**, **d**), CB2R (**b**, **e**), and GPR55 (**c**, **d f**) on cryosections of healthy (**a**, **b**, **c**) and laminitic (**d**, **e**, **f**) lamellae. Scale bar: 100 µm

portions of the HL and the ALL groups ($p < 0.0001$). No significant differences were found between the abaxial portions of the HL and the CLL groups ($p = 0.36$), or the axial portions of the HL and the CLL groups ($p = 0.56$) (Supplementary item 3). Moreover, significant differences were observed between the axial portions of the LL (acute vs. chronic laminitis) ($p < 0.0001$) and the abaxial portions of the LL (acute vs. chronic laminitis) groups ($p < 0.0001$) (Supplementary item 3).

Furthermore, CBR1-IR, CB2R-IR, and GPR55-IR were also observed in other cell types of the laminar junction, such as fibroblasts, inflammatory cells, and endothelial cells.

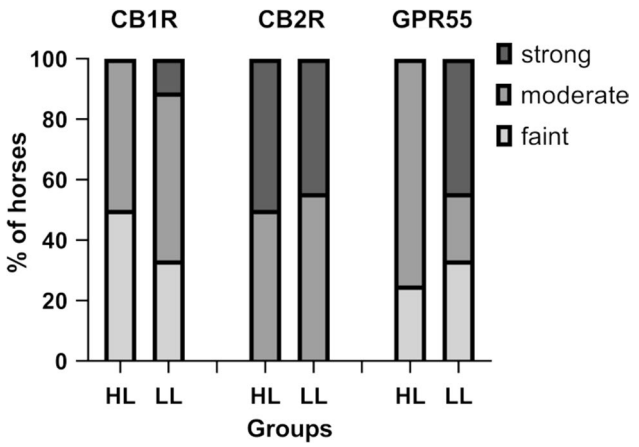

Fig. 5 The graph represents the frequency of the lamellae from the CTRL and the laminitic cases expressing faint, moderate, and strong immunoreactions of CB1R, CB2R, and GPR55

Discussion

Cannabinoid receptors in epidermal cells

The ultra-specialised dermo-epidermal junction of the hoof is of great interest to veterinarians, as inflammation and damage to this lamellar tissue will characterise laminitis (Belknap et al. 2007), a complex condition with multifactorial aetiology, making it challenging to treat effectively. Various therapeutic approaches have been explored, including pain management, anti-inflammatory medications, hoof support techniques, prolonged continuous cryotherapy (Van Eps and Pollitt 2004), and nutritional interventions (Pollitt 2010). However, the success rates of these therapies vary, and achieving complete resolution of laminitis remains a significant challenge. Treatment success often depends on early diagnosis, comprehensive management strategies, and addressing the underlying causes of laminitis, such as metabolic imbalances or systemic inflammation. The lamellar tissues are highly vascularised and innervated, and their dysfunction contributes to the development and progression of laminitis (Cassimeris et al. 2021). One potential therapeutic avenue is the targeting of cannabinoid receptors, specifically

