New insights in canine recurrent flank alopecia

Sophie Vandenabeele

Dissertation submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD), Faculty of Veterinary Medicine, Ghent University, 2014

**Promotors:**

Prof. dr. S. Daminet

Dr. H. De Cock

Prof. dr. L. Van Ham

Faculty of Veterinary Medicine

Department of Medicine and Clinical Biology of Small Animals
Vandenabeele, Sophie

New insights in canine recurrent flank alopecia

Ghent university, Faculty of Veterinary Medicine
Department of Medicine and Clinical Biology of Small Animals

Cover art by Simon, 2014

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

GENERAL INTRODUCTION

1. Introduction
2. Hair follicle structure and function
3. Hair cycling
   3.1 Systemic (extrinsic) influences on hair cycling
   3.2 Intrinsic (local) influences on hair cycling
4. Hair follicle cycling disorders
5. Canine recurrent flank alopecia
   5.1 Introduction
   5.2 Etiology and pathogenesis
   5.3 Clinical presentation
      Generalized form
      Flank alopecia without an episode of visual alopecia
      Flank alopecia with interface dermatitis
   5.4 Diagnosis
   5.5 Clinical management
5.6 Conclusion
References

SCIENTIFIC AIMS

CHAPTER 1
Atypical canine recurrent alopecia: a case report

CHAPTER 2
Immunohistochemical localization of FGF18 in hair follicles of healthy beagle dogs

CHAPTER 3
Immunohistochemical evaluation of FGF18 in canine recurrent flank alopecia
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 4</td>
<td>111</td>
</tr>
<tr>
<td>Study of the behaviour of lesional and non lesional skin of canine recurrent flank alopecia transplanted to athymic nude mice</td>
<td></td>
</tr>
<tr>
<td>GENERAL DISCUSSION</td>
<td>129</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>151</td>
</tr>
<tr>
<td>SAMENVATTING</td>
<td>157</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>163</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>167</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>173</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AEC  3-amino-9-ethylcarbazole
CRFA  canine recurrent flank alopecia
DNA  deoxyribonucleic acid
FGF  fibroblast growth factor
FGFR  fibroblast growth factor receptor
GVH  graft versus host
HE  haematoxylin and eosin
IHC  immunohistochemistry
IRS  inner root sheath
kg  kilogram
min  minute
mm  millimeter
µm  micrometer
mRNA  messenger ribonucleic acid
MSH  melatonin stimulating hormone
PBS  phosphate buffered saline
POMC  pro opiomelanocortin
PCR  polymerase chain reaction
SCID  severe combined immunodeficient
sec  second
T4  thyroxine
TSH  thyroid stimulating hormone
Wnt  Wingless
GENERAL INTRODUCTION
1. Introduction

The focus of this thesis is to further characterize canine recurrent flank alopecia (CRFA). First the hair follicle structure and function are briefly reviewed. Secondly the hair follicle cycle and factors influencing hair follicle cycling are described with an emphasis on fibroblast growth factor 18 (FGF18) in order to provide the reader with adequate background information for the scientific studies. Finally a review of the literature regarding CRFA is presented.

2. Hair follicle structure and function

The skin is the largest and most visible organ of the body. It forms an anatomic and physiologic barrier between the animal and it’s environment (Miller et al., 2013a). The hair follicle, one of nature’s most fascinating structures, resides in the skin and is a unique mammalian trait (Tobin, 2009).

Dogs generally possess a hair coat that covers the entire skin surface, with exception of the nasal planum, footpads, lips, nipples and anus. Hair has many important biological functions, including thermoregulatory, sensory and protective functions (Miller et al., 2013a). Hairs also function in camouflage, social and sexual communication. Hair follicles are usually positioned at a 30-60 degree angle to the skin surface. Although hair length, thickness, density and colour varies between individuals and especially between breeds, all hairs have the same basic structure (Paus et al., 1999).

The hair follicle has five major components: dermal hair papilla, hair matrix, hair, inner root sheath and outer root sheath (Figure 1). The hair and inner root sheath are formed by pluripotent stem cells in the hair matrix. The outer root sheath represents a downward extension of the epidermis (Miller et al., 2013a).

The anagen hair follicle (growing follicle) is divided in three anatomic segments: the upper portion or infundibulum, the middle portion or isthmus and the lower portion or inferior segment (Figure 1).
The infundibulum consists of the segment from the entrance of the sebaceous duct to the skin surface. The isthmus consists of the segment between the opening of the sebaceous duct and the attachment of the arrector pili muscle. The inferior segment extends from the attachment of the arrector pili muscle to the dermal hair papilla (Miller et al., 2013a).

Figure 1. Schematic depicting the five major components from the hair follicle and the three anatomic segments. Adapted from Lloyd and Patel, 2012.

In contrast to mice and humans which have single follicles, dogs have compound follicles (Figure 2). In single follicles each infundibulum contains one hair shaft that exits through the os, whereas in compound follicles, multiple hairs exit through one os. A compound follicle in dogs consists of one to five primary hairs surrounded by 5 to 20 smaller secondary hairs. The central primary hair is the largest primary hair and the smaller primary hairs are called lateral primary hairs. Each primary hair arises from its individual pore and has sebaceous glands, sweat glands and an arrector pili muscle.
Secondary hairs are only accompanied by sebaceous glands and emerge through a common pore (Miller et al., 2013a). Hair growth in dogs occurs in a mosaic pattern and is maximal in summer and minimal in winter (Miller et al., 2013a; Gunaratnam and Wilkinson, 1983).

Figure 2. Schematic depicting a simple and a compound follicle. Adapted from Credille, 2000.

3. Hair cycling

The hair follicle is a unique and complex mini organ. It is the only organ that undergoes life-long cycles of rapid growth (anagen), apoptosis-driven regression (catagen) and relative resting periods (telogen) (Miller et al., 2013a; Krause and Foitzik, 2006). Shedding of the hair shaft has recently been described as a fourth distinct cycle phase named exogen (Higgins et al., 2009). This sequence of anagen, catagen, telogen and exogen is defined as the hair growth cycle (Figure 3).
The histologic appearance of the hair follicle varies with the stage of the hair follicle cycle (Miller et al., 2013a; Al-Bagdadi et al., 1979; Mecklenburg, 2009a).

The anagen hair follicle extends into the deep dermis and often the subcutis. It is characterized by a well-developed spindle shaped hair dermal papilla capped by the hair matrix. Matrix cells can show mitotic activity and are often heavily melanised (Figure 4a).
The catagen hair follicle is characterized by retraction towards the surface. The hair follicle epithelium shrinks from the hair bulb to just below the entry of the sebaceous gland duct. The dermal papilla is round to oval shaped. The proximal hair shaft is pigmented, melanogenesis ceases and mitotic activity stops. The inner root sheath is partially replaced by trichilemmal keratinization (Figure 4b).

The telogen hair follicle is reduced to about one third of its former length. The dermal papilla is small and is separated from the matrix cells. There is no melanin and no mitotic activity. The inner root sheath is absent. A distinct club hair has now formed that demarcates the isthmus region of the follicle (Miller et al., 2013a; Al-Bagdadi et al., 1979; Mecklenburg, 2009a).

Directly below the isthmus there is a secondary hair germ. These cells will produce a new hair follicle once a new anagen phase is initiated (Mecklenburg, 2009a). The small and condensed dermal papilla is found directly below this secondary hair germ (Figure 4c). In exogen hair follicles the hair is shed from its follicle (Miller et al., 2013a; Higgins et al., 2009). This stage of the hair follicle cycle has received little attention to date.

Figure 4. Histologic pictures of an anagen (a), catagen (b) and telogen(c) follicle. A. Anagen follicle. Note dermal papilla, matrix with melanocytes. B. Catagen hair bulb with dermal papilla, prominent basement membrane and epithelial strand. C. Note telogen hair follicle with club hair and trichilemmal keratinization (white arrow). Prominent basement membrane separating dermal papilla (black arrow) and secondary germ. Adapted from Mecklenburg, 2009.
All the body hairs manifest this cycle, that is based on epithelial–mesenchymal interactions, although the duration of that cycle, the duration of the individual phases and the length of the individual shafts vary dramatically from site to site (Miller et al., 2013a; Gunaratnam and Wilkinson, 1983; Al-Bagdadi et al., 1977; Diaz et al., 2004; Tobin, 2007). Hair growth and shedding has been extensively studied in humans, sheep and laboratory rodents, but few studies have been published in in other mammalian species (Diaz et al., 2004).

Most mammalian species can be grouped into mammals with mainly telogen hairs or mammals with a long anagen phase. The predominant phase of the hair cycle is telogen and anagen respectively (Figure 5). Most dog breeds belong to the telogenic hair cycling type (Credille, 2000). Only a small percentage of dog breeds like the Poodle and Maltese dog have a long anagen phase (Tobin, 2007). This hair may not appear to have a predetermined hair length and may regularly need to be cut. Arctic breeds like the Malamute and Husky have a higher number of the hair follicles in the telogen phase (Tobin, 2007; Lloyd et al., 2012; Mecklenburg, 2006).

This can be viewed as an energy efficient mechanism to maintain an adequate coat in cold environments. Many dog breeds have often lost their ability to express seasonal coat changes through the process of domestication (Meyer, 2009). As a result, the seasonal moult may be influenced too. Dogs normally show a non-synchronized mosaic pattern of hair cycling, where the moult starts from the head and proceeds dorsoventrally and caudally (Meyer, 2009).
These growth waves require intimate interactions between epithelium and mesenchyme and are controlled by factors intrinsic to hair follicle groups. This inherent rhythm can be influenced by neighbouring follicles (paracrine factors) and systemic (e.g. endocrine) stimuli (Stenn and Paus, 2001).

### 3.1 Systemic (extrinsic) influences on hair cycling

Systemic (extrinsic) factors are those that are produced in another organ and are transported to the follicle via the peripheral blood. Hormones are the best defined extrinsic factors (Ebling et al., 1991).

In wild mammals environmental stimuli such as changes in the photoperiod induce a seasonal moult (Bissonnette, 1935). Similar reports in dogs demonstrate that day-length is the most important factor whereas variations in environmental temperature are considered less important (Miller et al., 2013a; Gunaratnam and Wilkinson, 1983; Favarato and Conceição, 2007).
Seasonal moulting is coordinated by the pineal gland, which transduces the environmental signals: long daylight hours (summer) induce short periods of melatonin production by the pineal gland thus stimulating the formation of a summer coat, while short day-length (winter) increases melatonin production resulting in a winter pelage.

Melatonin’s mechanism of action is not known. Some of the reported hair growth modulatory effects of melatonin might result from receptor independent, direct effects of melatonin (Fischer et al., 2008). Other effects are likely the result of functional melatonin receptors expressed by the hair follicles. Melatonin receptors are identified as cytosolic and nuclear receptors. While melatonin receptors are quite likely to exhibit functional effects on the human and canine hair follicle and growth regulation their precise expression pattern and proof of their functional activity is still lacking (Fischer et al., 2008). Surprisingly, melatonin can also interact with androgen- and estrogen receptor mediated pathways (Fischer et al., 2008; Paus and Cotsarelis, 1999). Indeed, in murine hair follicles melatonin has been shown to significantly downregulate estrogen receptor expression in a hair cycle dependent manner (Kobayashi et al., 2005). However, one study investigating the estrogen receptors in dogs with hair cycle arrest supplemented with melatonin was unable to demonstrate that the melatonin induced hair regrowth was correlated with a change in estrogen receptors (Frank et al., 2006).

Prolactin is the “other” environmental dependent hormone, and its circulating levels correlate inversely with melatonin, being high in the summer and low in the winter (Messenger, 1993). Prolactin receptors have been located in the outer root sheath of the hair follicle (Craven et al., 2001). Prolactin has been shown to inhibit growth of anagen follicles in mice and sheep (Nixon et al., 2002; Craven et al., 2006). It is shown to have inhibitory effects at different stages of the hair follicle cycle with the ability to reduce hair length, shorten anagen, induce shedding or lengthen the telogen phase (Nixon et al., 2002; Craven et al., 2006; Thompson et al., 1997).
In addition to melatonin and prolactin, other peripheral hormones are also known to influence the hair cycling activity: thyroid hormones, the sex steroids estradiol and testosterone and the adrenal steroids (Messenger, 1993).

Thyroid hormones pass through the target cell into the nucleus where they bind the nuclear receptors and initiate activity. Thyroid hormone receptors have been demonstrated in the outer root sheath and dermal papilla of the hair follicles (Dixon et al., 1999). They accelerate the onset of follicular activity and increase the duration of the anagen phase of the follicle (Ebling et al., 1991).

In humans an androgen excess results in accelerated hair growth in androgen-dependent areas, such as the beard, and male pattern baldness on the scalp. An androgen deficiency in men causes a sparse or absent beard growth (Zouboulis et al., 2007; Randall, 1994). In rats however androgens retard the initiation of anagen (Messenger, 1993). Documented hyperandrogenism is rare in the dog (Miller et al., 2013b).

Estrogens act directly on the hair follicle but are not essential for normal hair growth (Mecklenburg, 2009b; William et al., 1946). Estrogens act via the estrogen receptor, a nuclear receptor. Estrogen receptors have been demonstrated in the dermal papilla of hair follicles from different species, including mouse, dog and man (Oh and Smart, 1996; Bratka-Robia et al., 2002; Frank et al., 2006). The quantity of estrogen receptors varies during the hair growth cycle.

The biologic effects of estrogens on the hair follicle are very complex: major differences are seen between species, genders and anatomical skin sites.

