

## Alopecia areata in Eringer cows

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### Abstract

**Alopecia areata is a hair loss disorder in humans, dogs and horses with a suspected autoimmune aetiology targeting anagen hair follicles. Alopecia areata is only sporadically reported in cows. Recently, we observed several cases of suspected alopecia areata in Eringer cows. The aim of this study was to confirm the presumptive diagnosis of alopecia areata and to define the clinical phenotype and histopathological patterns, including characterization of the infiltrating inflammatory cells. Twenty Eringer cows with alopecia and 11 Eringer cows without skin problems were included in this study. Affected cows had either generalized or multifocal alopecia or hypotrichosis. The tail, forehead and distal extremities were usually spared. Punch biopsies were obtained from the centre and margin of alopecic lesions and normal haired skin. Histological examination revealed several alterations in anagen hair bulbs. These included peri- and intrabulbar lymphocytic infiltration, peribulbar fibrosis, degenerate matrix cells with clumped melanosomes and pigmentary incontinence. Mild lymphocytic infiltrative mural folliculitis was seen in the inferior segment and isthmus of the hair follicles. Hair shafts were often**

**unpigmented and dysplastic. The large majority of infiltrating lymphocytes were CD3<sup>+</sup> T cells, whereas only occasional CD20<sup>+</sup> lymphocytes were present in the peribulbar infiltrate. Our findings confirm the diagnosis of T-cell-mediated alopecia areata in these cows. Alopecia areata appears to occur with increased frequency in the Eringer breed, but distinct predisposing factors could not be identified.**

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### Introduction

Alopecia areata (AA) is a chronic, non-scarring dermatosis, which affects anagen hair follicles (HFs). It results in a clinically non-inflammatory alopecia, although an inflammatory infiltrate can be observed histologically. The disease is common in humans and has also been reported in dogs,<sup>1–5</sup> horses<sup>6–11</sup> and two cows.<sup>12,13</sup> The aetiology and pathogenesis of AA is still under debate. Autoimmunity, mediated by autoreactive, primarily CD8<sup>+</sup>, T cells,<sup>2,14,15</sup> neuroendocrine pathways inhibiting hair growth<sup>16,17</sup> and genetic predispositions<sup>1–22</sup> have been and are being investigated.

The Eringer breed is a small cattle breed, largely restricted to the canton Wallis in southern Switzerland. Most animals have a black hair coat, but rare variants with dark red to brown pelage occur. The breed can be used for meat and milk production, but nowadays, owing to a naturally highly developed aggressive potential, most cows are kept by part-time farmers for competition purposes. Female cows carry out fights within their herd to establish a hierarchy while out at mountain pastures and they also participate in organized fights.<sup>23</sup> During fights, two cows lock horns and test their strength by pushing their heads together without harming each other. The cow that evades first loses the fight.

Between 2004 and 2007, six Eringer cows presented with adult-onset, clinically non-inflammatory alopecia. The severity of hair loss varied between diffuse hypotrichosis, multifocal alopecia and generalized alopecia involving almost the entire body and head. Animals were otherwise healthy. Histopathology revealed peri- and intrabulbar infiltration of T lymphocytes related to anagen HFs, peribulbar fibrosis and pigmentary incontinence, and dysplastic hairs suggestive of AA.

The purpose of this prospective study was to confirm the presumptive diagnosis of AA in a larger number of Eringer cows with clinically non-inflammatory alopecia, characterize the clinical phenotype, define histological findings and compare these with AA in other species. We hypothesized that there is limited genetic diversity in the Eringer breed owing to the isolated alpine location of the

canton Wallis. This population structure makes the breed much more accessible to study genetic risk factors than human populations.

## Materials and methods

### Animals

Owners of alopecic Eringer cows were asked to contact one of the authors (K.T.) via distribution of information at a breeders' convention and in a breeders' journal. The inclusion criterion was the presence of a clinically non-inflammatory, non-pruritic alopecia. In the context of this study, the term 'clinically non-inflammatory alopecia' is defined by the absence of other macroscopically visible dermatological lesions, while histopathology reveals an inflammatory cellular infiltrate. Exclusion criteria were the presence of other dermatological diseases or of systemic illnesses. The owners were asked to complete a questionnaire (Table 1). For the control group, Eringer cows without a history of skin- or haircoat-related problems were recruited. The owners also completed a similar questionnaire. The study protocol was approved by the Committees on Animal Care and Use of the cantons of Berne and Wallis (request 57/08). All owners signed an informed consent form acknowledging the procedures performed in this study.

### Diagnostic protocol

Cases 1–18 were biopsied in April 2008, and a telephone follow-up was performed 1 year later. Cases 19 and 20 were sampled in April 2009. All the animals received physical and dermatological examinations to rule out systemic illnesses and the existence of other dermatological conditions, including dermatophytosis. The sites and degree of alopecia were recorded using a pictograph.

Eight millimetre punch biopsies were obtained after subcutaneous injection of 2–3 mL lidocaine (Lidocain 2% Streuli ad us. vet.<sup>®</sup>; Streuli Pharma AG, Uznach, Switzerland). Samples were collected at three different sites from the alopecic cows. One biopsy was taken from the centre of the alopecic lesions (lesional skin), one from the margin of a lesion (borderline skin) and one from grossly normal haired skin (unaffected skin). In the control group, two skin biopsies per cow were obtained from the lumbar region of the dorsum, a commonly affected site in cows with alopecia. All the specimens were fixed in 4% buffered formaldehyde solution. Blood samples were collected in EDTA tubes from affected and control cows and stored at 4 °C for genetic analysis.