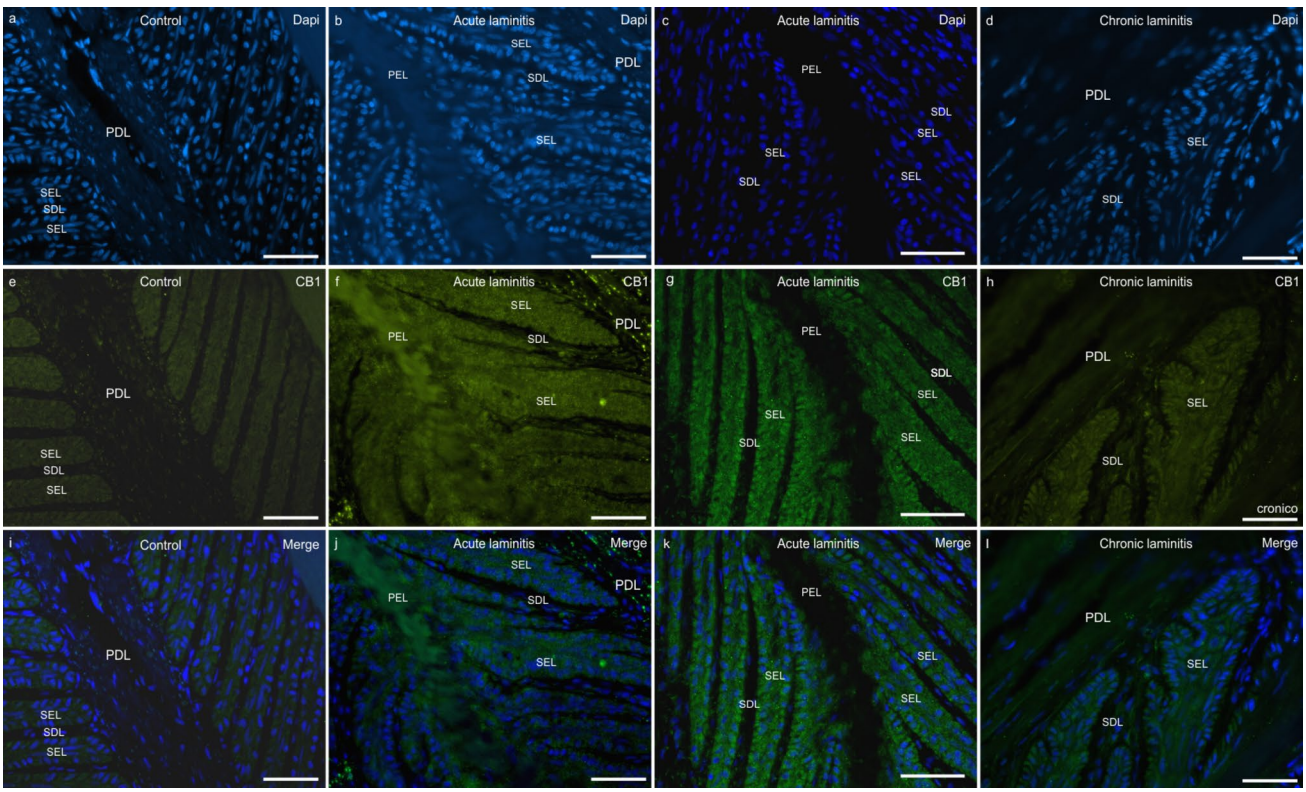

Fig. 6 Immunofluorescence staining for the CB1 receptor on cryosections of the lamellae of healthy (**a**, **e**, **i**) and laminitic (acute: **b**, **c**, **f**, **g**, **j**–**l**; chronic: **d**, **h**, **m**) horses. *PDL* primary dermal lamellae, *PEL* primary epidermal lamellae, *SDL* secondary dermal lamellae, *SEL* secondary epidermal lamellae. Scale bar: 50 µm