Differences in the regional distribution of estrogen receptors and/or variations in the metabolic capacity of the hair follicle may be responsible for the regional alopecia seen in estrogen related alopecias (Eigenman et al., 1984). Estrogens decrease hair growth by delaying the initiation of anagen and reducing the rate of hair growth (Oh and Smart, 1996).
The cellular response to glucocorticosteroids is mediated through specific intracellular receptors that have been demonstrated in the hair follicle bulge keratinocytes and hair follicle matrix cells of humans and rodents (Beato et al., 1995). Glucocorticoids also affect the expression of estrogen receptors (Thornton et al., 2006). They are potent suppressors of hair growth. Another way to describe their action on the hair follicle cycle is a suspension of the anagen stimulus (Stenn and Paus, 2001).

The hair follicle is not only a target organ for numerous hormones but also produces many of these. For example, the hair follicle is both a target and a source of melatonin, prolactin, estrogen, cortisol and thyroid hormones (Fischer et al., 2008).

### 3.2 Intrinsic (local) influences on hair cycling

In the hair follicle, intrinsic factors are produced by and act on a variety of different cell types of the hair follicle, fibroblasts surrounding the follicle and endothelial cells of the blood vessels that supply the follicles (Stenn and Paus, 2001).

The exact mechanism by which local signals control hair follicle induction, development, regression and reactivation remain obscure. However, multiple growth factors and/or their receptors have been identified and localized to hair follicles and surrounding mesenchyme.

Indeed, it is thought that, during the hair cycle, the epithelium and mesenchyme are regulated by a distinct set of molecular signals that are unique for every distinct phase of the hair cycle. In telogen hair follicles epithelial-mesenchymal interactions are characterized by a predominance of inhibitory signals that retain the hair follicle in quiescent state. During anagen a large variety of growth stimulatory pathways are activated in the epithelium and mesenchyme, the coordination of which is essential for proper hair fiber formation (Botchkarev and Kishimoto, 2003).
During catagen phase, the termination of anagen specific signalling interactions between the epithelium and the mesenchyme leads to apoptosis in the hair follicle epithelium, while activation of selected signalling pathways promotes transition of the dermal papilla into a quiescent state. The signalling exchange between follicular epithelium and the mesenchyme is modulated by cytokines, locally produced hormones, neuropeptides and growth factors (Stenn and Paus, 2001; Paus, 1998).

Six major molecular family systems are recognized to be important in hair follicle cycling: sonic hedgehog, Wingless or Wnt pathway, neutrotrophins, homeobox (hox) gene families, transforming growth factor beta (TGF-β), and the fibroblast growth factors (FGF) (Cotsarelis, 1998; Oro and Scott, 1998; Philpott and Paus, 1998; Stenn and Paus, 2001). A complete description of these molecules and their follicular functions is beyond the scope of this thesis. As we studied FGF18, a member of the FGF family, the next paragraph will focus on the knowledge of the influence of the different FGF’s on the hair follicle.

The fibroblast growth factors are a large family of 23 multifunctional peptide growth factors that have pivotal roles in many cellular processes (Beenken and Mohammadi, 2009; Haque et al., 2007). Among them FGF1, 2, 5, 7, 10, 13, 18 and 22 are known to be expressed in dermal and follicular cells and to regulate hair growth and skin regeneration (du Cros, 1993; du Cros et al., 1993; Herbert et al., 1994; Danilenko et al., 1995; Guo et al., 1996; Petho-Schramm et al., 1996; Rosenquist and Martin, 1996; Mitsui et al., 1997; Suzuki et al., 2000; Nakatake et al., 2001; Stenn and Paus, 2001; Beyer et al., 2003; Kawano et al., 2004).

These FGF’s have been identified through immunohistochemistry, mouse mutants with defects in hair follicle cycling and by characterizing gene expression profiles of distinct murine hair cycle stages (Schneider et al., 2009). Currently, there are no data available on FGF’s in the canine follicle. Members of the FGF family that impact the control of the normal hair cycle are listed in table 1.
At the time of starting this thesis, the presence of FGF18 mRNA had just been reported in murine hair follicles (Kawano et al., 2005). It was shown that the injection of FGF18 in uniform telogen stage mice induced anagen hair growth. As the intention of this work was to find a therapeutic agent to reverse the temporary alopecia in CRFA, and because CRFA is histologically characterized by a telogenisation of hair follicles (Gross et al., 2005), FGF18 was considered a very interesting target to study in the canine hair follicle and CRFA affected hair follicle.
FGF18 is a growth factor first reported in 1998 in mice and humans (Hu et al., 1998). The first amino acids of the FGF18 protein are hydrophobic residues, which are believed to be signal peptides for secretion. It is suggested to be a glycoprotein, but the importance of the glycosylation to its function is not well understood (Hu et al., 1998). It has a wide variety of expression and is involved in many cellular processes during embryogenesis and adult life including morphogenesis, angiogenesis and development of a variety of cells (Haque et al., 2007). FGF18 induces proliferation in many cell types and in tissues of both epithelial and mesenchymal origin (Hu et al., 1998). The action of FGF18 is dependent on the presence of heparan sulphate proteoglycans. These retain the FGF18 in the vicinity of the FGF18 producing sites, making FGF18 act in a paracrine manner (Hu et al., 1998). The proliferation promoting function of FGF18 also depends on the spatial and temporal expression of the FGF receptors and the presence of heparan sulphate proteoglycans (Hu et al., 1998; Haque et al., 2007; Marie, 2003). The FGF receptor (FGFR) family consists of four distinct tyrosine kinase receptors (FGFR1-4). One receptor can be activated by several FGFs implicating that receptor ligand binding is not unique (Haque et al., 2007; Marie, 2003; Dailey et al., 2006).

However, FGF18 was found to have a greater receptor selectivity than other FGFs (Haque et al., 2007). There are many conflicting reports on the cognate receptors for FGF18 in the hair follicle. The expression of FGFR1, FGFR2, FGFR3 and FGFR4 has been characterized and all these receptors have been found in the murine hair follicle in different stages of the hair growth cycle (Rosenquist et al., 1996). FGFR2, FGFR3 and FGFR4 have all been implicated as being receptors for FGF18 in the murine hair follicle (Rosenquist et al., 1996; Kimura-Ueki et al., 2012). FGF18 mRNA levels peaked during the telogen phase in murine hair follicles. In situ hybridization techniques demonstrated that FGF18 mRNA is mainly expressed in the transient portion of the inner root sheath and telogen bulge of the murine hair follicles, close to the hair bulb. FGF18 also induces DNA synthesis in human hair follicle dermal papilla cells, dermal fibroblasts, epidermal keratinocytes and vascular endothelial cells. Finally, when FGF18 was administered subcutaneously to mice in a uniform telogen stage, anagen hair growth was observed. Therefore FGF18 was suggested to be important for the regulation of hair growth in mice (Kawano et al., 2005).
General introduction

Information on the presence and localization of the FGF’s, and also of FGF18 in canine hair follicles is lacking. There is increasing support for the involvement of local paracrine factors in the regulation of the hair growth cycle (Moore et al., 1991). FGF18 acts in a paracrine manner, is mainly present at the end of the telogen phase and was able to induce anagen when injected in uniform telogen stage mice. Therefore the knowledge of the presence and the localization of FGF18 in the canine hair follicle is important. It could be crucial to a better understanding of the molecular regulation of the normal cycling dog follicle and it’s function in canine alopecic diseases caused by hair cycling arrest, such as canine recurrent flank alopecia.

4. Hair follicle cycling disorders

Primary alopecia is a common problem in dogs. It is histologically characterized as hair cycle arrest. Despite the frequent occurrence of these non-inflammatory alopecia disorders in practice, the pathogenesis for this group of skin diseases is still unclear (Müntener et al., 2012). It is caused by either an endocrine imbalance (hypothyroidism, hypercorticism, hyperestrogenism) or yet unknown factors (alopecia X, CRFA). One opinion is that these hair cycle disorders either lack anagen induction, as suggested for hypercorticism, have an impaired anagen promotion as suggested for hypothyroidism or are prematurely forced into catagen as suggested for alopecia X and CRFA (Mecklenburg, 2006). A more recent opinion is that in CRFA there is an impaired anagen promotion (Müntener et al., 2012). The unique feature of CRFA is that there is a spontaneous regrowth. This “temporary” hair cycle arrest is what makes CRFA so important for studies focusing on aberrant hair follicle cycling.
5. Canine recurrent flank alopecia

5.1 Introduction

Canine recurrent flank alopecia is a visually striking disease characterized by cyclic episodes of non-inflammatory hair loss (or coat changes) that can recur annually (Miller et al., 2013c). Several names have been proposed for this unique canine alopecic disease (canine flank alopecia, seasonal flank alopecia, idiopathic cyclic flank alopecia, cyclic follicular dysplasia) but none of the names fit perfectly: visually complete hair loss is not always seen, alopecia is not always confined to the flank area and some dogs only experience one episode throughout their entire life (Miller et al., 2013c; Paradis, 2009). In this thesis, the term “canine recurrent flank alopecia” will be used, as this is the most specific terminology for this disease.

This intriguing disease was first reported in 1990 by Scott (Scott, 1990). He described a clinically distinct form of waxing and waning non scarring alopecia in five ovariohysterectomized dogs. Later it became evident that dogs of either sex and reproductive status could be affected (Paradis, 2009). Although the disease is well-recognized in practice, it remains poorly documented in the veterinary literature.

5.2 Etiology and pathogenesis

The exact etiology of CRFA remains unknown. Studies evaluating thyroid function, reproductive hormones and growth hormones in affected dogs have not revealed abnormalities (Curtis et al., 1996; Daminet and Paradis, 2000). However, a systemic factor as well as a localized change in the amount or sensitivity of the hair follicle receptors still cannot be excluded (Miller et al., 2013c).
In addition so far no studies have evaluated possible roles for growth factors such as FGF18 despite their importance in the hair growth cycle function in other animals.

Because the disease is more prevalent in certain breeds, a genetic predisposition is suspected (Paradis, 2009; Waldman, 1995; Fontaine et al., 1998). Duration of daylight exposure or changes in light exposure appear to play a role in the development of the lesions. Several observations support the role of light in the pathogenesis of this disease. First, there is the seasonal nature and often annual recurrence of the disease. Interestingly the onset of CRFA in the northern hemisphere is the reverse of what is seen in Australia and New Zealand. Which means that in both hemispheres the onset of alopecia coincides with the months with shorter duration of daylight (Miller et al., 2013c; Paradis, 2009). Secondly, some cases that were reported in the literature describe development of lesions in dogs that were kept in abnormal light conditions (Ando and Nagata, 2000) and one dog in the northern hemisphere developed lesions in the summer when kept in a dark room (Waldman, 1995).

Light therapy has anecdotally been tried with success as a preventative therapy. Dogs exposed to a light source of 100-200 Watt during 15 to 16 hours from September until April did not develop alopecia (Paradis, 1998).

There are two important photo-dependent hormones in the body: melatonin and prolactin. Melatonin is primarily synthesized in the pineal gland and acts at the level of the pars tuberalis of the pituitary. Production of melatonin is proportional to the length of the dark period. Decreased retinal day light exposure results in increased melatonin production. Melatonin is important for reproduction, thermoregulation, coat colour and hair cycling (Paradis, 2000; Stankov et al., 1994). It is known to be involved in the moulting of several mammalian species. Melatonin implants have been used in foxes and minks to manipulate seasonal coat changes (Valtonen et al., 1995; Rose et al., 1984).

Because of the familial incidence, the association with light exposure and the positive effects of melatonin supplementation, a decreased endogenous melatonin production in genetically predisposed animals is suggested to play a role in the pathogenesis of this disease (Paradis, 1995).
Melatonin could act directly on the hair follicle or it’s effect could be through modulation of melatonin stimulating hormone (MSH) and/or prolactin (Paradis, 1995; Fischer et al., 2008). Prolactin levels are known to inversely correlate with melatonin levels (Messenger, 1993). Increase of melatonin levels and subsequent decrease in prolactin levels induces the formation of a winter coat in sheep (Nixon et al., 2002). Hair follicle cycling is governed by seasonal changes to produce a summer and winter moult and the other photo-dependent hormone, prolactin, has been implicated as a principal regulator of this process (Messenger, 1993).