### Evaluation of clinical parameters

The clinical parameters that were evaluated for descriptive statistical analysis are depicted in Table 1. The degree of alopecia was defined as follows: severe, cases with extensive alopecia involving more than 50% of the body surface and head; moderate, either several large, well-demarcated areas of alopecia or extensive hypotrichosis; and mild, either a few small, well-demarcated alopecic areas or multifocal hypotrichosis. A few single hairs may have been present in alopecic skin, but they were so sparse that the skin had a 'naked' appearance. Hypotrichosis was defined as regions where more hair was present than in alopecic skin, but the skin was visible through the remaining pelage.

### Histopathological examination

Specimens fixed in 4% buffered formaldehyde were embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin (H&E), and with periodic acid Schiff (PAS) for exclusion of dermatophytosis. All sections were evaluated by one of the authors (K.T.), and random samples were examined by two experienced veterinary dermatopathologists (C.v.T., V.F.B.) to verify the findings. All examiners were blinded to the site of origin of the biopsies. The histopathological findings concerning hair follicles were recorded and are depicted in Table 2. Furthermore, the presence of anagen and telogen HF and of an inflammatory infiltrate was recorded. The severity of the inflammatory infiltrate was subjectively evaluated.

**Table 1.** Summarized results of the questionnaires and clinical parameters of 20 Eringer cows with alopecia areata and 11 control animals

	Affected group (%)	Control group (%)
Questionnaire		
Age at onset >5 years	17/19 (89.4)	NA
Duration >1 year	11/19 (57.9)	NA
Season at onset		
Spring	3/19 (15.8)	NA
Summer	1/19 (5.3)	NA
Autumn	8/19 (42.1)	NA
Winter	7/19 (36.8)	NA
Leukotrichia at onset	4/20 (20.0)	NA
Continuous progression	19/20 (95.0)	NA
Partial regression	1/20 (5.0)	NA
Localization at onset		
Rump	8/18 (44.4)	NA
Neck	4/18 (22.2)	NA
Other	6/18 (33.4)	NA
Rank in herd hierarchy		
High	14/19 (73.7)	6/11 (54.5)
Middle or low	5/19 (26.3)	5/11 (45.5)
Fertility problems	4/19 (21.1)	3/11 (27.3)
Participation at fights	14/19 (73.7)	8/11 (72.7)
Alopecia influenced by		
Season	3/19 (15.8)	NA
Pregnancy	1/19 (5.3)	NA
Fights	1/19 (5.3)	NA
Alopecia in related cows	2/19 (10.5)	NA
Clinical parameters		
Degree of alopecia		
Severe	13/20 (65.0)	NA
Moderate	4/20 (20.0)	NA
Mild	3/20 (15.0)	NA
Rare involvement of		
Tail	2/20 (10.0)	NA
Forehead	1/20 (5.0)	NA
Distal extremities	3/20 (15.0)	NA
Bridge of nose	6/20 (30)	NA
Mid-line of back and neck	6/20 (30)	NA
Leukotrichia present	14/20 (70.0)	NA
Situation 1 year later		
No change	12/20 (60)	NA
Mild improvement	4/20 (20.0)	NA
Strong improvement	4/20 (20.0)	NA
Regrowth of white hair	7/8 (87.5)	NA

A high rank in the hierarchy was defined as one of the top three positions in the herd.

NA, not available.

### Immunohistochemistry

Immunohistochemistry was performed with polyclonal rabbit antibodies against CD3<sup>24</sup> (1:600 dilution; A0452; DAKO, Glostrup, Denmark) and CD20 (1:100 dilution; NeoMarker RB-9013P; P.H. Stehelin & Cei AG, Basel, Switzerland). Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylol and rehydrated in 100% ethanol. After a 30-min incubation time in methanol containing 3% hydrogen peroxide to block the endogenous peroxidase, slides were washed three times in phosphate-buffered saline (PBS). Tissue sections were blocked with 5% normal goat serum for 30 min. The anti-CD20 primary antibody was used without pretreatment, whereas the CD3 antibody required antigen retrieval by enzymatic digestion with 0.5% trypsin and 0.5% chymotrypsin at 37 °C for 10 min. After antigen retrieval, sections were immersed once in water and twice in PBS for 5 min each time. Sections were incubated with the primary antibodies either overnight at 4 °C (CD3) or for 1 h at room temperature (CD20). In negative control sections, the primary antibodies were replaced by non-specific immunoglobulins from

**Table 2.** Summary of the most characteristic histological findings in Eringer cows with alopecia areata, and the incidence of histological changes in the different biopsy sites from affected cows and control cows