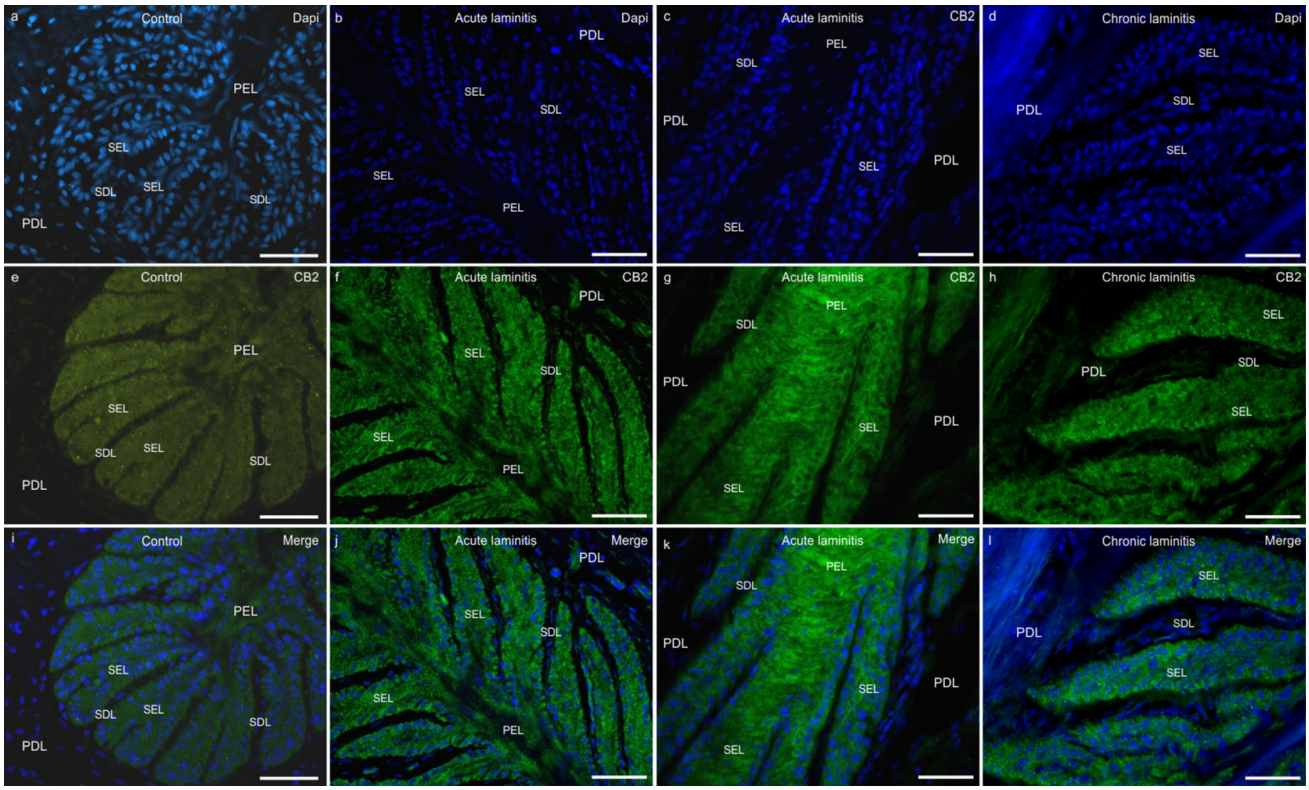

Fig. 7 Immunofluorescence staining for CB2 receptor on cryosections of the lamellae of healthy (**a**, **e**, **i**) and laminitic (acute: **b**, **c**, **f**, **g**, **j**–**l**; chronic: **d**, **h**, **l**) horses. *PDL* primary dermal lamellae, *PEL* primary epidermal lamellae, *SDL* secondary dermal lamellae, *SEL* secondary epidermal lamellae. Scale bar: 50 µm

CB1R, CB2R, and GPR55, which this study showed to be expressed in the lamellar tissues of horses.

In the current study, the interdigitation of the dermal and the epidermal lamellae samples were taken specifically from the dorsal region of the hoof (Douglas and Thomason 2000; Fails 2020), where during the breakover (the period in the stance phase after the heels have left the ground but before the dorsal distal margin does so) the forces will be concentrated. Therefore, the epidermal–dermal junction at this point experiences strong force transmission and deformation, and with an increased area of lamellar junctions when compared to other hoof regions (Douglas and Thomason 2000). Targeting these cells can provide therapeutic benefit for equine patients suffering from lamellar disease, enhancing clinical outcomes regarding hoof diseases.

The ECS regulates multiple aspects of human epithelial physiology, including inhibition/stimulation of keratinocyte proliferation/apoptosis, respectively (Sugawara et al. 2021). Activation of the CB1R by cannabinoids has resulted in promising effects on various cellular processes within the epidermis, including regulation of inflammation, proliferation, differentiation, and barrier function (Wilkinson and Williamson 2007; Czifra et al. 2012). Cannabinoid receptor 1 signalling regulates inflammation by inhibiting the release of pro-inflammatory cytokines and modulating immune responses (Wilkinson and Williamson 2007).

The current study reported dysregulation of CB1R in laminitic horses, with two tendencies to the chronicity of the disease, i.e. higher or lower protein expression. The presence of the two tendencies may be due to different underlying causes of the disease, the different disease time frames, and the chronicity and severity of the disease; the lack of these correlations is a limitation of the present study when dealing with a small number of horses. It is plausible to hypothesise that CB1R may play a role in the cellular turnover process, with pathways to promoting proliferation and apoptosis, at the epidermal junction of the hoof lamellae and may be implicated in modulating the analgesic effects during disease, specifically during the onset period.

Cannabinoid receptor 2 exhibited widespread and robust expression in the epidermal cells at the lamellar junction, in line with the findings of Kupczyk et al. (2022), who showed that keratinocytes of the equine skin exhibit bright CB2R expression. Moreover, CB2R expression has been found to be upregulated in inflamed human skin (Wang et al. 2016), in the skin of dogs with atopic dermatitis (Chiocchetti et al. 2022), and in the oral mucosa of cats with gingivostomatitis (Polidoro et al. 2021), indicating its involvement in the