5.3 Clinical presentation

The age of onset has a wide range: from 1 year of age to 11 years, with most cases developing clinical signs for the first time between 3 and 6 years of age (Miller et al., 2013c; Paradis, 2009; Paradis, 2012). Numerous breeds can be affected, but there seems to be a breed predilection in the Boxer, English bulldog, Airedale, Griffon Korthals, Affenpinscher, Labrador Retriever, Bouvier des Flandres, Doberman and Schnauzer (Miller et al., 2013c; Paradis, 2009; Waldman, 1995; Fontaine et al., 1998; Cerundolo, 1999). Dogs of either sex and reproductive status can be affected. In practice, the typical clinical presentation of CRFA is a bilateral symmetrical, geographic shaped, non-scarring and non-inflammatory alopecia in the thoracolumbar area (Figure 6). Lesions frequently are bilateral symmetrical, but one side is commonly more affected than the other. Unilateral lesions have been recognized. At the time of onset there is increased epilation in the affected areas (Miller et al., 2013c). It is further characterized by a rapid onset of alopecia between the months of November and April in the northern hemisphere. The actual month of onset does not appear to be related to breed, age, sex or reproductive status (Miller et al., 2013c; Paradis, 2009). Hair regrowth generally occurs within 4 to 8 months in most cases but occasionally may take up to 18 months. More rarely permanent alopecia can be seen in chronic recurrent patients.
The area of alopecia often remains visually recognisable because regrown hair has a slightly different texture and/or colour (Miller et al., 2013c; Paradis, 2009). Skin hyperpigmentation in the alopecic area can be striking but is not always present. The presence or absence of hyperpigmentation in response to light exposure depends on the breed and within certain breeds depends on the individual pigmentation profile of the dog. Indeed, in some breeds and some individuals, hypermelanization of the skin resulting from endogenous production of factors that stimulate the melanocytes is never noticed. Breed related lack of hyperpigmentation is usually seen in the Wirehaired Pointer, Dalmatian, Doberman, Vizsla and Weimaraner (Paradis, 2009; Declercq 2008).
Other clinical presentations have been recognized in practice. A generalized presentation, flank alopecia without an episode of visual flank alopecia and flank alopecia with interface dermatitis have already been documented. The factors that unify all of these cases are the often recurrent nature of rapid onset of the non-pruritic lesions between the months of November - April and the spontaneous hair regrowth (Declercq, 2008; Cerundolo and Rest, 2013). However, as no exact etiology or good diagnostic test is available for CRFA, it is possible that these so called different presentations of CRFA have different etiologies or pathomechanisms, and are truly different diseases.

**Generalized form**

In these dogs alopecia is present in the thoracolumbar area and other regions such as the dorsal muzzle, periocular regions, base of the ears, perineum and base of the tail (Miller et al., 2013c; Declercq, 2008; Cerundolo and Rest, 2013). This multifocal non scarring alopecic form has been described in some Airedales, Golden Retrievers, Griffon Korthals, Dobermans, Wirehaired Pointers and Giant Schnauzers (Paradis, 2009). Spontaneous regrowth is seen simultaneously in all of the affected areas.

**Flank alopecia without an episode of visual alopecia**

Coat colour changes and/or changes in texture of the coat are sometimes observed in the flank and thoracolumbar area, without a visual episode of alopecia. These coat colour changes are irregular in distribution and can have a geographic pattern (Figure 7). In the literature, aurotrichia was described in Schnauzers without preceding alopecia (White et al., 1992). Interestingly the onset of the discolouration of the coat from silver or black hairs turning into a gold coloured coat was during the months of April-September. This is later than what is seen with the other forms of flank alopecia. One of the Schnauzers described by White had two consecutive episodes of aurotrichia (White et al., 1992). Idiopathic aurotrichia has also been described in a Bichon frisé (Miller et al., 2013d). It is currently unknown why in these dogs the coat colour change occurs at that time of the year. Possibly the changes in the hair coat represent the recovery phase of the disease and are actually newly grown hairs.
Flank alopecia in non-related dogs in the same household has been anecdotally noted. The case description of the flank alopecia in the Affenpinschers mentions that multiple Affenpinschers developed flank alopecia in the winter, when kept in the conservatory, where there was no artificial heating or lighting (Waldman, 1995).

**Flank alopecia with interface dermatitis**

This entity was first described in 2003 by Rachidi in Boxers and is characterized by a combination of flank alopecia and interface dermatitis/folliculitis (Rachid et al., 2003; Mauldin, 2005). In Europe this presentation of flank alopecia has been reported by Van der Luer in an English Bulldog (Van der Luer and Bonestroo, 2010). The distribution of the lesions is very similar to the classical presentation of flank alopecia, with lesions confined to the thoracolumbar area. The difference is the concurrent presence of non-painful and non-pruritic multifocal circular scaly and crusted depigmented plaques within the alopecic area (Figure 8).
The alopecia and the interface dermatitis demonstrated concurrent courses of remission and recurrence in these patients. The relationship between the two types of lesions is not known (Rachid et al., 2003; Mauldin, 2005). The possibility of a superimposed erythema ab igne (chronic radiant heat dermatitis) on CRFA lesions in some of those cases has been suggested (Paradis, 2009). Erythema ab igne is diagnosed by a history of chronic access to heat sources such as a heating pad, electric blanket or heat lamp, with clinical signs that are non-seasonal and typical dermatopathological changes (Declercq and Vanstapel, 1998; Walder and Hargis, 2002). The distribution of the lesions and the irregular alopecia of erythema ab igne are similar to CRFA with interface dermatitis. However, the hypopigmentation bordered by the hyperpigmentation is unique to erythema ab igne (Declercq and Vanstapel, 1998). Moreover, histopathological changes typical of erythema ab igne such as epidermal keratinocyte atypia and karyomegaly and a variable number of wavy eosinophilic elastic fibres (“red spaghetti”) are not seen in CRFA with interface dermatitis (Declercq and Vanstapel, 1998; Walder and Hargis, 2002; Rachid et al., 2003; Mauldin, 2005).
5.4 Diagnosis

If a dog is presented with a history of annual recurring alopecia presenting with the typical lesions from November to April and spontaneous regrowth is evident the diagnosis of CRFA can be made based on the history and striking clinical findings (Miller et al., 2013c; Paradis, 2009).

If a dog is presented for a first episode of CRFA with the typical clinical presentation endocrinopathies such as hypothyroidism, breed specific hair cycle abnormalities, colour dilution alopecia, post shaving arrest, erythema ab igne (chronic radiant heat dermatitis), glucocorticoid injection reaction and postrabies vaccination panniculitis need to be ruled out (Declercq, 2008).

It is of interest that certain breeds that are predisposed for CRFA such as the Boxer, Airedale and German Pointers are also predisposed for hypothyroidism (Dixon et al., 1999; Paradis, 2009). Hypothyroidism and CRFA could both occur in the same dog (Daminet and Paradis, 2000). Hypothyroidism usually presents as a slowly progressive hypotrichosis, as opposed to the rapid onset of alopecia in CRFA. Usually other coat changes are present in hypothyroid dogs such as a scaly or a dull brittle hair coat. Concurrent pyoderma is an occasional complaint in hypothyroid dogs (Paradis, 2009). Slow hair regrowth in clipped areas and a rat tail are other clinical findings suggestive of hypothyroidism. In CRFA the quality and quantity of the coat in the non lesional skin are normal. Another difference is that metabolic signs (weight gain, lethargy) are generally seen with hypothyroidism, but not in dogs with CRFA (Paradis, 2009).

If an owner does not want to wait for spontaneous regrowth, a biopsy and histopathological examination are warranted. In active lesions the fairly typical histopathological changes consist of infundibular hyperkeratosis extending to secondary follicles and sometimes even into the sebaceous gland ducts (Figure 9). The hair follicles demonstrate an atrophic base and can be malformed. These fore-mentioned changes create a specific dysplastic appearance of the hair follicles resembling a malformed foot, hence called “witch’s feet” or “octopus-like hair follicles”.

30
The size of the adnexae is normal, but sebaceous glands can be melanized. Melanin aggregates can also be present in the follicular lumen (Bagladi et al., 1996; Miller and Dunstan, 1993). The timing of the biopsies greatly influences the histopathological changes (Fontaine et al., 1998; Gross et al., 2005). When patients are biopsied early in the disease process most follicles will be in the telogen or catagen phase. However, more often patients are biopsied when the alopecia has been present for several months and is in the resolution phase. If biopsied then, the follicles will often already be in anagen phase and the infundibular orthokeratotic hyperkeratosis might not be prominently present (Fontaine et al., 1998). The described follicular changes are suggestive, but not pathognomonic for CRFA (Paradis, 2009; Scott, 1990). Indeed, dysplastic hair follicles and abnormal melanin aggregation occur in both follicular dysplastic diseases and endocrine skin diseases (Rothstein et al., 1998).

Figure 9. Photomicrograph. Haematoxylin and eosin stain, X50 magnification. Note infundibular hyperkeratosis extending into the secondary follicles, creating the shape of a witches’ foot (arrow).
5.5 Clinical management

Dogs with this disease are otherwise healthy and the disease should be considered as a cosmetic disease. As spontaneous hair regrowth does occur (albeit potentially incomplete with recurrent episodes) benign neglect can be a valid treatment option (Miller et al., 2013c; Paradis, 2009).

Because of variable timing of the spontaneous regrowth and the unpredictable course of the alopecic periods, evaluation of treatment, either curative or as a preventive measure is very difficult to assess objectively.

Melatonin is considered the initial treatment of choice if treatment is requested, but is not readily available in many countries (Paradis, 2009; Miller et al., 2013c). The optimal dose, best route of administration, and the duration of treatment and best time of initiation of treatment are currently unknown, as placebo controlled studies have not yet been published. A success rate of 50-75% has been reported based on anecdotal information (Paradis, 2009). Melatonin implants at 12mg/dog have been successfully used as a preventative treatment in dogs with recurrent episodes of CRFA (Paradis, 2000). Oral melatonin can be administered at a dose of 3 to 6 mg per dog twice to three times daily during 4 to 6 weeks. This duration of treatment is based on a study in mink showing that melatonin induces the anagen hair cycle within a 4 to 6 week period (Paradis, 2000). However, once the hair cycle is restarted melatonin is no longer necessary for continuous growth and maturation of the pelage (Valtonen et al., 1995). Treatment should be initiated shortly after the onset of the alopecia or 1 to 2 months before the anticipated onset of the alopecia. Melatonin is a safe drug, without side effects, but due to its interaction with reproductive hormones it should not be used in breeding animals (Valtonen et al., 1995).
6. Conclusion

Hair cycling has been extensively studied in humans, sheep and laboratory animals. The hair follicle demonstrates a unique intrinsic rhythmic activity (hair follicle cycling) that is orchestrated by an interplay of systemic factors and less well understood local factors. Despite the existence of many hair follicle diseases in dogs, the most important being CRFA, little is known of the existence or importance of these intrinsic and local factors in hair cycling. In order to prevent or treat this disease in an optimal way there is a high need for more information on elements influencing the hair cycle in dogs.

Aberrations in hair follicle cycling are seen in non-inflammatory symmetrical alopecia, such as CRFA. To date, no single systemic factor disturbance has been demonstrated in this unique canine disease, but still cannot be excluded with certainty. Local factors such as steroid receptors have been examined and no significant changes were found. More recently discovered local factors such as the FGF’s that play an important role in hair cycling in other species have not been evaluated in normal and diseased hair growth in dogs. As FGF18 induced anagen hair growth when injected in mice, it would especially be interesting to investigate if FGF18 has a potential therapeutic use in CRFA.
References


Bissonnette, TH. Relations of hair cycles in ferrets to changes in the anterior hypophysis and to light cycles. The Anatomical Record 1935; 63: 159-168.

General introduction


Dixon RM, Reid SW, Mooney CT. Epidemiological, clinical, haematological and biochemical characteristics of canine hypothyroidism. Veterinary Record 1999; 145: 481.


Favarato ES and Conceição LG. Hair cycle in dogs with different hair types in a tropical region of Brazil. Veterinary Dermatology 2007; 19: 15-20.


Frank LA, Donnell RL, Kania SA. Oestrogen receptor evaluation in Pomeranian dogs with hair cycle arrest (alopecia X) on melatonin supplementation. Veterinary Dermatology 2006; 17: 252-258.


Oh H and Smart RC. An estrogen receptor pathway regulates the telogen-anagen hair follicle transition and influences epidermal cell proliferation. Proceedings of the National Academy of Sciences, USA, 1996; 93: 12525-12530.


Rachid MA, Demaula CD, Scott DW, Miller WH, Senter DA, Myers S. Concurrent follicular dysplasia and interface dermatitis in Boxer dogs. Veterinary Dermatology 2003; 14: 159-166.


Rosenquist TA and Martin GR. Fibroblast growth factor signalling in the hair growth cycle: expression of the fibroblast growth factor receptor and ligand genes in the murine hair follicle. Developmental Dynamics 1996; 205: 379-386.


SCIENTIFIC AIMS
Canine recurrent flank alopecia is a frequently observed non-inflammatory alopecic disease. It has only been documented in dogs and has some unique features: there is a seasonal occurrence of the alopecia followed by spontaneous regrowth. Although the typical presentation of the disease is clinically well described, there are different atypical presentations recognized in practice that have not yet been documented in the literature. To date, no underlying hormonal disease or single factor systemic disturbance could be documented in these dogs. Thus the exact pathogenesis of the hair cycle arrest causing the alopecia remains unknown. Studies focusing on local paracrine factors influencing this hair follicle cycle aberration are needed in order to characterize the local follicular events that lead to the hair cycle arrest and in order to potentially provide a new treatment option for this disease. FGF18 is a local growth factor that acts in a paracrine manner. Furthermore FGF18 was able to induce anagen when injected in uniform telogen stage mice. As CRFA is histologically characterized by a telogenisation and catagenisation of hair follicles, the knowledge of the presence and the localization of FGF18 in the canine hair follicle could be crucial to a better understanding of the molecular regulation of the normal cycling dog follicle and it’s function in canine alopecic diseases caused by hair cycling arrest, such as CRFA.

The working hypothesis of this thesis was: could FGF18 be used as a therapeutic agent in CRFA?