	(a) Lesional (%)	(b) Borderline (%)	(c) Unaffected (%)	(d) Control (%)
<b>Hair bulbs</b>				
Intrabulbar lymphocytes	14/20 (70) <sup>C,D</sup>	9/20 (45) <sup>c,d</sup>	2/20 (10) <sup>A,b</sup>	0/11 (0) <sup>A,b</sup>
Degeneration of matrix cells	10/20 (50) <sup>b,c,D</sup>	3/20 (15) <sup>a</sup>	2/20 (10) <sup>a</sup>	0/11 (0) <sup>A</sup>
Loss of bulbar pigment	20/20 (100) <sup>C,D</sup>	20/20 (100) <sup>C,D</sup>	10/20 (50) <sup>A,B,D</sup>	0/11 (0) <sup>A,B,C</sup>
Peribulbar lymphocytes	20/20 (100) <sup>B,C,D</sup>	11/20 (55) <sup>A,D</sup>	5/20 (25) <sup>A</sup>	0/11 (0) <sup>A,B</sup>
Peribulbar fibrosis	20/20 (100) <sup>B,C,D</sup>	11/20 (55) <sup>A,d</sup>	8/20 (40) <sup>A</sup>	0/11 (0) <sup>A,b</sup>
<b>Hair follicles</b>				
Mural folliculitis, inferior segment	12/20 (60) <sup>c,D</sup>	5/20 (25)	4/20 (20) <sup>a</sup>	0/11 (0) <sup>A</sup>
Mural folliculitis, isthmus	11/20 (55) <sup>b,c</sup>	4/20 (20) <sup>a</sup>	4/20 (20) <sup>a</sup>	3/11 (27)
Dysplasia of hair follicles	12/20 (60) <sup>C,D</sup>	8/20 (40) <sup>d</sup>	2/20 (10) <sup>A</sup>	0/11 (0) <sup>A,b</sup>
Perifollicular pigmentary incontinence	20/20 (100) <sup>C,D</sup>	20/20 (100) <sup>C,D</sup>	10/20 (50) <sup>A,B,D</sup>	0/11 (0) <sup>A,B,C</sup>
Infundibular hyperplasia, exocytosis	15/20 (75) <sup>D</sup>	14/20 (70) <sup>D</sup>	10/20 (50)	4/11 (36) <sup>A,B</sup>
<b>Hair shafts</b>				
Present in infundibulum	19/20 (95)	16/20 (80)	20/20 (100)	11/11 (100)
Lack of internal detail	19/20 (95) <sup>C,D</sup>	17/20 (85) <sup>c,D</sup>	7/20 (35) <sup>A,B,d</sup>	0/11 (0) <sup>A,B,c</sup>
Depigmentation	17/20 (85) <sup>C,D</sup>	11/20 (55) <sup>d</sup>	4/20 (20) <sup>A</sup>	0/11 (0) <sup>A,b</sup>
Melanin clumping	10/20 (50) <sup>D</sup>	9/20 (45) <sup>d</sup>	7/20 (35) <sup>d</sup>	0/11 (0) <sup>A,b,c</sup>
<b>CD3<sup>+</sup> T cells</b>				
Matrix of hair bulb	19/20 (95) <sup>C,D</sup>	20/20 (100) <sup>C,D</sup>	10/20 (50) <sup>A,B,d</sup>	0/11 (0) <sup>A,B,c</sup>
Dermal papilla	19/20 (95) <sup>C,D</sup>	16/20 (80) <sup>c,D</sup>	7/20 (35) <sup>A,b,d</sup>	0/11 (0) <sup>A,B,c</sup>
Peribulbar infiltrate	20/20 (100) <sup>C,D</sup>	17/20 (85) <sup>c,D</sup>	10/20 (50) <sup>A,b,D</sup>	0/11 (0) <sup>A,B,C</sup>
Inferior segment	17/20 (85) <sup>c,D</sup>	18/20 (90) <sup>c,D</sup>	11/20 (55) <sup>a,b,d</sup>	1/11 (9) <sup>A,B,c</sup>
Isthmus	17/20 (85) <sup>d</sup>	17/20 (85) <sup>d</sup>	14/20 (70)	4/11 (36) <sup>a,b</sup>
Infundibulum	18/20 (90) <sup>D</sup>	18/20 (90) <sup>c,D</sup>	17/20 (85) <sup>b,D</sup>	5/11 (45) <sup>A,B,C</sup>

The letters a–d refer to the lesional, borderline, unaffected and control columns, respectively.

Highly significant statistical results with a *P* value < 0.01 are marked with capital letters. Results of lower statistical significance with a *P* value < 0.05 are marked with lower case letters.

non-immunized rabbits. Positive controls consisted of lymph node sections. Slides were washed three times with PBS and incubated with a secondary biotinylated goat anti-rabbit antibody (1:2000 dilution; Jackson 111-065-003; Jackson Immuno Research, Newmarket, UK) for 45 min at room temperature. Three further washing steps in PBS were followed by incubation with peroxidase-conjugated streptavidine (1:2000 dilution; Jackson 016-030-0849; Jackson Immuno Research) for 30 min at room temperature. Afterwards, slides were again washed three times in PBS and stained for 5 min with 3-amino-9-ethyl-carbazol (AEC Staining-Kit, AEC 101-1KT; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), which produced a red colour at the site of reaction. After two washes in water, the slides were counterstained in Ehrlich's haematoxylin, rinsed in water and mounted in Aquatex<sup>®</sup> (Merck, Darmstadt, Germany).

All sections were scored by the same examiner (K.T.), who was blinded to the origin of the sample (affected or control group and biopsy site). Scoring for the different localizations was defined after preliminary review of six case samples for CD3<sup>+</sup> cells (two samples each from alopecic lesions of clinically severe, moderate and mildly affected animals). A graticule was used to count the number of positively stained cells. The same scoring scheme was used to evaluate CD3<sup>+</sup> and CD20<sup>+</sup> lymphocytes. At first, each anagen hair bulb was scored separately for the presence of positively stained lymphocytes in the matrix of the hair bulb, dermal papilla and peribulbar region. In addition, the inferior segment, isthmus and infundibulum of all present HF's, and the perivascular infiltrate were also evaluated. The different structures were scored as unaffected, mildly, moderately or severely affected according to the number of positive cells. On the basis of the overall pattern of scores at the different anatomical sites, the complete biopsy was scored as unaffected, mildly, moderately or severely affected.

### Pedigree analysis

The full pedigrees for the 20 affected cows and 11 control animals were obtained from the herd book. In total, the pedigrees for the 31

individuals consisted of 2976 ancestors. Preliminary pedigree analysis was conducted using CFC 1.0.<sup>25</sup> To test for differences in the average numerator relationship between the two groups, a non-parametric *t*-test was performed in NCSS 2007<sup>®</sup> (NCSS, Kaysville, UT, USA).