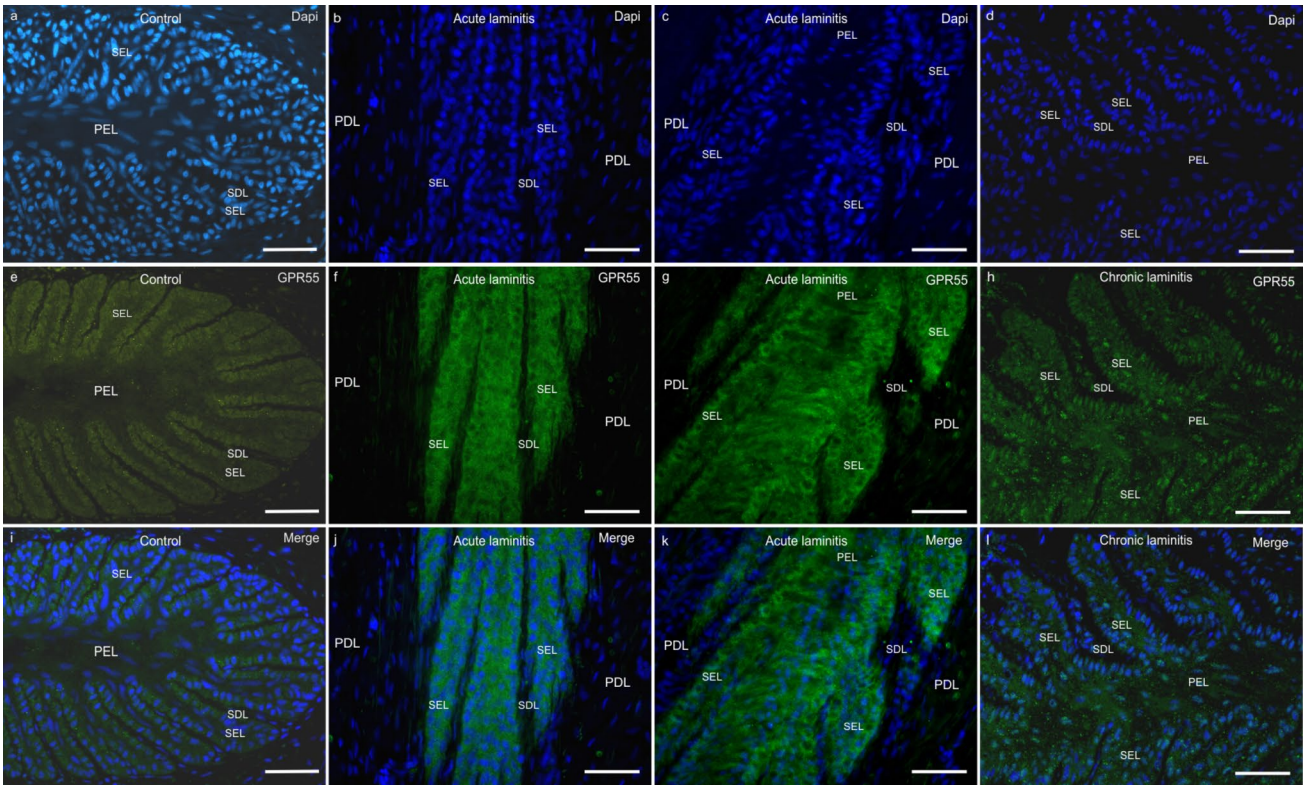

Fig. 8 Immunofluorescence staining for GPR55 on cryosections of the lamellae of healthy (**a**, **e**, **i**) and laminitic (acute: **b**, **c**, **f**, **g**, **j**–**l**; chronic: **d**, **h**, **m**) horses. *PDL* primary dermal lamellae, *PEL* primary epidermal lamellae, *SDL* secondary dermal lamellae, *SEL* secondary epidermal lamellae. Scale bar: 50 µm

disease pathogenesis. These findings are consistent with the present results, where CB2R-IR showed strong labelling on the control tissue and was upregulated at diseased epidermal lamellae of horses with acute and chronic laminitis. Activation of the CB2R pathway has also been shown to mitigate the damage caused by inflammatory cytokines and to preserve cellular permeability in the colon epithelium (Harvey et al. 2013).

Considering the present findings of CB2R expression in epidermal cells of the hoof lamellae and its dysregulation at the epidermal lamellae affected by tissue aggression, one can speculate that targeting this receptor during equine laminitis may potentially slow disease progression by modulating the response of the epidermal lamellae to inflammatory infiltrates and the production of pro-inflammatory molecules by the damaged tissue, consequently reducing lamellar damage and reducing the inflammatory infiltrate and lamellar degeneration.