The main scientific aims of this thesis therefore were:

1. To localize and quantify FGF18 protein in the normal canine hair follicle in different cycle stages, body locations, sexes and seasonal changes

2. To compare FGF18 protein in CRFA affected hair follicles with normal canine hair follicles

3. To determine the feasibility of a reliable animal model of this disease. This would enable the differentiation between local or systemic factor(s) inducing CRFA and enable the assessment of FGF18 as a hair growth inducer.
CHAPTER 1

Atypical canine recurrent alopecia: a case report

Sophie Vandenabeele\textsuperscript{1}, Jan Declercq\textsuperscript{1}, Sylvie Daminet\textsuperscript{1}, Ilona Schwarzkopf\textsuperscript{2}, Hilde De Cock\textsuperscript{3}

\textsuperscript{1}Department of Medicine and Clinical Biology of Small Animals, University of Ghent, Merelbeke, Belgium

\textsuperscript{2}DAC Malpertuus, Heusden, Belgium

\textsuperscript{3}Veterinary Pathology Services/Medvet, Antwerpen, Belgium

Adapted from: Veterinary Dermatology 2014; 25: 56-58.
Abstract

Canine recurrent flank alopecia has been reported in a large number of breeds. The current literature describes a non-pruritic, non-inflammatory alopecia of the thoracolumbar area sometimes in conjunction with the dorsal muzzle, base of the ears, base of the tail and perineum. The unique features of this disease are that it is recurrent in most cases and that spontaneous regrowth is seen.

Canine recurrent flank alopecia can present with non-inflammatory alopecia on the face, without involvement of the thoracolumbar area.

One privately owned 5-year-old male Cane Corso presented with complete alopecia on the dorsal muzzle, facial folds and ear pinnae. Spontaneous regrowth was noted. Lesions recurred annually for three consecutive years.

Detailed history, complete blood count, serum chemistry profile, histopathological examination and long term follow up were used to establish a diagnosis of CRFA with an atypical distribution of the lesions.

This is the first case report of CRFA without involvement of the classical thoracolumbar area. Atypical presentations of this disease can be seen in practice, and are not yet well documented in the current literature.
Introduction

Canine recurrent flank alopecia has been reported in a number of breeds (Miller et al., 2013a; Paradis, 2009; Fontaine et al., 1998; Miller and Dunstan, 1993; Waldman, 1995). This unique canine alopecic disease is characterized by a rapid onset of bilateral symmetrical alopecia most often confined to the thoracolumbar area between the months of November and April in the Northern Hemisphere (Miller et al., 2013a; Paradis, 2009). Paradis describes that in certain breeds such as the Airedales, Golden Retrievers, Griffon Korthals, Dobermans Wirehaired Pointers and Giant Schnauzers alopecia of the thoracolumbar area has been observed in conjunction with alopecia of the bridge of the nose, base of the ears, base of the tail and/or perineum (Paradis, 2009). We report here on a non-colour linked cyclical follicular dysplasia confined to the dorsal muzzle, facial folds and pinnae in an intact male Cane Corso.

Case report

A five-year-old intact male Cane Corso was presented in May 2012 for the investigation of alopecia on the face which started 8 weeks prior to consultation. The owner mentioned that the alopecia had started in March of 2010 and that spontaneous hair regrowth had been seen in July of 2010. The next year an identical episode of alopecia and hair regrowth had occurred. The dog was otherwise healthy. On examination the dog was bright and alert and general examination revealed no abnormalities. The dermatological examination revealed a complete alopecia on the dorsal muzzle, facial folds and pinnae. The rest of the hair coat had a normal quality. A trichogram did not show any melanin clumping or fractured hair shafts. Blood was collected for biochemistry, haematology and basal thyroxine and thyroid stimulating hormone levels. The results were within normal limits. Skin biopsy was declined by the owner at this time.
In March of 2013 the dog was presented again for recurrence of the alopecia. The owner mentioned that now, for three consecutive years the dog developed alopecia on the face in the month of March or April with spontaneous regrowth in the month of July (Figure 1). The main reason for consultation was that the regrowth of hairs since the last episode was incomplete, with about 75% of hair regrowth. The regrown hairs were identical to hairs in the non-alopecic areas and had the same colour. There were no abnormalities on general examination.

Figure 1. Dog in the month of August, demonstrating the hair regrowth
Dermatological examination revealed a complete alopecia on the dorsal muzzle, facial folds, ear pinnae (Figure 2). There was no alopecia or difference in coat colour or texture on the flanks. The presence of hyperpigmentation in the affected areas was hard to assess as this dog had an already heavily pigmented skin.

![Figure 2. Clinical picture demonstrating the striking non inflammatory alopecia of the dorsal muzzle and facial folds](image)

At this point the owner permitted skin biopsies to be performed. Two 8mm tissue samples were taken from the alopecic area on the dorsal muzzle and one 8mm tissue sample of the facial fold.

Histopathology demonstrated similar changes in the three submitted tissue samples. A uniform melanosis of the epidermis was noted. The hair follicles demonstrated hair cycle arrest, being in catagen or telogen phase.
There was marked infundibular hyperkeratosis extending to the secondary hair follicles. In addition there was mild follicular dysplasia (Figure 3).

![Histopathology, skin biopsy from the muzzle. Note the hair cycle arrest with follicular hyperkeratosis extending to the secondary follicles and the melanisation of the sebaceous glands (arrowhead). Haematoxylin and eosin stain.](image)

Some perifollicular pigmentary incontinence was noted. The sebaceous glands were melanized. There was no fracture of hair shafts present in these tissue samples.

Given the cyclical occurrence of the alopecia and the spontaneous regrowth, combined with the histopathological features, a diagnosis of an atypical presentation of canine recurrent flank alopecia was made. The owners elected not to treat the dog at this point.
Discussion

To the authors' knowledge, this is the first case report of CRFA, where the lesions are confined to the dorsal muzzle, facial folds and pinnae without alopecia of the flanks. The current literature states that alopecia of the bridge of the nose, base of the ears, base of the tail and/or the perineum can be seen in conjunction with alopecia of the thoracolumbar area. The authors have seen a similar clinical presentation in several Bordeaux dogs with non-inflammatory seasonal alopecia on the dorsal muzzle, facial folds and pinnae without flank involvement and with spontaneous regrowth in practice but unfortunately the owners never allowed skin biopsy in these patients. It is interesting to note that the Cane Corso breed belongs to the Molosser group of breeds according to the American Kennel Club. This group also includes other breeds that are known to be predisposed to developing CRFA: Bulldog, Boxer and Bordeaux dog.

Vandenabeele did report on a Golden Retriever with CRFA that only had dorsal muzzle involvement, however the clinical presentation in the dog described here is different as in addition to the bridge of the nose involvement, the pinnae and facial folds were also involved (Vandenabeele, 2009).

When the patient was presented during the first alopecic episode, the main differential diagnosis was hypothyroidism. Indeed, hypothyroidism can present with alopecia on the bridge of the nose and alopecia in areas of friction (Paradis, 2009). Thyroid tests in this patient where within normal limits, and more importantly, spontaneous regrowth as reported in this dog is never seen in hypothyroid dogs.

Currently there is a debate if CRFA should be classified as a follicular cycling disorder with follicles forced into premature catagen in CRFA or a follicular dysplasia (Mecklenburg, 2009b; Mecklenburg, 2006). The term dysplasia is commonly used in veterinary dermatology, and is used on both disorders of hair cycling, and true anagen based structural defects in hair shaft production (Roperto et al., 1995; Miller and Scott, 1995).
Follicular dysplasias can be divided in three broad groups, as described by Laffort-Dassot et al.: colour linked follicular dysplasia, non-colour linked follicular dysplasia and non coulour-linked cyclical follicular dysplasia (Laffort-Dassot et al., 2002).

Colour linked follicular dysplasias include colour dilution alopecia and black hair follicular dysplasia (Mecklenburg, 2009a; Carlotti, 1990). Diagnosis of these two conditions is based on clinical signs, trichogram and skin histopathology (Miller et al., 2013b; Gross et al., 2005). Clinical signs consisting of alopecia or progressive hypotrichosis start at young age, where astute breeders recognize this disease in puppies as young as two weeks of age (Schmutz et al., 1998). Hair examinations show melanin clumps in the cortex and medulla of hairs and fractures of hair shafts. The histopathological changes consist of follicular hyperkeratosis, dystrophy of hair follicles and hair shafts and peribulbar melanophages (Miller et al., 2013b; Gross et al., 2005). Our patient had a black hair coat.

The later age of onset, and the distribution of lesions in this patient are not compatible with black hair follicle dysplasia. Moreover, patients with black hair follicle dysplasia do not demonstrate seasonal spontaneous regrowth and the alopecia is slowly progressive, whereas in this case the distribution of lesions and the extend of the lesions remained unchanged in the alopecic periods during the years we were able to follow the dog.

Non colour linked follicular dysplasias have a strong breed predisposition and have been described in the Irish Water Spaniel, Portuguese Water Dog, Curly coate Retriever, Pont Audemer Spaniel and Chesapeake Bay Retrievers (Miller and Scott, 1995; Cerundolo et al., 2009; Cerundolo et al., 2005).

These follicular dysplasias occur in young adult dogs and have a distribution of lesions that is breed related. Some dogs show some initial waxing and waning of the alopecia, but the noted alopecia and regrowth are never cyclical. Moreover, the alopecia seen in these dogs is progressive and permanent (Cerundolo et al., 2009; Cerundolo et al., 2005). The age of onset of our patient is similar to what is described in the dogs with non-colour linked follicular dysplasia, but again the distribution of the lesions and the cyclical episodes of alopecia and hair regrowth do not fit with the description of the non-colour linked follicular dysplasias.
Non colour linked cyclical follicular dysplasia is seen in CRFA. The age of onset is quite diverse, with a range from 8 months to 11 years of age. The histopathological changes seen when skin biopsies are taken from alopecic areas demonstrate infundibular hyperkeratosis spreading to the secondary follicles and hair follicle arrest, with most follicles being in telogen phase. Sebaceous gland hyperpigmentation and melanin aggregates in the follicular lumen can also be observed (Miller et al., 2013a; Paradis, 2009; Gross et al., 2005; Bagladi et al., 1996).

Our patient had histopathological changes that were suggestive of CRFA. It is important to note that histopathological changes are suggestive but not pathognomonic for this disease (Paradis, 2009). Indeed, the histological changes seen in the follicular dysplasia syndrome of the Chesapeake Bay Retrievers are very similar to those described in CRFA. The main clinical difference is that in Chesapeake Bay Retrievers the hair regrowth does not cycle with the seasons, as in our patient (Cerundolo et al., 2005). Less than complete hair regrowth is seen occasionally in dogs with CRFA when multiple episodes of alopecia have occurred (Miller et al., 2013a; Paradis, 2009; Miller and Dunstan, 1993; Paradis, 2012). This was also seen in this patient, where approximately 75% of hair regrowth was noted following the third alopecic episode.

In conclusion, we report on a patient with an atypical distribution of lesions of canine recurrent flank alopecia, without involvement of the flanks. This emphasises what is written by Paradis, namely that none of the currently and previously used names for this disease (seasonal flank alopecia, idiopathic cyclic flank alopecia, cyclic follicular dysplasia, recurrent flank alopecia) fit perfectly.
References

Bagladi MS, Scott DW, Miller WH. Sebaceous gland melanosis in dogs with endocrine skin disease or follicular dysplasia. A retrospective study. Veterinary Dermatology 1996; 7: 85-90.


CHAPTER 2

Immunohistochemical localization of FGF18 in hair follicles of healthy Beagle dogs

Sophie Vandenabeele\textsuperscript{1}, Sylvie Daminet\textsuperscript{1}, Luc Van Ham\textsuperscript{1}, Thomas Farver\textsuperscript{2}, Hilde De Cock\textsuperscript{3}

\textsuperscript{1}Department of Medicine and Clinical Biology of Small Animals, University of Ghent, Merelbeke, Belgium
\textsuperscript{2}Population Health and Reproduction, University of California, Davis, California
\textsuperscript{3}Veterinary Pathology Services/Medvet, Antwerpen, Belgium

This study was presented in part at the 24\textsuperscript{th} Annual Congress of the ESVD-ECVD in Bled, Slovenia, September 2009 and published as an abstract in Veterinary Dermatology 2010; 21: 211.

Abstract

Increasing emphasis is being placed on the role of FGF’s in hair follicle cycling. In mice expression of FGF18 mRNA peaks during the late telogen phase leading to the hypothesis that FGF18 plays a role in anagen induction. There are no data on the presence of FGF18 in dogs. The main objective of this study was to identify and locate FGF18 in the canine hair follicle. The second objective was to assess potential differences in FGF18 concentration between biopsies taken in winter and summer, shoulder and flank regions, and between different genders. The skin tissue from ten healthy beagle dogs (3 intact females, 3 spayed females and 4 intact males) was collected from the shoulder and flank. The biopsies were collected in February and August, on day 0 after which the dogs were clipped and biopsies collected again from the shoulder and flank on day 1, day 3, day 7 and day 17. Four µm paraffin sections of the biopsies were stained with an anti-FGF18 antibody. FGF18 positive cells were counted in the hair follicle epithelium from seven follicular units of each biopsy. FGF18 was detected as granular cytoplasmatic staining in follicles at the level of the inner root sheath, rarely at the outer root sheath and dermal papilla. It was also detected in the apocrine glands, arrector pili muscles and in vascular endothelial cells. There was no statistical difference in the number of FGF18 positive cells or follicles between genders, different anatomical locations, seasons or the consecutive days of sampling.
Introduction

Canine endocrine diseases commonly present with bilateral symmetrical non-inflammatory alopecia (Scott et al., 2001a). There is a pattern of alopecia, with hair loss at the predilection sites of flanks, perineum and neck, while other areas are typically spared. Patterns of alopecia have been explained by regionalisation of hormone receptor numbers, but recent studies could not detect significant differences in the number of hormone receptors in different anatomical locations (Bratka-Robia et al., 2002; Rosychuk, 1998). The reason that the alopecia affects those areas remains unknown. Alopecia develops because follicles prematurely enter telogen or catagen phases of the hair cycle (Rosychuk, 1998). A normal hair follicle demonstrates an intrinsic rhythmic activity that is influenced by an interplay of systemic factors and local regulatory processes. Of the systemic factors, hormonal systems are known to facilitate seasonal changes in follicular activity in response to environmental factors such as photoperiod and environmental temperatures (Tobin, 2009).