### Statistical analysis

Cross-tabulations and chi-squared test statistics were used to compare frequencies between the two groups for different biopsy sites as well as the degrees and duration of alopecia. Owing to small cell frequencies, final comparisons were performed using Fisher's exact test after merging adjacent categories (histological score levels) of the respective categorical variables. All analyses were performed in NCSS 2007<sup>®</sup>. The threshold value for statistical significance was set at *P* < 0.05.

## Results

### Clinical parameters of affected animals

Detailed results can be found in Table 1. Figures 1 and 2 show severely and moderately affected cows, respectively. Twenty black affected Eringer cows between 3 and 13 years of age were included in the study. The location of alopecia at the time of onset was unknown for one animal. For another cow, the duration, age, season, leukotrichia and location at the time of onset, occurrence of alopecia in related cows and the influence of seasonality, pregnancy, fertility problems and fighting events could not be obtained.

Abnormalities were restricted to the skin in all animals. All the affected animals presented with clinically non-inflammatory, non-pruritic alopecia or hypotrichosis and had not received any previous treatment. The claws and



**Figure 1.** Eringer cow with severe alopecia areata showing extensive alopecia involving large areas of the rump, neck, proximal extremities and the head.



**Figure 2.** Eringer cow with moderate alopecia areata displaying multifocal alopecic patches.

horns were unremarkable. Herds ranged from six to 35 cows, but other animals in the herd were not affected.

At the time of sampling, most cows exhibited multifocal leukotrichia. Regrowth of white hair consisted of either patches (Figure 3) or irregularly dispersed individual hairs. It was often more wire-haired and longer than the normal black hair coat. The tail was rarely affected, and alopecia was only seen at the dorsal base.

There were no statistically significant differences in the rank in herd hierarchy (high rank was defined as one of the top three positions), history of fertility problems and participation at fights between the affected and the control groups. There was a non-significant tendency for cows from the affected group to be high ranking ( $P = 0.425$ ). There was no correlation between the duration (longer or shorter than 1 year) and the degree of alopecia ( $P = 0.126$ ).

All owners were contacted again 1 year later by telephone. Eight cows showed mild (four of eight) or good improvement (four of eight). Three of the animals with good improvement had earlier presented with only mild-to-moderate alopecia. However, one cow which had exhibited severe hair loss at the time of sampling also showed extensive regrowth of black hair 1 year later,



**Figure 3.** Alopecic area with a patch of re-grown white hair.

with only a few white spots. In the other improved cows, most of the regrown hair was white.

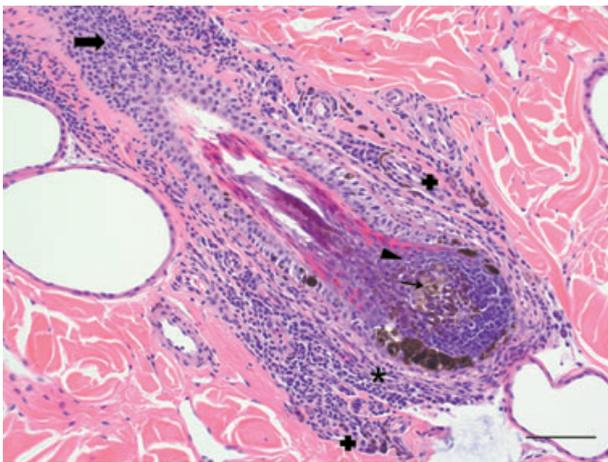
#### Clinical parameters of control animals

Eleven Eringer cows, aged between 7 and 9 years, were recruited for the control group. Two control cows were from the same farm as an affected cow. None of the cows in the control group was related to the cows in the affected group. There were neither abnormalities on physical and dermatological examination nor any history of skin disease. Details concerning rank in herd hierarchy, history of fertility problems and participation at fights can be found in Table 1.

#### Histopathology

The most characteristic histological features, their incidence and statistical analysis are shown in Table 2. Most of the highly significant statistical differences ( $P < 0.01$ ) were seen between lesional and unaffected skin, and lesional and borderline skin from the affected group compared with biopsies from the control group.

Frequent findings were intrabulbar lymphocytic infiltration and degeneration of matrix cells, defined by vacuolation and collapse of matrix cells (Figure 4). Peribulbar lymphocytic infiltration and peribulbar fibrosis was seen in all biopsies from lesional skin, and highly significant differences ( $P < 0.01$ ) were found compared with borderline biopsies. A few eosinophils, plasma cells and rare mast cells could also be found in the peribulbar infiltrate. Loss of bulbar pigment was seen in all biopsies from lesional and borderline skin, resulting in pigment accumulation in degenerate matrix cells and pigmentary incontinence with melanophagia and melanin clumping. These fea-



**Figure 4.** Histopathology of lesional skin of an Eringer cow with alopecia areata. Anagen hair bulb with peribulbar (asterisk) and intra-bulbar lymphocytic infiltration (arrowhead), degenerate matrix cells with intracellular pigment accumulation (thin arrow), pigmentary incontinence with melanophagia (crosses) and lymphocytic infiltration of the inferior segment (thick arrow). H&E; scale bar = 100  $\mu$ m.