G protein-coupled receptor 55 controls crucial physiological processes and is involved in the pathogenesis of various mammalian diseases including cancer, cardiovascular disease, and diabetes (Henstridge et al. 2011; Andradas et al. 2013). It has been linked to insulin resistance and directly affects the regulation of glucose homeostasis (Tudurí et al. 2017), which is one of the causes of equine laminitis (Elliott and Bailey 2023). The expression of GPR55 has been observed in the sensory ganglia, ileum, and synoviocytes of horses (Galiazzo et al. 2021, 2022; Zamith Cunha et al. 2023a, d). However, its precise role and pathways within these cell types have not been fully understood.

In this study, GPR55-IR was widely and prominently present in the PEL of the hoof, more strongly expressed by the basal cells, which undergo mitosis and proliferation in order to generate mature secondary lamellae and subsequently keratinisation. The role of GPR55 in regulating cell proliferation (Piñeiro et al. 2011; Liu et al. 2015; Tudurí et al. 2017; Hill et al. 2018) and energy metabolism (Romero-Zerbo et al. 2011; Simcocks et al. 2014) is well established. Based on the results obtained, we hypothesised that GPR55 plays a role in regulating cell proliferation and glucose metabolism in the basal cells of the primary and secondary epidermal lamellae.

Epithelial cells were shown not to express the insulin receptor, and to express the insulin-like growth factor-1 (IGF-1) receptor (Burns et al. 2013); the effect of insulin is mediated by the IGF-1 receptor in the basal cells (Baskerville et al. 2018), and hyperinsulinemia and insulin resistance are a known cause of equine laminitis. The stronger

expression of GPR55 at the basal cells of the secondary epidermal lamellae may indicate a specific role of this receptor in this cell subtype in the potential absence of an insulin receptor. Therefore, targeting this receptor could have beneficial effects for horses suffering from laminitis and from degeneration of the hoof wall caused by sepsis, vasculitis, and insulin resistance, by promoting hoof wall growth and lamellae proliferation as well as modulating the cellular response to insulin and promoting normal glucose influx.

The present results on dysregulation of the cannabinoid receptors in the abnormal lamellae provide insight into their role within the development of laminitis. Knowing that CBD can inhibit metalloproteinase (MMP) secretion and activity (Ligresti et al. 2016), one could hypothesise that cannabinoids exert their effect in retarding lamellar disease and slowing the course of the disease through cannabinoid receptor pathways. Under acute conditions, these receptors are involved in modulating inflammatory responses, reducing pain, and controlling immune cell activation and migration (Ligresti et al. 2016). In chronic diseases, cannabinoid receptors continue to regulate inflammation, but also influence tissue remodelling and fibrosis (Pryimak et al. 2021). In one in vivo study of a mouse model of type I cardiomyopathy, it was demonstrated that CBD treatment led to a significant decrease in collagen deposition and the expression of pro-fibrotic genes including *MMP-2*, *MMP-9*, *TGF-β*, connective tissue growth factor (*CTGF*), fibronectin, and collagen-1 (Montecucco and Di Marzo 2012). It has been shown that tetrahydrocannabinol (THC) also inhibits the proliferation of liver myofibroblasts and stellate cells via CB2R, leading to their programmed cell death. Therefore, THC may also possess anti-fibrotic properties (Tam et al. 2011; Pryimak et al. 2021). These receptors offer potential therapeutic targets for managing both the immediate and the long-term consequences of laminitis.

The use of cannabinoids such as CBD, THC, CBG (cannabigerol), CBN (cannabinol), and CBC (cannabichromene) in veterinary medicine has gained significant attention. These compounds interact with CB1R, CB2R, and GPR55 and can promote anti-inflammatory, analgesic, and anti-oxidant effects (Atalay et al. 2019). The use of cannabinoids with positive therapeutic effects in horses has already been described by equine clinicians for mechanical allodynia (Ellis and Contino 2021) and cribbing (Zamith Cunha et al. 2023a), and has been shown to be safe and tolerable in a different range of doses for horses (Williams et al. 2022; Yocom et al. 2022). Preclinical and clinical studies support the anti-oxidant and anti-inflammatory effects of CBD, which are linked to its skin protective effects; CBD exerts protective effects on human keratinocytes via the modulation of the caspase-1–IL-1β axis, supporting its potential skin health applications (Liu et al. 2021). At present, no studies targeting patients with hoof diseases have yet been reported.