The local regulatory processes in the follicle are numerous and poorly understood. They include a variety of factors such as cytokines, locally produced hormones, neuropeptides and growth factors (Stenn and Paus, 2001; Paus, 1998). A variety of polypeptide growth factors, including various members of the fibroblast growth factor family are involved in the dynamics of hair growth regulation. Among them FGF1, 2, 5, 7, 10, 13, 18 and 22 are known to be expressed in dermal and follicular cells and to regulate hair growth and skin regeneration in mice (Kawano et al., 2005).

FGF18 is a growth factor first reported in 1998 (Hu et al., 1998). It has a wide variety of tissue expression and is involved in many cellular processes throughout the body. In mouse skin FGF18 is involved in regulating hair growth and skin maintenance (Hu et al., 1998). In mice FGF18 mRNA is expressed virtually exclusively in the transient portion of the inner root sheath of the hair follicles, close to the hair bulb. FGF18 also induces DNA synthesis in human hair follicle dermal papilla cells, dermal fibroblasts, epidermal keratinocytes and vascular endothelial cells.
Finally, when FGF18 was administered subcutaneously to mice in a uniform telogen stage, anagen hair growth was observed. Therefore FGF18 was suggested to be important for the regulation of hair growth in mice (Kawano et al., 2005).

The main objective of this pilot study was to demonstrate the presence and the specific location of FGF18 protein in canine hair follicles. The second objective was to compare immunohistochemical FGF18 protein expression of normal canine skin in the shoulder and flank areas, during two different seasons (winter and summer) and between different genders.

**Materials and methods**

**Study population**

All procedures dealing with animal care, handling and sampling were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Ghent (Approval EC2006/88).

Ten healthy beagle dogs (3 intact females, 3 spayed females and 4 intact males) without any dermatological problems were included in this study. The age of the animals ranged from 2 years to 7 years (median 4 years).

The dogs underwent a complete physical examination in February and again in August. A complete blood cell count and chemistry profile, including thyroxine and thyroid stimulating hormone serum concentrations, were performed before commencing the study. The physical examinations, complete blood cell counts and chemistry profiles did not reveal any clinically relevant abnormalities. All dogs were housed similarly in a non-climate controlled area with outdoor access, thus being exposed to the same fixed length of photoperiod and fed the same commercial diet.
Biopsy collection

In February, on day 0, two skin biopsies were taken from the left shoulder and two from the left flank using an eight mm biopsy punch. Then, in order to attempt to induce synchronization of the hair follicles, the left shoulder and left flank were clipped over an area of 14 by 7 cm using a 2 mm clipper head and a plastic grid (Stenn and Paus, 2001).

On days 1, 3, 7 and 17, 8 mm skin biopsies were taken again, two from the left shoulder and two from the left flank on each occasion, in the previously clipped areas. The biopsies were taken 2 cm from each other, in order to avoid inflammatory infiltrates from one biopsy site in the skin samples from the previously obtained biopsies.

The same procedure was repeated in August on the right shoulder and right flank. Prior to the biopsy procedure a line was drawn on the skin surface parallel to the direction of hair growth with a permanent marker in order to facilitate trimming of the biopsy sample in the direction of the hair growth.

From the two skin samples taken of the shoulder and flank of each occasion, one skin sample was stored in a cryovial with RNA later and snap-frozen in liquid nitrogen for later use and the other skin sample was fixed in 4% buffered formalin for 48 h. After formalin fixation the tissue samples were bisected and after embedding in paraffin wax, 4μm sections were cut from each tissue block. Control sections were stained with haematoxylin and eosin (HE) in order to detect any histopathological abnormalities.

Immunohistochemical staining

After deparaffinizing in xylene and rehydration (four steps : 100% alcohol, 95% alcohol, 50% alcohol to distilled water), an antigen retrieval technique was performed. In this procedure, slides were placed in citrate buffer (0.1M with 2% urea) and heated at 96°C for one period of 15 min in a microprocessor-controlled pressure chamber.
The tissues were then kept in the citrate buffer-urea solution for 15 min at 4°C (cooldown period). Distilled water was added if necessary to prevent the slides from drying out. Endogenous peroxidase activity was blocked by incubation with 12% H$_2$O$_2$ in methanol (10 min). The slides were then processed using a DAKO autostainer. Subsequently the slides were incubated with 30% normal rabbit serum (RUO, DAKO, Heverlee, Belgium) in Phosphate Buffered Saline (PBS) for 30 min to reduce non-specific antibody binding. After investigating various dilutions and incubation times the following optimal protocol was used: a polyclonal antibody to human FGF18 (SC-16830, Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:50 in PBS was applied for 60 min. After rinsing the slides were incubated for 30 min with secondary antibodies (P0160, DAKO, Heverlee, Belgium) diluted 1:200.

After another rinse of 10 min, the slides were incubated for 5 min with 3-amino-9-ethylcarbazole (AEC) (DAKO, Heverlee, Belgium). Counterstain was achieved by incubation with Mayer’s haematoxylin for 10 seconds.

Negative controls were performed by replacing the first antibody with PBS. Positive controls were added in each run of the DAKO stainer and consisted of mouse lung tissue. Within the skin sections apocrine glands served as an internal positive control.

One slide was evaluated for the shoulder and the flank for each sampling day. For each slide positive staining was evaluated in the first seven follicular units encountered when evaluating the slide from left to right using a x400 magnification. The number of positive cells in the hair follicle epithelium were counted and the number of follicles with positive cells were recorded.

**Statistical analysis**

The cell counts were logarithmically transformed to stabilize the variability in the data, but untransformed data revealed the same results after analysis.
A four factor analysis of variance was used to determine if there was a significant difference in counts between the sexes (intact female, female spayed, intact male), the anatomical locations (shoulder and flank), the seasons (winter and summer) or between the days (days 0, 1, 3, 7 and 17). The day factor was considered a subject factor and the remaining factors were considered repeated measures factors. All statistical tests were performed at the 5% significance level.

Results

Hematoxylin and eosin (HE) stains

None of the samples had significant morphological changes. There was no inflammatory infiltrate present in any biopsy. All HE stains were considered to be consistent with normal skin.

Immunohistochemistry (IHC) stains

1. Distribution and location of FGF18

Positive staining was detected as fine granular cytoplasmatic staining. The cells of the arrector pili muscles showed moderate to marked staining in all tissue samples. Therefore this helped serve as an internal control. Moderate specific staining was also detected in the myoepithelial cells of the apocrine glands and in vascular endothelial cells (Figure 1). There was no staining of keratinocytes in the epidermis, sebaceous glands or dermal fibroblasts in any of the samples. Positive cytoplasmic staining was present within some hair follicles. When positive staining was seen, it was always in the inferior segment of the follicle.
Figure 1. Photomicrograph. Positive staining of the myoepithelial cells of the apocrine glands (black arrow), cells of the arrector pili muscles (asterisk) and vascular endothelial cells (red arrow). Immunohistochemistry for FGF18. Bar = 0.2 mm.

Figure 2. Photomicrograph. Positive cytoplasmic staining present in the inner root sheath transverse section (long arrow). Note positive staining of the myoepithelial cells of the apocrine glands (short arrow). Immunohistochemistry for FGF18. Bar = 0.1 mm.
More specifically it was visualised in the inner root sheath and only rarely in the outer root sheath and dermal papilla (Figures 2 and 3). The positive follicles were found in the deep dermal and superficial panniculus. These positive follicles were anagen follicles, indicated by the presence of an inner root sheath (Figure 4). No marked difference in immunohistochemical FGF18 expression was found when subjectively assessing the different tissue samples under the microscope.
Figure 4. Photomicrograph. Positive cytoplasmic staining present in the inner root sheath longitudinal section indicated with arrow. Immunohistochemistry for FGF18. Bar = 0.1 mm.
2. Quantification of FGF18

The total counts of the number of FGF18 positive follicles and FGF18 positive cells per 7 follicular units per dog and per sampling day and area are provided in table 1.

No effects (sex, location, season and day) were statistically significant for the number of positive follicles or the number of positive follicular cells except for the 3 factor interaction season-day-sex. This factor 3 interaction was significant with \( p < 0.008 \) for the number of positive follicles. The 3 factor interaction season-day-sex for the number of positive cells in the follicles had a \( p < 0.021 \). This indicates a considerable lack of parallelism in those data.

3. Macroscopical evaluation of hair regrowth

During the study notes were taken on the visual hair regrowth. Actual hair growth rates were not measured. In both February and August hair regrowth in the shoulder area was faster than in the flank area in all the dogs. Dog 4 (female intact) had the fastest hair regrowth in both seasons whereas dog 10 (male intact) had the slowest hair regrowth in both seasons.
Table 1: number of positive cells and positive follicles in seven follicular units per sampling occasion and area. Dog 1, 2 and 3 are female spayed, dog 4, 5 and 6 are female intact and dog 7, 8, 9 and 10 are male intact. F: February, A: August, D: day, / no seven follicular units available for counts.

<table>
<thead>
<tr>
<th>Dog 1</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>13</td>
<td>77</td>
</tr>
<tr>
<td>F D1</td>
<td>17</td>
<td>151</td>
</tr>
<tr>
<td>F D3</td>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td>F D7</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>F D17</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>A D0</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>A D1</td>
<td>7</td>
<td>108</td>
</tr>
<tr>
<td>A D3</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td>A D7</td>
<td>12</td>
<td>61</td>
</tr>
<tr>
<td>A D17</td>
<td>10</td>
<td>75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 2</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>F D1</td>
<td>6</td>
<td>45</td>
</tr>
<tr>
<td>F D3</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>F D7</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>F D17</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>A D0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>A D1</td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td>A D3</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>A D7</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>A D17</td>
<td>5</td>
<td>33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 3</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>F D1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>F D3</td>
<td>4</td>
<td>88</td>
</tr>
<tr>
<td>F D7</td>
<td>5</td>
<td>128</td>
</tr>
<tr>
<td>F D17</td>
<td>15</td>
<td>87</td>
</tr>
<tr>
<td>A D0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A D1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A D3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>A D7</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>A D17</td>
<td>8</td>
<td>51</td>
</tr>
</tbody>
</table>
Table 1 continued

<table>
<thead>
<tr>
<th>Dog 4</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>15</td>
<td>70</td>
</tr>
<tr>
<td>F D1</td>
<td>31</td>
<td>176</td>
</tr>
<tr>
<td>F D3</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>F D7</td>
<td>17</td>
<td>61</td>
</tr>
<tr>
<td>F D17</td>
<td>17</td>
<td>83</td>
</tr>
<tr>
<td>A D0</td>
<td>14</td>
<td>84</td>
</tr>
<tr>
<td>A D1</td>
<td>14</td>
<td>71</td>
</tr>
<tr>
<td>A D3</td>
<td>10</td>
<td>83</td>
</tr>
<tr>
<td>A D7</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>A D17</td>
<td>14</td>
<td>94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 5</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F D1</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>F D3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>F D7</td>
<td>9</td>
<td>55</td>
</tr>
<tr>
<td>F D17</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A D0</td>
<td>6</td>
<td>73</td>
</tr>
<tr>
<td>A D1</td>
<td>7</td>
<td>88</td>
</tr>
<tr>
<td>A D3</td>
<td>6</td>
<td>61</td>
</tr>
<tr>
<td>A D7</td>
<td>12</td>
<td>219</td>
</tr>
<tr>
<td>A D17</td>
<td>6</td>
<td>62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 6</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>F D1</td>
<td>11</td>
<td>57</td>
</tr>
<tr>
<td>F D3</td>
<td>12</td>
<td>99</td>
</tr>
<tr>
<td>F D7</td>
<td>11</td>
<td>69</td>
</tr>
<tr>
<td>F D17</td>
<td>23</td>
<td>188</td>
</tr>
<tr>
<td>A D0</td>
<td>10</td>
<td>121</td>
</tr>
<tr>
<td>A D1</td>
<td>9</td>
<td>77</td>
</tr>
<tr>
<td>A D3</td>
<td>10</td>
<td>41</td>
</tr>
<tr>
<td>A D7</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>A D17</td>
<td>10</td>
<td>82</td>
</tr>
</tbody>
</table>
Table 1 continued

<table>
<thead>
<tr>
<th>Dog 7</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>15</td>
<td>104</td>
</tr>
<tr>
<td>F D1</td>
<td>8</td>
<td>56</td>
</tr>
<tr>
<td>F D3</td>
<td>10</td>
<td>114</td>
</tr>
<tr>
<td>F D7</td>
<td>6</td>
<td>27</td>
</tr>
<tr>
<td>F D17</td>
<td>9</td>
<td>78</td>
</tr>
<tr>
<td>A D0</td>
<td>8</td>
<td>67</td>
</tr>
<tr>
<td>A D1</td>
<td>19</td>
<td>278</td>
</tr>
<tr>
<td>A D3</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>A D7</td>
<td>14</td>
<td>78</td>
</tr>
<tr>
<td>A D17</td>
<td>10</td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 8</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>F D1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>F D3</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>F D7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>F D17</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>A D0</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>A D1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>A D3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A D7</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>A D17</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 9</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F D1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F D3</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>F D7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F D17</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>A D0</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>A D1</td>
<td>11</td>
<td>60</td>
</tr>
<tr>
<td>A D3</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>A D7</td>
<td>12</td>
<td>51</td>
</tr>
<tr>
<td>A D17</td>
<td>14</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 10</th>
<th>Shoulder</th>
<th>Flank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># follicles</td>
<td># cells</td>
</tr>
<tr>
<td>F D0</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>F D1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F D3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F D7</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>F D17</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>A D0</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>A D1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>A D3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A D7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A D17</td>
<td>4</td>
<td>17</td>
</tr>
</tbody>
</table>
Discussion

To the authors’ knowledge, this is the first report describing the identification and localisation of FGF18 in the canine hair follicle. This study demonstrates positive FGF18 staining in the arrector pili muscle, myoepithelial cells of the apocrine glands and vascular endothelial cells.