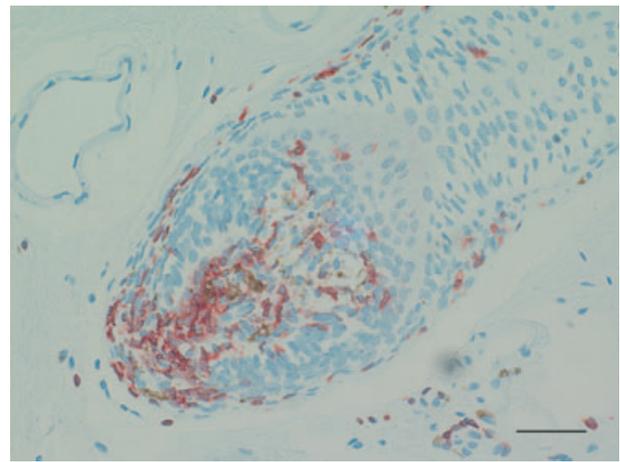
tures, as well as perifollicular pigmentary incontinence, were also seen in 50% of the biopsies from unaffected skin, resulting in a highly significant difference ( $P < 0.01$ ) for all biopsy sites from the affected group compared with the control group.

Anagen and telogen HFs were present in all specimens, and the number of HFs did not vary between the different biopsy sites from affected cows or between the two groups. There were, however, highly significant differences ( $P < 0.01$ ) between the frequency of dysplastic HFs in affected skin compared with unaffected or control skin. Furthermore, a mild infiltrative lymphocytic mural folliculitis with accompanying perifollicular lymphocytic accumulation was sometimes observed in the inferior segment and in the isthmus. This mild mural folliculitis was significantly ( $P < 0.01$ ) more frequent in lesional than in control skin. Hyperplasia of the HF infundibulum with mild exocytosis of mononuclear cells was a frequent finding in biopsies of both groups, but was statistically more likely ( $P < 0.01$ ) to be seen in lesional and borderline skin compared with control samples.

Hair shafts (HSs) were present in all biopsies from the affected group, but they were often dysplastic and showed an irregular structure with variable fragmentation and lack of internal detail, and melanin clumping and depigmentation, characterized by diffuse eosinophilic staining. Dysplasia of HSs was primarily present in lesional biopsies ( $P < 0.01$ ) compared with unaffected skin and control biopsies.

Except for the presence of a mild infiltrative lymphocytic mural folliculitis of the isthmus and infundibular hyperplasia (with exocytosis in a few samples), the biopsies of control skin did not show any changes in the anagen hair bulbs, HFs or HSs.

A further finding in all biopsies from the affected and the control group was a mild to moderate perivascular dermatitis consisting of mainly lymphocytes and eosinophils, few plasma cells and mast cells and rare neutrophils. The PAS-stained sections did not reveal fungal structures in any of the specimens.



**Figure 5.** Immunohistochemistry of lesional skin of an Eringer cow with alopecia areata. Detailed photomicrograph of an anagen hair bulb with positively stained peri- and intrabulbar lymphocytes. Scale bar = 50  $\mu$ m.

There were no significant statistical differences in histological features between cows that were alopecic for more or <1 year. The only statistically significant difference associated with the degree of alopecia was found in the amount of bulbar pigmentary incontinence between mild and moderately affected cows ( $P < 0.05$ ).

#### Immunohistochemistry

Detailed results are given in Table 2. An increased number of CD3<sup>+</sup> T lymphocytes was present in all biopsies from the affected group, while in the control group only occasional positively stained cells were seen. No CD3<sup>+</sup> T cells were seen in anagen hair bulbs, but cells were sometimes seen in the isthmus and infundibulum. In the affected group, immunohistochemistry staining revealed that the lymphocytic peribulbar and perifollicular inflammation as well as the infiltrate seen in the bulbar matrix, the dermal papilla and the follicular wall were composed almost exclusively of CD3<sup>+</sup> T lymphocytes (Figure 5). Positively stained cells were most frequent in biopsies from lesional and borderline skin, but numbers were also increased in unaffected skin. There was a highly significant difference ( $P < 0.01$ ) between the number of CD3<sup>+</sup> T cells in the bulbar matrix, the dermal papilla and the peribulbar infiltrate in lesional and unaffected, and lesional and control skin. There was also a significant difference ( $P < 0.01$ ) between borderline and control biopsies. There were, however, no significant differences between lesional and borderline biopsies.

The perivascular infiltrates and the perifollicular accumulations of lymphocytes were also almost exclusively T lymphocytes. The number of CD3<sup>+</sup> cells in the infiltrate was more frequently scored 'high' in the affected group compared with the control group, where 'medium' scores were most common ( $P < 0.05$ ).

Immunohistochemistry for CD20<sup>+</sup> B lymphocytes identified occasional cells in the peribulbar and perivascular infiltrate. In one lesional and one borderline biopsy, there were rare B lymphocytes in the dermal papilla, hair bulb matrix and in the peribulbar inflammatory infiltrate. There

were, however, no significant statistical differences in CD20 staining.

There were no significant associations between the number of CD3<sup>+</sup> cells at any site and the clinical severity or the duration of alopecia.

### Pedigree analysis

In four affected animals, the parents were unknown. Within the 16 remaining affected cows, there were three paternal half-sib groups. The largest group contained five affected cows with the same father, followed by two groups containing two affected animals each with the same father. There was no paternal half-sib structure found in the control animals. Based on the pedigree analysis, the affected cows showed on average a higher numerator relationship than the control animals. However, the differences were not significant ( $P = 0.05$ ; results not shown).