The present findings a revealed an inverse trend between the gene expression data and the protein expression of CB2R and GPR55 in the different laminitic groups. Specifically, mRNA levels for both receptors were significantly higher in the control and chronic laminitic groups than in the acute laminitic group (Table 2). This was evidenced by significant *p*-values when comparing the control group to the acute laminitic group and to the chronic laminitic group. Conversely, receptor protein expression was significantly higher in the acute laminitic group, with significant differences observed between the same groups. This apparent discrepancy is consistent with evidence that mRNA and protein expression levels can exhibit asynchronous dynamics, especially under stress or inflammatory conditions.

Previous study have shown that mRNA concentrations can display transient, pulse-like patterns that return to baseline, while protein concentrations may establish a new steady state and remain elevated due to prolonged synthesis or reduced degradation (Cheng et al. 2016). Furthermore, it has been demonstrated that protein expression may increase despite a decrease or plateau in mRNA levels, reflecting differential control of translation and protein stability (Hargrove and Schmidt 1989; Cheng et al. 2016). This divergence is further supported by modeling studies suggesting that the rates of synthesis and degradation of both mRNA and protein influence final protein concentrations and their kinetics (Hargrove and Schmidt 1989; Liu et al. 2016). Thus, in the context of acute laminitis, the inflammatory environment may promote rapid gene translation and higher protein expression, leading to a temporary depletion of mRNA levels. This hypothesis warrants further investigation to elucidate the downstream regulatory pathways involved.

The present experiment was unable to obtain consistent quantitative data due to the low number of samples of CB1R mRNA detected. This limitation is likely due to the insufficient sample size inherent to the protocol used. Specifically, the 14-micron sections of the dermo-epidermal junction may not have contained enough mRNA of CB1R-expressing cells to reach detectable levels. Cannabinoid receptors type 1 are predominantly localised in the epidermal cells, sensory nerve fibres, and rare endothelial cells. Given the sparse distribution of these cells within the 14-micron samples, the amount of specific mRNA was likely too low for accurate measurement. Future studies should focus on isolating and phenotyping specific cell populations, followed by cell culture, to ensure adequate sample representation for mRNA analysis and could also take samples from the coronary band and heels. This approach could provide a more accurate assessment of CB1R expression and its role in laminitis, ultimately leading to focused therapeutic strategies. Despite the relevance of our findings, other limitations must be acknowledged. The study included a relatively small and uneven number

of samples across groups and lack of older horses in the control group, which may limit the statistical power, generalizability, and give bias to the results. Additionally, the absence of complete clinical histories for all animals, particularly those from abattoirs, hindered a more robust correlation between the etiology and severity of laminitis and the observed patterns of protein expression. The study design was not blinded, which could introduce potential observer bias during immunohistochemical analysis. The heterogeneity in the underlying causes of laminitis could influence receptor distribution, and this was not stratified or analyzed separately in the current design.

Conclusions

The present findings revealed the presence of cannabinoid receptors CB1R, CB2R, and GPR55 in healthy hoof lamellar epithelial cells. These receptors not only exist in healthy tissues but also show increased and dysregulated expression in pathological lamellae. Interestingly, the mRNA levels for CB2R and GPR55 were higher in the control and the chronic laminitic groups than in the acute group, suggesting a potential compensatory mechanism. This dysregulation in lamellar epithelial cells underscores their crucial role in hoof homeostasis and response to injury. Modulating CB1R, CB2R, and GPR55 pathways could enhance hoof growth, aid in lamellar healing in laminitis cases, and reduce inflammation. Future research should explore these mechanisms with larger animal cohorts to establish correlations between cause, severity, and protein expression. Targeting these receptors with cannabinoids may hold promise for improving hoof disease management, ultimately improving equine health and performance.

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Author contributions R.Z.C., R.C. and A.G. contributed to the study design. The epidermal lamellae of horses were fixated and processed for immunohistochemical experiments by R.Z.C, C.M. and L.D. The immunohistochemical experiments were conducted by M.M. and F.G. R.Z.C. and A.Z. contributed to the quantitative real-time PCR experiments. Acquisition of data: R.Z.C. and R.C. All the authors interpreted the data. Drafting of the manuscript: R.Z.C. and R.C. All the authors contributed to the study execution and approved the final manuscript.

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Material availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval and consent to participate According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 regarding the protection of animals used for scientific purposes, Italian legislation (D. Lgs. n. 26/2014) did not require any approval by competent authorities or ethics committees as this study did not influence any therapeutic decisions.

Consent for publication All the authors have approved the publication of this manuscript.

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