In the hair follicle, FGF18 is found mainly in the inner root sheath and rarely in the outer root sheath. The positive follicles are found in the deep dermal and superficial panniculus. In addition most of the positive follicles were anagen follicles, indicated by the presence of an inner root sheath (Scott et al., 2001b). These findings are comparable to those reported by Kawano et al. (Kawano et al., 2005) in which the site of FGF18 mRNA expression in mouse skin moved to the transient portion of the inner root sheath with progression of hair follicle growth; with a peak of mRNA expression at the bulge region of follicles in the late telogen phase, just prior to anagen initiation. This led to the hypothesis that FGF18 is involved in regulating hair growth in mice (Kawano et al., 2005). We were unable to demonstrate marked FGF18 presence in telogen follicles. As our study was an IHC study of FGF18 demonstrating the protein it is possible that there was a discrepancy between the FGF18 mRNA expression site and the localization of FGF18 protein.

It seems not unreasonable that gene transcription during the late telogen phase in mice, as detected by PCR, precedes the stage in which FGF18 protein is found (anagen phase in our dog study). Although our study was conducted in dogs, and no mRNA expression was measured it is generally thought that the most basic controls of hair follicle cycling are not different between mammalian species (Tobin, 2009).

Further evidence for the role of FGF18 in the anagen follicle is found in human patients with alopecia areata, where FGF18 was found to be down-regulated in lesional skin (Subramanya et al., 2010).
Moreover, FGF18 is found to be a direct target of the canonical Wnt signalling, an essential signalling pathway for hair formation (Subramanya et al., 2010; Shimokawa et al., 2003; Van Mater et al., 2003).

Although the specific antibody used in this study was a polyclonal human antibody positive staining was considered to be a true reflection of the canine FGF18 in the skin because there is a high degree of antibody cross-reactivity. Indeed, there is a reported 99% identical amino acid sequence between mouse and human, and the nucleotide sequence is 90% conserved (Hu et al., 1998). The advantage of a polyclonal antibody is that a broader spectrum of epitopes can be recognized, which is preferable in a pilot investigation such as this one. Moreover, with our control samples we demonstrated that the antibody cross-reacted sufficiently with canine antigen in a specific manner.

The function of FGF18 is dependent on the spatial and temporal expression of FGF receptors (FGFr) (Haque et al., 2007). There are four known FGFr in the murine hair follicle: FGFr1, FGFr2, FGFr3, FGFr4 (Rosenquist and Martin, 1996). In a study conducted by Rosenquist and Martin, FGFr4 was found to be expressed in the inner root sheath, outer root sheath and hair matrix (Rosenquist and Martin, 1996). FGFr4 is a known ligand for FGF18 in human lung (Falvella et al., 2009).

Studies of specific receptor selectivity for FGF18 in the hair follicles have not yet been reported. Indeed, investigating the specific interaction of FGF18 with the four known FGFr in the hair follicle might further elucidate the exact function of FGF18 in the hair follicle.

This study was performed with beagle dogs because hair growth has been well described in this breed (Al-Bagdadi et al., 1977; Gunaratnam and Wilkinson, 1983; Scott et al., 2001c). Beagle dogs have a coat typified as a “normal coat” where the coat is composed of coarse guard hairs and a high proportion of fine secondary hairs (Al-Bagdadi et al., 1977; Gunaratnam and Wilkinson, 1983).
For this study, the two anatomical sites of the shoulder and flank were chosen because they are large enough to have ten samples taken with 2cm margins from each other from each site. The flank is an anatomical region that is often affected in dogs with non-inflammatory alopecia, such as seasonal recurrent flank alopecia or hypothyroidism.

The shoulder is rarely affected in non-inflammatory alopecia (Scott et al., 2001a). Several studies have demonstrated that hair follicles in the flank region exhibit a different hair growth pattern than for example the shoulder (Scott et al., 2001c; Gunaratnam, 2008). This is in accordance with our visual evaluation of the hair regrowth, where we had the clinical impression that hairs in the shoulder area had a faster growth rate than those in the flank area.

No statistical differences were found when examining the number of positive cells or the number of positive follicles in the shoulder and flank. Different growth rates have been reported for different anatomical regions. Gunaratnam and Wilkinson found that hair growth was slightly more rapid in the shoulder region, when compared to the flank region and the forehead in three out of four male normal coated dogs. They found that in those areas where the final length of the hairs was longer, the hair grew faster (Al-Bagdadi et al., 1977; Gunaratnam, 2008). The duration of anagen dictates the final length of the hair shaft (Diaz et al., 2004). On a molecular level termination of anagen hair growth has been linked to an increase in transforming growth factor beta and induction of catagen has been linked to FGF5 (Alonso and Fuchs, 2006).

No statistical difference in cell counts of FGF18 at the cellular or follicular level on the different days of sampling (days 0, 1, 3, 7 and 17) were found. In taking consecutive biopsies after clipping the beagle dogs we wanted to synchronise follicles and induce anagen hair growth. In this way we were able to monitor possible changes in the amount of immunohistochemical FGF18 protein expression during the different hair cycle stages.
Clipping without injury to the skin surface and follicle epithelium was found not to be traumatising enough to the follicles to induce synchronous growth in dogs. Indeed, in order to induce anagen hair growth more trauma needs to be done, for example by vigorous shaving, plucking of the hairs or chemical exposure. Our study suggests that the traumatic stimulus needs to exceed a certain minimum threshold before synchronized growth occurs. Plucking a single hair for example will not initiate hair growth, as at least 1000 hair shafts need to be plucked (Stenn and Paus, 2001). Taking the biopsies was also found not to be traumatising enough to synchronise the follicles, nor did it induce sufficient inflammation to be detected microscopically in the skin biopsies taken on days 1, 3, 7 or 17.

No statistical differences were found when examining the number of positive cells or the number of positive follicles in the male, intact female and female spayed dogs.

Different growth rates have been reported when intact female or male dogs are compared to spayed or neutered dogs. Butler and Wright found that spayed females showed good growth during most of the year, while entire males and females showed virtually no growth in late winter and summer. In that study it was suggested that there are seasonal effects on growth rate that are unrelated to those caused by seasonal variations in hormone levels in intact dogs (Butler and Wright, 1981). It is possible that those hormonal influences in normal dogs act as modifiers of hair growth and are not basic to all hair growth or hair follicle cycling.

No statistical differences were found when examining the number of positive cells or the number of positive follicles at the two different times of sampling. Dogs were sampled in February correlating to winter and during August for summer. Peak growth has been reported in summer in intact dogs; indeed environmental effects of hair growth have been reported to be most marked in intact animals (Al-Bagdadi et al., 1977; Butler and Wright, 1981).
Although the seasonal moulting has been shown to be influenced by the environment (light, temperature and nutrition) and systemic (endocrine factors) parameters, it is generally thought that there is considerable innate local control of hair shedding (Stenn and Paus, 2001).

Limitations of the study are that only a small number of dogs from one particular breed were included. Also, FGF18 was counted in only seven follicular units per slide. This number of follicular units was chosen because these are always present in an 8mm biopsy. An 8mm diameter piece of skin is small and is a reflexion of the follicular action in that particular area at that exact moment in time, thus representing a snapshot of the follicular events. As consecutive biopsies were taking in a 17 day time period, we do think that the findings are representative of what occurs at the follicular level of a normal coated, healthy beagle. It would be interesting to investigate the FGF18 presence in different breeds, particularly in dogs with a long anagen hair period, such as the poodle, and dogs with a long telogen hair period such as the husky. Also further studies should be conducted to profile possible dynamic changes in immunohistochemical FGF18 protein expression and FGF18 mRNA expression over the course of the canine hair follicle growth cycle.

In summary, the results of the present study confirm that FGF18 is present in the normal canine hair follicle. FGF18 is mostly found in the inner root sheath of anagen hair follicles. This may indicate a role in hair follicle cycle regulation, but the exact role remains obscure. The results of this study set the basis for further investigation in the potential of induction of anagen follicles or maintenance of the anagen phase by FGF18.
References


CHAPTER 3

Immunohistochemical evaluation of FGF18 in canine recurrent flank alopecia

Sophie Vandenabeele¹, Sylvie Daminet¹, Luc Van Ham¹, Thomas B. Farver², Hilde De Cock³

¹Department of Medicine and Clinical Biology of Small Animals, University of Ghent, Merelbeke, Belgium
²Population Health and Reproduction, University of California, Davis, California
³Veterinary Pathology Services/Medvet, Antwerpen, Belgium

This study was presented in part at the 24th Annual Congress of the ECVD-ESVD in September 2009 in Bled. An abstract was published in Veterinary Dermatology Volume 21, Number 2, April 2010.

Adapted from: Veterinary Dermatology 2012; 23: 461-462.
Abstract

Canine recurrent flank alopecia is a localized cyclic follicular dysplasia. Recently, FGF18 has been found to be important in the regulation of hair regrowth in adult mice. With this information available on the role of FGF18 it is interesting to look at this form of canine alopecia at the level of growth factor differences.

The objective of this study was to review the histopathological changes in dogs with CRFA and to study the presence of immunohistochemical FGF18 protein expression in affected follicles of dogs with CRFA.

Eight mm punch biopsies were taken from the flank of ten healthy Beagle dogs and from the lesional skin (flank) of 19 dogs with CRFA. All skin samples were routinely processed. Four µm paraffin sections of each biopsy were immunohistochemically stained using an anti-FGF18 antibody. Positive staining was seen as a fine granular cytoplasmatic staining. The amount of FGF18 was evaluated by counting the positive cells and the number of positive follicles in seven follicular units per biopsy.

FGF18 was detected in the hair follicles in the inner root sheath and seldomly at the level of the outer root sheath and dermal papilla. There was a statistically significant higher amount of FGF18 positive cells and positive follicles in the healthy dogs compared to the dogs with CRFA (2 sample t-test, p= 0.004).

These findings justify further studies to investigate the role of FGF18 as an anagen inducing factor in non-inflammatory alopecic diseases such as CRFA.
Introduction

Canine recurrent flank alopecia (also known as seasonal flank alopecia, seasonal growth hormone deficiency, canine idiopathic cyclic flank alopecia or follicular dysplasia) is a visually striking disease. It is characterized by often recurrent episodes of non-inflammatory, usually bilateral symmetrical alopecia most often confined to the thoracolumbar skin. The alopecic area has well demarcated borders and can be markedly hyperpigmented. The diagnosis is based on history, clinical signs and ruling out other causes of noninflammatory alopecia especially hypothyroidism (Paradis, 2009; Scott et al., 2001a).

A genetically influenced dysregulation of melatonin has been suggested to play a role in the pathogenesis of this disease, but the exact pathogenesis of CRFA remains unclear (Paradis, 2009). What causes the follicular arrest and what initiates the hair regrowth in these patients?

Studies suggest that growth waves are controlled by factors intrinsic to the hair follicle groups. This inherent rhythm, however, is influenced by neighboring follicles and/or systemic (e.g., endocrine) stimuli. So, although the cycle is intrinsic and essentially autonomous, it is influenced by environmental, systemic and local factors (Stenn and Paus, 2001; Paus, 1998). Previous studies have been unable to demonstrate underlying systemic abnormalities such as endocrinopathies, including hypothyroidism in CRFA (Daminet and Paradis, 2000; Paradis, 2012).

Hair growth is intrinsically regulated by several cytokines and growth factors (dual mode regulation). Indeed, studies suggest that growth factors are responsible for inductive properties of the papilla (Stenn, 2003). Of these growth factors we do not yet know how any one impacts the follicle cycling. The human/mouse FGF family consists of at least 23 members exhibiting a variety of biologic activities (Itoh and Ornitz, 2011). Among these FGF FGF18 has been reported to be involved in hair growth in mice. It was shown that FGF18 mRNA peaked during telogen in mice, just prior to anagen initiation (Kawano et al., 2005). It would thus be interesting in alopecic diseases with hair follicle arrest to investigate the presence of FGF18. Possibly a peak in FGF18 protein could be demonstrated prior to hair regrowth.
Vandenabeele et al. demonstrated the presence of FGF18 protein in the canine hair follicle of normal Beagle dogs. In that study the FGF18 protein was mainly found in anagen hair follicles (Vandenabeele et al., 2011).

The objective of this study was to review histopathological changes in patients with CRFA and to compare immunohistochemical FGF18 protein expression in normal canine hair follicles from flank skin with hair follicles of flank skin of dogs affected with CRFA.

**Materials and methods**

All procedures dealing with animal care, handling and sampling were reviewed and approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine University of Ghent (Approval EC2006/88).