### Discussion

This paper describes the occurrence of AA in a group of 20 Eringer cows. The clinical findings, histopathology and immunohistochemical identification of T lymphocytes in the inflammatory infiltrate confirmed the diagnosis. There were strong similarities in clinical and histological features between these cattle and other species. Previous case reports on bovine AA in two 4-year-old Holstein cows showed well-demarcated, round-to-oval alopecic lesions of varying size.<sup>12,13</sup> In one case, the forehead was the only affected area,<sup>12</sup> in contrast to our study, where this area was rarely involved. The histopathology in both cases was restricted to focal lymphocytic accumulation around anagen hair bulbs and inferior segments of the HFs. The rest of the skin was histologically normal. One cow also showed regrowth of white hair.<sup>13</sup>

Bovine follicular dysplasia has been described.<sup>26–28</sup> Clinically, this entity is difficult to differentiate from AA. It involves black-haired areas of the body, suggesting a similarity with black-hair follicular dysplasia described in dogs. There are also certain histological similarities to the findings in our study, such as distorted HFs and HSS, melanin clumping, melanophagia and occasional degenerate hair bulbs. Unlike AA, however, an inflammatory infiltrate is not present.

Alopecia areata in cows, dogs and horses usually develops in adult animals. Of the examined cows, 89.4% were older than 5 years at the time of onset, and the median age of the examined 25 dogs in one study was also 5 years.<sup>4</sup> In humans, AA is also seen in newborns and children and is classified as congenital or acquired in human medicine.<sup>29</sup>

There are species differences in the distribution of alopecia in AA. In humans, AA is characterized by patchy hair loss on the scalp. Two subgroups of AA exist in more severe cases. Alopecia totalis describes the loss of all scalp hair, and the term alopecia universalis is used if all scalp and body hair is lost.<sup>30</sup> Nail involvement is also a common feature of human AA.<sup>31</sup> In contrast to humans, dogs predominantly show a facial distribution of AA with well-demarcated areas of alopecia.<sup>4</sup> Claw or hoof

changes have only been described in one dog<sup>32</sup> and one horse.<sup>11</sup> In our Eringer cows, 13 animals showed severe alopecia with involvement of more than 50% of the body surface. Patchy hair loss was a rare finding, in contrast to the two cows described previously.<sup>12,13</sup> Areas of the body that were usually spared were the tail, forehead, distal extremities, bridge of the nose and the mid-line of the back and neck. Involvement of claws or horns was not detected.

Alopecia areata lesions are most commonly seen in black-haired areas in all species. This is caused by the activation of cytotoxic T cells by melanocyte-associated peptides.<sup>21</sup> In acute AA, damage of hair bulb melanocytes can even be detected before hair bulb keratinocytes are damaged.<sup>33</sup> The attack on melanocytes also explains the regrowth of depigmented hairs and the occurrence of leukotrichia without alopecia as a primary sign of AA. In one study, 12 of 16 dogs regrew white hair and two of 25 dogs developed leukotrichia prior to the onset of alopecia.<sup>4</sup> This is very similar to the Eringer cows, of which 70% exhibited multifocal leukotrichia. Four breeders also reported the presence of leukotrichia as the first lesion.

Spontaneous remission of AA can occur in humans,<sup>30</sup> dogs<sup>4,5</sup> and horses.<sup>9</sup> In Eringer cows, this seems to be uncommon, since only four animals improved to any great degree. The duration of alopecia does not seem to influence the prognosis, since four of eight improved cows had already been alopecic for 2–3 years.

The histological features observed in Eringer cows with AA are similar to the dermatopathological description of AA in humans,<sup>30,31,34,35</sup> dogs<sup>2–5,36</sup> and horses.<sup>8–11</sup> The most striking histopathological features are related to the anagen HFs.

Bulbar pigmentary incontinence with melanophagia and a lymphocytic infiltrate in the peri- and intrabulbar region of anagen HFs are the most characteristic findings.

Degeneration of anagen hair bulbs is also found in the other species, and there is a direct association between the presence of immune cells and degenerating matrix cells.<sup>34</sup> However, the presence of clumped melanosomes inside degenerate matrix cells has not been described in other species and seems to be characteristic for Eringer cows with AA. Another frequent finding in the cows was HS dysplasia with depigmentation and melanin clumping.

Peri- and intrabulbar as well as mural infiltration of the HF with CD3<sup>+</sup> T cells proved to be more prevalent with immunohistochemistry than observed with the H&E stain, owing to better visualization of lymphocytes (Table 2). It is remarkable that there were no significant statistical differences in CD3<sup>+</sup> lymphocyte infiltration between lesional and borderline biopsies (Table 2). This implies that the samples taken at the margin were involved in the disease to the same extent as the ones from the centre of lesions. However, on H&E sections there was less peribulbar fibrosis, peribulbar inflammation, degeneration of hair bulbs and mural folliculitis of the isthmus in borderline samples compared with lesional skin. This suggests that lymphocyte infiltration precedes the development of other histological changes.

Another important feature is the presence of some histopathology characteristic of AA in unaffected skin. This was not as pronounced as in lesional or borderline skin, but loss of bulbar pigment, perifollicular pigmentary incontinence and infiltration of the peribulbar area and the infundibulum with CD3<sup>+</sup> T cells reached high statistical significance compared with the control group (Table 2). Mild AA-like changes in unaffected skin of a dog with AA have been reported.<sup>2</sup> Thus, histological changes do not seem to be restricted to alopecic areas, although generalized macroscopic alterations may not be perceived.

There are some limitations to the statistical evaluation of our data. Owing to the relatively low number of cases, it was not possible to investigate possible associations between different parameters within single biopsies of each location. Therefore, it cannot be excluded that certain parameters may not be independent but may be interconnected. The large number of tests performed means that the data are also susceptible to type 1 (i.e. false positive) statistical errors.