The control group consisted of ten healthy Beagle dogs without any dermatological problems: three intact female, 3 female spayed and 4 male. The age of the animals ranged from 2 years to 7 years (median 4 years). All control dogs were assessed as being healthy based on a physical exam and results of a complete blood cell count and chemistry profile, including thyroxine (T4) and thyroid stimulating hormone (TSH) levels. These dogs were all housed similarly in a non-climate controlled area with outdoor exposure, thus being exposed to the same fixed length of photoperiod and fed a same commercial diet.

The second group consisted of 19 client owned dogs with CRFA. Clinical diagnosis of CRFA was based on compatible history and clinical signs, spontaneous regrowth in the alopecic area and absence of other clinical signs suggestive of endocrinopathies such as hypothyroidism. For this study only dogs with lesions on the flanks and thoracolumbar area were included.
Five different breeds were represented (Boxer n=6, Dalmatian n=2, Dobermann n=2, English Bulldog n=6, Bordeaux dog n=1, mixed breed dogs n=2). Seven were intact female, 9 female spayed, and 3 male neutered. The age of the animals ranged from 2 years to 9 years (mean 4.5 years). Biopsy specimens were obtained from lesional skin.

**Skin biopsies**

The Beagle dogs were biopsied under local anesthesia using an 8 mm biopsy punch on the flank in February. Prior to performing the biopsy a fine line was drawn with a permanent marker indicating the direction of hair growth.

The CRFA dogs were biopsied under local anesthesia on the flank with an 8 mm biopsy punch in the alopecic area of the flank.

The skin samples were fixed in 4% buffered formaldehyde for 48 h, bisected in the direction of hair growth visualized by the drawn line and embedded in paraffin wax. Four μm sections were cut from each tissue block. Control sections were stained with haematoxylin and eosin in order to detect any histopathological abnormalities in both groups.

**Immunohistochemical staining**

After deparaffinizing in xylene and rehydration (four steps: 100% alcohol, 95% alcohol, 50% alcohol to distilled water), an antigen retrieval technique was performed. In this procedure, slides were placed in citrate buffer (0.1M with 2% urea) and heated at 96°C for one period of 15 min in a microprocessor-controlled pressure chamber. The tissues were then kept in the citrate buffer-urea solution for 15 min at 4°C (cool down period).
Distilled water was added if necessary to prevent the slides from drying out. Endogenous peroxidase activity was blocked by incubation with 12% H2O2 in methanol (10min). The slides were then processed using a DAKO autostainer. Subsequently the slides were incubated with 30% normal rabbit serum (RUO; DAKO, Heverlee, Belgium) in phosphate-buffered saline (PBS) for 30 min to reduce non-specific antibody binding. A polyclonal antibody to human FGF18 (SC-16830; Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:50 in PBS was applied for 60 min. After rinsing the slides were incubated for 30 min with secondary antibodies (P0160; DAKO) diluted 1:200. After another rinse of 10 min, the slides are incubated for 5 min with AEC (AEC, DAKO, Merelbeke, Belgium). Counterstain was achieved by incubation with Mayer’s hematoxylin for 10 sec.

Negative controls were performed by replacing the first antibody with PBS. Positive controls were added in each run of the DAKO stainer and consisted of mouse lung tissue.

In each slide positive staining, evidenced by a fine granular cytoplasmic staining, was evaluated in seven follicular units using x400 magnification. One slide was counted per animal. The number of positive cells in the hair follicle epithelium and the number of follicles with positive cells were recorded.

**Statistical analysis**

The counts were logarithmically transformed to stabilize the variability in the data, but untransformed data revealed the same results after analysis.

A 2 sample t-test was performed to compare immunohistochemical FGF18 protein expression in normal canine hair follicles from flank skin with hair follicles of flank skin of dogs affected with CRFA.
Chapter 3

Results

HE stains

None of the samples of the Beagle dogs had an inflammatory infiltrate present. There were no changes present in these biopsies and they were consistent with normal haired skin.

Histopathologic findings observed in the lesional skin biopsies of the CRFA patients were consistent with CRFA and they were classified as non-inflammatory alopecia. The histopathological findings are summarized in table 1. Briefly, the epidermis was of normal thickness without evidence of hyperkeratosis. Epidermal hyperpigmentation was present in all but 4 samples (Dalmatian n=2, Bordeaux dog n=1, mixed breed dog n=1). Sebaceous and apocrine glands were normal sized.

The arrector pili muscles showed no degenerative changes. Hair follicle changes included dilated and truncated hair follicles with infundibular orthokeratotic hyperkeratosis extending to secondary hair follicles (Figure 1).
Melanin aggregates were present in the follicular lumen in 12 samples. Pigmentary incontinence evidenced by melanin spill out of the hair follicles and presence of perifollicular melanophages was noticed in 9 biopsies. Melanin aggregates were observed in sebaceous gland ducts and lumens (n=10). Pigmentation of the epithelium of the sebaceous glands and the apocrine glands (n=5) was also noticed. Anagen follicles were present in 6 tissue samples.
<table>
<thead>
<tr>
<th>Histopathologic changes</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermis</strong></td>
<td></td>
</tr>
<tr>
<td>Hypermelanosis</td>
<td>16</td>
</tr>
<tr>
<td><strong>Follicles</strong></td>
<td></td>
</tr>
<tr>
<td>Distorted follicles</td>
<td>8</td>
</tr>
<tr>
<td>Follicular hyperkeratosis</td>
<td>19</td>
</tr>
<tr>
<td><strong>Melanin aggregates</strong></td>
<td></td>
</tr>
<tr>
<td>Follicular lumen</td>
<td>12</td>
</tr>
<tr>
<td>Perifollicular pigmentary incontinence</td>
<td>7</td>
</tr>
<tr>
<td>Sebaceous gland</td>
<td>9</td>
</tr>
<tr>
<td>Sebaceous gland duct</td>
<td>9</td>
</tr>
<tr>
<td>Apocrine gland</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1. Summary of histopathological changes in the CRFA patients.
IHC stains

1. Localisation of FGF18

In all the samples of the control group and the CRFA group FGF18 was detected as a fine granular cytoplasmatic staining in the hair follicles at the level of the inner root sheath and seldomly outer root sheath and the dermal papilla. The follicles showing positive staining were located in the deep dermis or superficial panniculus and were anagen follicles (Figure 2).

Figure 2. Photomicrograph of flank skin of normal Beagle dog. Positive cytoplasmic staining present in the inner root sheath transverse section (long arrow). Note positive staining of the myoepithelial cells of the apocrine glands (short arrow). Immunohistochemistry for FGF18 (400x).
FGF18 positive staining was also detected in the myoepithelial cells of the apocrine glands, m.arrector pili and in vascular endothelial cells (Figure 3).

Figure 3. Photomicrograph of flank skin of dog with CRFA. Positive staining present in the myoepithelial cells of the apocrine glands (arrow) and arrector pili muscle (asterisk). Note the hyperpigmentation of the epidermis. Immunohistochemistry for FGF18 (100x).

In all samples arrector pili muscles had positive staining. Therefore this helped serve as an internal control. There was no staining of the nuclei within the epidermis or dermal fibroblasts in any tissue samples.

2. Quantification of FGF18 statistical analysis

The number of FGF18 positive cells in the hair follicle epithelium of seven follicular units of the control group ranged from 0 to 135 with an average of 42.6. The number of FGF18 positive follicles in seven follicular units in the control group ranged from 0 to 15 with an average of 5.4.
The number of FGF18 positive cells in the hair follicle epithelium of seven follicular units of the CRFA group ranged from 0 to 47 with an average of 7.79. The number of FGF18 positive follicles in seven follicular units in the CRFA group ranged from 0 to 12 with an average of 1.47.

There was a significant higher amount of FGF18 positive cells in the hair follicle epithelium of the control group compared to the follicles of CRFA affected skin (2 sample t-test, p< 0.05) (Figure 4A and Figure 4B).
Figure 4. Number of FGF18 positive cells in seven follicular units in normal dogs (A) and dogs with CRFA (B).
There was a significant higher amount of FGF18 positive follicles in the control group compared to the affected skin of the CRFA group (2 sample t-test, \( p < 0.05 \)) (Figure 5A and Figure 5B).

Figure 5A. Number of FGF18 positive follicles in 7 follicular units in normal dogs.

Figure 5B. Number of FGF18 positive follicles in 7 follicular units in CRFA dogs.

Figure 5. Number of FGF18 positive follicles in seven follicular units in normal dogs (A) and dogs with CRFA (B).
Discussion

Canine recurrent flank alopecia is described to occur at a variable age range with a mean age of 4 years (Paradis, 2009). The median age of the dogs in the present study was 4.5 years. As in previous reports, dogs of either sex were affected, but no intact male dogs with CRFA were present in this study. Most of the affected dogs were female intact and female spayed, as seen in a study reported by Miller et al. (Miller and Dunstan, 1993). There is a strong breed predilection for CRFA, and it is reported to be most commonly seen in Boxers, Airedale Terriers and English Bulldogs (Paradis, 2009; Scott et al., 2001a; Paradis, 2012; Stenn, 2003; Itoh and Ornitz, 2011). In the present study Boxers and English Bulldogs represent more than half of the enrolled patients. Two of the other included breeds have also been reported to be predisposed: the Dalmatian and the Doberman. To our knowledge, there have been no reports describing CRFA in the Bordeaux dog. Two of the patients in this present study were Bordeaux dogs. It is interesting to note that Bordeaux dogs have a same coat type being the fine short coat as the Boxer and the Bulldogs (Scott et al., 2001b). Indeed, CRFA is mainly reported in dogs with this type of coat, and is rare to absent in plush coated Nordic breeds, normal coat type German Shepherds, Beagle dogs and Cocker Spaniels (Paradis, 2012).

The prominent histopathological features of the CRFA in this study were the infundibular follicular hyperkeratosis with keratin extending into the primary and secondary follicles (witch’s feet) and sometimes even into the sebaceous gland ducts. Histologic changes were fundamentally the same as described in previous reports (Paradis, 2009; Scott et al., 2001a; Paradis, 2012; Miller and Dunstan, 1993; Gross, 2005).

It is interesting to note however that some patients develop epidermal hyperpigmentation and others do not. This seems to be a breed specific finding, where especially the Dalmatian patients do not present with epidermal hyperpigmentation. Moreover, the authors have the clinical impression that CRFA patients that do not present with epidermal hyperpigmentation or perifollicular pigmentary incontinence have a shorter period of alopecia.
An article discussing seasonal flank alopecia in Boxers and Airedale Terriers found that the mean duration of the alopecic period was longer in Airedales than in Boxers. It was assumed that because the Boxers have a shorter hair coat, the anagen period would have to be shorter and a full length coat regrowth would take less time. Another finding in that study was the more pronounced hyperpigmentation and perifollicular pigmentary incontinence of the Airedale Terriers (Miller and Dunstan, 1993).

Epidermal hyperpigmentation in dogs can be genetic or acquired. Acquired hyperpigmentation can be present as a postinflammatory lesion (Scott et al., 2001c). As CRFA is a non-inflammatory alopecia this cannot explain the epidermal hyperpigmentation. Acquired hyperpigmentation can also develop when there is an increase in exposure to UV radiation (for example in alopecic skin) (Scott et al., 2001c). As hyperpigmentation seen in patients with CRFA is present before the development of complete alopecia ultraviolet exposure seems an unlikely cause for the hyperpigmentation. Moreover, the lesions most commonly occur between November and March, months in which the daylight time is very sparse. Diffuse hyperpigmentation can result from metabolic or hormonal disorders too. The hyperpigmentation is proposed to be caused by a direct hormonal effect on the melanocytes (Scott et al., 2001c). The hyperpigmented areas in CRFA are well demarcated, making it difficult to link a systemic hormonal disorder to a regional hyperpigmentation. A regional melanocyte stimulation could explain the pattern of hyperpigmentation in the epidermis. Recently, it was shown that melanogenesis and melanocyte stimulation can also occur in a paracrine and autocrine fashion (Fischer, 2008). Melanin aggregates were commonly observed in the follicular lumen and many sebaceous gland ducts and within sebocytes. Melanin was also found in the apocrine cells and perifollicular. The melanin in the periadnexal structures is probably caused by spill over and stasis from the follicular lumens. These findings are consistent with an event where sudden hair cycle arrest without previous arrest of follicular pigmentation results in uneven distribution of the melanin. Melanin will then spill out of the follicles and will be noticed as perifollicular melanin aggregates (pigmentary incontinence) (Mecklenburg, 2009).
Currently, there is still a debate whether CRFA should be classified as an anagen dysplasia or a hair cycle abnormality (Mecklenburg, 2009).

The control group consisted of flank skin of healthy Beagles. Another possibility would have been to use breed matched samples. As the etiology of CRFA probably implies a genetic predisposition to have a decreased pro opiomelanocortin (POMC) production due to less light exposure it is difficult to compare affected dogs to breed matched samples too, as those privately owned dogs would have had completely different light exposures. Using the Beagle dog population enabled us to have a control group where all environmental and nutritional parameters were the same for every dog in the control group, however, CRFA has never been reported in Beagle dogs (Paradis, 2009).

To our knowledge investigations of FGF18 in alopecic canine skin have not been performed previously.