A surprising finding in the Eringer cows with AA was the mild infiltrative lymphocytic mural folliculitis of the isthmus, which was observed in 31.7% of all H&E-stained biopsies of the affected group and 85% of immunohistochemistry-stained biopsies from lesional and borderline skin. Dermatophytosis and demodicosis (which are differential diagnoses for mural folliculitis) were ruled out by negative PAS stains and absence of mites in the specimens, respectively. Dermatophytosis, however, cannot definitely be excluded by histopathology alone. Fungal cultures and trichograms were negative in preliminary examinations of Eringer cows with AA (data not presented). These tests were not repeated in this study because of the atypical long duration of clinical signs, the lack of suggestive clinical signs in contact animals, especially in young cows, and financial constraints. Deep skin scrapes to further rule out demodicosis were not taken. Clinically, bovine demodicosis usually presents as a popular-to-nodular disease and was therefore not a clinical differential in these cows.<sup>37</sup> In the two cows reported previously, accumulation of lymphocytes was only seen around the hair bulb and inferior segment.<sup>12,13</sup> Extension of the inflammatory infiltrate to the level of the sebaceous glands can occur in humans with AA but does not usually infiltrate the HF wall.<sup>38</sup> In horses, lymphocytic invasion of the inferior segment has been described.<sup>9</sup> Primary targeting of the follicular isthmus without involvement of the hair bulb has been described in one biopsy of a horse with AA, but this might have been associated with a different pathogenesis.<sup>10</sup> Since three of 11 control cows also had a mild infiltrative lymphocytic mural folliculitis of the isthmus and four of 11 control cows had a mild lymphocytic exocytosis into the infundibular wall on H&E-stained samples, the significance of these observations in affected and control cows is unclear.

The aetiology and pathogenesis of AA have been intensively investigated. The favoured hypothesis is that it is an autoimmune disease, which is probably mediated by autoreactive, primarily CD8<sup>+</sup>, T cells.<sup>2,14,15</sup> Circulating antifollicular IgG antibodies directed against trichohyalin, a protein of the inner root sheath of anagen HFs, and

against other HF structures have also been detected in humans,<sup>39</sup> rodents,<sup>34</sup> dogs<sup>2,3</sup> and horses<sup>4</sup> with AA.

In humans and rodents, the influence of psychoemotional stress on the development of AA has also been intensively studied. Substance P and nerve growth factor are neuroendocrine mediators of stress-induced hair growth inhibition.<sup>16,17</sup> The 'immune privilege collapse model' sees the HF as an organ that is naturally protected from immunological attacks by several suppressive mechanisms. Various factors, including stress, can lead to collapse of the immune privilege via upregulation of the expression of interferon- $\gamma$  and major histocompatibility complex (MHC) class Ia molecules in the normally MHC I-negative matrix of anagen hair bulbs. This renders the HF more susceptible to autoimmunity. The attack of cytotoxic T cells on the hair bulb and resulting secondary, follicle-damaging autoimmune reactions can lead to the initiation of AA. The presence of melanocyte-associated autoantigens may also be important, because active melanogenesis is a distinct feature of the anagen phase.<sup>40</sup> Nevertheless, potential stress events, such as calving, abortions, fertility problems or participation at fighting events, did not seem to influence the development of AA. However, the majority of the affected cows held a high place in the herd hierarchy. Having to defend their position within the herd may be a cause of continual stress.

Only two owners knew of related cows that developed similar alopecic lesions. A genetic predisposition to AA is proposed in humans. Genes involved in the susceptibility and progression of AA were identified using the C3H/HeJ mouse model,<sup>18,19</sup> and an association with certain human leukocyte antigens has been demonstrated.<sup>20–22</sup> The results obtained based on the preliminary pedigree analysis of affected and control Eringer cows may indicate that there is a genetic component to AA in cattle. However, a more detailed analysis based on a larger sample size is required for a better understanding of the genetic aspects of AA and autoimmune responses in general in cattle.

In conclusion, this report defines clinical and histological features of AA in Eringer cattle. Further immunopathological studies, including antifollicular antibodies and investigations of genetic aspects of the disease in this breed, are planned.

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**Résumé** L'alopecia areata (AA) est une cause fréquente de perte de poils chez l'homme, le chien et les chevaux dont l'origine auto-immune supposée cible les follicules pileux en phase anagène. L'AA est sporadiquement rapportée chez les vaches. Nous avons récemment suspecté plusieurs cas d'AA chez des vaches Eringer. Le but de cette étude était de confirmer notre diagnostic d'AA, de définir le phénotype clinique et les patrons histopathologiques, ainsi que de caractériser les cellules inflammatoires. Vingt Eringer alopeciques et 11 Eringer sans trouble cutané ont été incluses dans l'étude. Les vaches atteintes présentaient de l'alopecie multifocale ou généralisée ou de l'hypotrichose. La queue, le chanfrein et les extrémités distales étaient généralement épargnés. Des biopsies punch ont été réalisées sur peau normale, au centre et en marge des lésions alopeciques. L'examen histologique a révélé plusieurs anomalies des bulbes de poils en phase anagène. Celles-ci comprenaient une infiltration lympho-

cytaire péri- et intrabulbaire, une fibrose périlbulbaire, une dégénérescence des cellules matricielles avec des amas de mélanosomes et une incontinence pigmentaire. Une folliculite murale lymphocytaire modérée était présente dans la portion inférieure et l'isthme des follicules pileux. Les tiges pilaires étaient souvent dépigmentées et dysplasiques. La grande majorité de l'infiltrat lymphocytaire était constituée de cellules T CD3+, alors que seulement quelques lymphocytes CD20+ étaient présents dans l'infiltrat périlbulbaire. Ces résultats confirment le diagnostic d'alopecia areata médiate par les cellules T chez ces vaches. L'AA semble plus fréquente dans la race Eringer bien que les facteurs prédisposant n'aient pu être identifiés.

**Resumen** La alopecia areata (AA) es una enfermedad con pérdida de pelo común en humanos, perros y caballos, para la cual se sospecha una etiología autoinmune con los pelos en fase anagena como diana inmunológica. AA tan solo ha sido documentada esporádicamente en vacuno. Recientemente hemos observado varios casos sospechosos de AA en vacas Eringer. El propósito de este estudio fue confirmar el presunto diagnóstico de AA, y definir el fenotipo clínico y los patrones histopatológicos, incluidas las características de las células inflamatorias. Veinte vacas Eringer con alopecia y once vacas Eringer sin problemas en la piel fueron incluidas en el estudio. Las vacas afectadas tenían alopecia generalizada o multifocal o hipotrichosis. La cola, frente y extremidades distales no estaban generalmente afectadas. Se obtuvieron biopsias de piel del centro y de los márgenes de las áreas de alopecia y de piel aparentemente normal. El examen histopatológico reveló varias alteraciones en los bulbos pilosos anagenos. Estas incluían infiltración linfocítica peri- e intrabulbar, fibrosis peribulbar, células de la matriz degeneradas con melanosomas agrupados e incontinencia pigmentaria. Se observó foliculitis mural linfocítica leve en el segmento inferior y en el istmo de los folículos pilosos. Los pelos estaban a menudo depigmentados y displásicos. La mayoría de los linfocitos eran linfocitos T CD3 positivos, mientras que tan solo se observaron algunos linfocitos CD20 positivos en el infiltrado peribulbar. Nuestros hallazgos confirman el diagnóstico de alopecia areata mediada por linfocitos T en estas vacas. AA parece ocurrir con mayor frecuencia en la raza Eringer, pero no se identificaron factores para justificar esa predisposición.

**Zusammenfassung** Die Alopecia areata (AA) ist eine bei Menschen, Hunden und Pferden vorkommende Haarausfallerkrankung, bei der eine autoimmune Ätiologie, die auf anagene Haarfollikel abzielt, vermutet wird. AA wird bei Kühen nur sporadisch beschrieben. Unlängst beobachteten wir bei Eringer-Kühen mehrere Fälle mit der Verdachtsdiagnose AA. Das Ziel dieser Studie war es, die Verdachtsdiagnose von AA zu bestätigen und den klinischen Phänotyp und die histopathologischen Charakteristika, inklusive der infiltrierenden Entzündungszellen, zu definieren. Zwanzig Eringer-Kühe mit Alopezie und 11 Eringer-Kühe ohne Hautprobleme wurden in die Studie aufgenommen. Die betroffenen Kühe zeigten entweder eine generalisierte oder eine multifokale Alopezie oder Hypotrichose. Der Schwanz, die Stirn und die distalen Extremitäten waren üblicherweise nicht betroffen. Stanzbiopsien wurden aus dem Zentrum und vom Rand der haarlosen Stellen und von normaler Haut genommen. Die histologische Untersuchung zeigte mehrere Veränderungen der anagenen Haarfollikel. Diese bestanden aus peri- und intrabulbärer Lymphozyteninfiltration, peribulbärer Fibrose, degenerierten Matrixzellen mit verklumpten Melanosomen und Pigmentinkontinenz. Eine milde lymphozytäre murale Follikulitis wurde im unteren Segment und im Isthmus der Haarfollikel gefunden. Die Haarschäfte waren oft unpigmentiert und dysplastisch. Die große Mehrheit der infiltrierenden Lymphozyten waren CD3<sup>+</sup> T-Zellen, während im peribulbären Infiltrat gelegentlich CD20<sup>+</sup> Lymphozyten vorkamen. Unsere Ergebnisse bestätigen die Diagnose einer T-Zell medierten Alopezia areata bei diesen Kühen. AA scheint bei den Eringer-Kühen mit einer größeren Häufigkeit aufzutreten, obwohl genaue prädisponierende Faktoren nicht identifiziert werden konnten.

**要約** 円形脱毛症 (AA) はヒトでは一般的な脱毛症であり、犬や馬では病因として成長期毛包を標的とする自己免疫疾患が疑われている。AA は牛で散発的に報告されているだけである。近年、我々は AA が疑われるエリンガー種の牛数例に遭遇した。この研究の目的は AA と推定的診断した症例を確定診断し、臨床的な表現系と浸潤する炎症細胞などの病理組織学的パターンを明らかにすることであった。この研究には 20 頭の脱毛症を示すエリンガー種の牛と 11 頭の皮膚病変のないエリンガー種の牛をこの研究に用いた。罹患した牛は全身性や多病巣性の脱毛、または貧毛であった。尾、前頭部、四肢遠位部には通常症状はみられなかった。脱毛部位の中心と辺縁を含む病変部と正常部からパンチ生検を行った。組織学的検査により成長期毛の毛球部に複数の変化が認められた。それらの変化は毛球部周囲と毛球部内へのリンパ球浸潤、毛球部周囲の線維化、集塊状のメラノソームの存在と色素失調を伴う毛母細胞の退行である。軽度のリンパ球浸潤性毛包壁炎が毛包の下部と峽部に認められた。毛幹はしばしば色素を欠き異形成を示していた。大多数の浸潤しているリンパ球は CD3<sup>+</sup>T 細胞であるのに対し、CD20<sup>+</sup>リンパ球はときに毛根周囲に浸潤するのみであった。我々の所見はそれらの牛の症例を T 細胞介在性の円形脱毛症と診断した。AA はエリンガー種でより頻繁に発生しているようであるが、明確な AA の素因は特定できなかった。