The present work demonstrates a statistically significant difference in immunohistochemical FGF18 protein expression in the epithelium of hair follicles when comparing the control group consisting of flank skin of healthy Beagles biopsied in the same season as when CRFA is seen with affected skin of dogs with CRFA. FGF18 was present in the hair follicles virtually exclusively in the inner root sheath and seldomly in the outer root sheath or dermal papilla. It was also found in the apocrine glands, arrector pili muscles and vascular endothelial cells. CRFA typically is characterized histopathologically by a catagenisation and telogenisation of follicles (Paradis, 2009; Scott et al., 2001a; Paradis, 2012; Miller and Dunstan, 1993; Gross, 2005). This implies that in those follicles an IRS is absent (Scott et al., 2001d). The absence of FGF18 in the CRFA patients is thus not unexpected. In some patients with CRFA FGF18 was visualised immunohistochemically in the hair follicles. When follicular FGF18 was present, it was seen in the IRS of follicles, either from biopsies taken from the margin of the alopecic skin or biopsies exhibiting microscopic hair regrowth that was not evident macroscopically.
Indeed, if biopsies are taken during the resolution phase most follicles will be in early or late stages of anagen (Paradis, 2009).

These results are similar to what has been described previously in the study by Vandenabeele at al. where FGF18 protein was found in anagen hair follicles of healthy Beagle dogs and a study by Kawano et al. in mice where FGF18 mRNA expression is present in the transient portion of the inner root sheath close to the hair bulb (Kawano et al., 2005; Vandenabeele et al., 2011).

Our study could not demonstrate any FGF18 in the telogen hair bulb, as described by Kawano et al. Indeed, Kawano et al described a peak in FGF18 mRNA just prior to anagen initiation in mice. As our study is an immunohistochemical study, it is not impossible that the presence of FGF18 protein in anagen follicles is preceded by FGF18 mRNA expression in canine hair follicles.

CRFA is a fairly common skin disorder in areas of the world around or north of the 45°parallel. Although this skin disease occurs in otherwise healthy animals and could thus be considered a cosmetic disease, many owners find the entity unpleasing and demand a treatment.

Treatment with melatonin has been reported to be beneficial, but large placebo controlled studies have not been performed (Paradis, 2000; Paradis, 1995). Moreover, melatonin is not widely available. FGF18 has been reported to induce anagen hair growth in mice and has been reported to be potentially useful in the treatment of non-inflammatory alopecia (Kawano et al., 2005; Beenken and Mohammadi, 2009). FGF18 could thus potentially be an alternative treatment for CRFA.

In order to evaluate its potential therapeutic usage, it was important to demonstrate differences in immunohistochemical FGF18 protein expression in CRFA when compared to normal skin.
In conclusion we can state that FGF18 is present in actively cycling follicles and virtually absent in CRFA affected skin when biopsies are taken before the resolution phase. The exact role of FGF18 in the hair follicle cycle is unknown, but as this fibroblast growth factor is present in anagen follicles, it may play a role in inducing anagen or maintaining follicles in an anagen fase. FGF18 injections were able to induce anagen from telogen stage hair follicles in mice (Kawano et al., 2005). Moreover FGF18 has been proposed as a possible therapeutic agent for non-inflammatory alopecia (Kawano et al., 2005; Beenken and Mohammadi, 2009). These findings justify further studies to investigate the role of FGF18 as an anagen inducing factor in non-inflammatory alopecic diseases such as CRFA.
References


CHAPTER 4

Study of the behaviour of lesional and non lesional skin of canine recurrent flank alopecia transplanted to athymic nude mice

Sophie Vandenabeele\textsuperscript{1}, Hilde De Cock\textsuperscript{2}, Luc Van Ham\textsuperscript{1}, Evelyne Meyer\textsuperscript{3}, Sylvie Daminet\textsuperscript{1}

\textsuperscript{1}Department of Medicine and Clinical Biology of Small Animals, University of Ghent, Merelbeke, Belgium

\textsuperscript{2}Veterinary Pathology Services/Medvet, Antwerpen, Belgium

\textsuperscript{3}Department of Pharmacology, Toxicology and Biochemistry, University of Ghent, Merelbeke, Belgium.

This study was presented in part in the non-pruritic hair loss workshop at the 7th World Congress in Veterinary Dermatology In Vancouver, Canada.

Adapted from: Veterinary Dermatology 2013; 24: 507-512.
Abstract

Background - Recurrent flank alopecia is a clinically well described skin disorder in dogs. The pathomechanism of the disease is difficult to study because it undergoes spontaneous resolution.

Objectives - To study the behaviour of xenografts in mice in order to assess the feasibility of a reproducible experimental model and to investigate local or systemic causes of canine recurrent flank alopecia (CRFA).

Animals - Two client-owned dogs with CRFA and five athymic nude mice from a research facility.

Materials and methods - Skin biopsies were taken from lesional and non lesional skin of dogs with CRFA and grafted onto five athymic mice. The lesional skin and xenografted skin were evaluated histologically on day 0 and day 30 respectively.

Results - Transplanted lesional and non lesional canine skin regrew hair within 30 days, while the donor dogs were still alopecic in the lesional areas post skin biopsy procedure on day 30. Graft rejection was evidenced histologically in two xenografted athymic mice. Lesional hyperpigmentation disappeared in the athymic mice.

Conclusions and clinical importance - This study showed that hair follicles from dogs with CRFA quickly regenerated and regrew hair once grafted onto the mice. Our data indicates that the pathogenesis of CRFA is likely mediated by systemic rather than local factors. While this xenograft approach might be less valuable for the study of CRFA it has potential value for the study of other causes of canine alopecia due to systemic factors.


**Introduction**

Canine recurrent flank alopecia is a visually striking skin disorder characterized by recurrent episodes of well demarcated non inflammatory alopecia most often confined to the thoracolumbar region (Paradis, 2009). In the Northern Hemisphere alopecia develops between November and March. Spontaneous regrowth of hair usually occurs within three to eight months but a number of cases progress to a permanent flank alopecia with marked hyperpigmentation (Gross et al., 2005; Paradis, 2012). The exact aetiopathogenesis of this disease is currently unknown. The seasonal nature and recurrence of CRFA suggest that the photoperiod may be involved. A genetically influenced melatonin deficiency also has been proposed (Paradis, 2009). Melatonin is a hormone synthesized primarily in the pineal gland and is secreted at night. In human and murine skin extra epiphyseal melatonin production has also been demonstrated (Fischer et al., 2008). Research into the exact pathogenesis of CRFA is complicated due to its seasonal nature and unpredictable duration of alopecic phases. There is a recognized need for a reliable animal model to study the pathogenesis and to evaluate treatment options. In recent years athymic (nude) mice and severe combined immunodeficient (SCID) mice have been used to study a variety of human as well as canine dermatoses (Croy et al., 2001; Krajcik et al., 2003). Xenograft experiments with these animals are attractive as they allow the differentiation of extrinsic and/or systemic from localized factors in the pathogenesis of alopecic skin disorders (Gilhar and Etzioni, 1994).

The objectives of the current study were to study the behaviour of lesional and non lesional skin of dogs with CRFA on athymic mice in order to investigate whether it is caused by a systemic or local factor. Also, the study was designed to assess the feasibility of grafting to immunodeficient mice in order to create a reproducible model of this entity.
**Materials and methods**

**Approval**

All procedures dealing with animal care, handling and sampling were reviewed and approved by the University of Ghent Institutional Animal Care and Use Committee (Approval EC2009/128).

**Animals**

Five athymic nude mice (CD1-foxn1nu) were used (Harlan laboratory, Boxmeer, The Netherlands). The animals were 12 weeks of age at receipt and were allowed to adjust to laboratory conditions for two weeks before the onset of the study. All mice were singly housed in individual ventilated cages with granulated wood and cage enrichment and maintained on a photoperiod of 14 hours of light and 10 hours of dark. Food and sterile distilled water were provided ad libitum.

Skin samples of two privately owned dogs with CRFA were used to transplant on the athymic nude mice. Donor dog one was a three year old female spayed Boxer (dog A). This dog had a brown coloured coat with sparsely pigmented skin. The lesional area was markedly hyperpigmented. The second donor was a three year old male neutered cross breed dog (dog B). This dog had a black coat and grey pigmented skin. Lesional hyperpigmentation was not seen. Lesional hyperpigmentation in CRFA is usually prominent in Boxer dogs while it can be absent in certain dog breeds such as the Wirehaired Pointer (Paradis, 2009).
The diagnosis of CRFA in both donor dogs was confirmed by a compatible history (both dogs had exhibited the same clinical signs with spontaneous hair regrowth in the previous year), clinical signs (well demarcated areas of non-inflammatory alopecia confined to the flanks), dermatopathology and absence of other clinical signs suggestive of endocrinopathies such as hypothyroidism. Complete blood cell count and serum chemistry profile, including thyroxine (T4) and thyroid stimulating hormone (TSH) levels were within normal limits for both dogs.

Harvesting and preparation of skin grafts and transplantation onto nude athymic mice was performed as follows:

First the hair coat from the left shoulder of both dogs was clipped. This area and the lesional skin area on the left flank were scrubbed with a chlorhexidine (Hibiscrub®, Regent Medical, Two omega drive, Manchester, M445BJ, United Kingdom) and ethanol solution (Ethanol VWR®, Stella, Geldenaaksebaan 464, 3001 Leuven, Belgium).

Full thickness canine skin tissue was harvested using a 6 mm skin biopsy punch using local anesthesia (0.5ml injection of 2% lidocaine (Xylocaine®, Astra Zeneca, Rue Egide Van Ophem 110, 1180 Brussel, Belgium) subcutaneously). Three and two skin samples from the shoulder and four and three skin samples from the flank area from dog A and B respectively, were obtained.

One skin sample from the flank of each dog was directly fixed in 4% neutral buffered formalin. The subcutaneous tissue was dissected from the remaining skin samples and tissues where protected on a gauze moistened with sterile 0.9% NaCl solution (Mini Plasco NaCl B.Braun 0.9%®, B.Braun Medical NV, Woluwelaan 140b, 1831 Diegem, Belgium) prior to transplantation onto the athymic mice.
The mice were anesthetized (intraperitoneal injection of xylazine at 10mg/kg (Xyl-M 2%, VMD, Hoge Mauw 900, 2370 Arendonk, Belgium) and ketamine 100mg/kg (Anesketin®, Eurovet, Poorthoevestraat 4, 3550 Heusden-Zolder, Belgium) according to body weight) and received an intraperitoneal injection of buprenorphine 0.1 mg/kg (Vetergesic®, Reckitt Benckiser Healthcare, Dansom Lane, Hull, HU8 7DS, United Kingdom) for pain management. The dorsal skin of each mouse was cleansed with chlorhexidine scrub (Hibiscrub®, Regent Medical, Two omega drive, Manchester, M445BJ, United Kingdom) and ethanol solution (Ethanol VWR®, Stella, Geldenaaksebaan 464, 3001 Leuven, Belgium). After this two small incisions were made in vertical line in the intrascapular area. The trimmed skin samples were transplanted onto the mice. The most cranial skin transplant was from the shoulder (normal haired skin of donor dog) and the most caudal transplant was from lesional flank skin. Both lesional and non lesional skin transplanted onto an individual mouse was obtained from the same dog. Mouse 1, 2 and 3 were grafted with skin from dog A and mouse 4 and 5 received skin from dog B. A small amount of topical skin adhesive (Dermabound Ethicon®, Computerweg 14, 3800 Amersfoort, Netherlands) was applied to the margins of the tissue samples in order to close the incision. The animals were closely monitored throughout the postoperative period.

The mice were humanely euthanized 30 days after transplantation and the xenografts were removed entirely along with the surrounding mouse skin and underlying musculature and fixed in 4% neutral buffered formalin for 48 h. After embedding in paraffin wax, 4μm sections were cut from each tissue block (10 from the athymic mice and two skin tissue samples collected from the lesional skin of each of the dogs) and stained with haematoxylin and eosin (HE).
Results

At day 7 all the xenografts developed a thick dry crust containing all the hair shafts. This crust showed progressive shrinkage from the graft periphery over the next 21 days. At day 30, hair regrowth was evident in all but one xenograft (mouse 5). That specific graft of non lesional skin was partially extruded from the mouse skin and was seen as a thick crust. There was no macroscopic difference in the amount of hair regrowth of the grafts from lesional skin versus non lesional skin (Figure 1 and Figure 2).

Figure 1. Macroscopic pictures of dog A on day 0, athymic mouse 2 on day 0 and day 30. A. Clipped shoulder area of dog A on day 0 demonstrating the site of sampling for the non lesional skin and demonstrating the normal pigmentation of the skin. B. Lesional flank area of dog A on day 0 demonstrating lesional hyperpigmentation. C. Athymic nude mouse 2 on day 0 demonstrating the position of the xenografts in the intrascapular area. Note the difference in pigmentation between the non lesional skin (most cranial graft) and the lesional alopecic skin (most caudal graft). D. Photomicrograph: athymic nude mouse 2 on day 30. Note the hair regrowth in both grafts and similar skin pigmentation in both grafts.
Hair regrowth seemed to start at the periphery of the graft. The canine donors exhibited no macroscopic hair regrowth of lesional skin 30 days post biopsy procedure. The dogs did show hair regrowth at the flanks on approximately day 90.

Furthermore, the lesional skin of dog donor A still exhibited marked hyperpigmentation on day 30, whereas the skin grafts from lesional and non lesional skin on the athymic mice (mouse 1, 2 and 3) showed similar pigmentation on day 30.
This was in marked contrast to day 0 when hyperpigmentation of the lesional transplanted skin was very pronounced and a clear difference in the amount of pigmentation between lesional and non lesional skin was macroscopically observed.

Histological examination of the lesional canine skin at day 0 of both dogs revealed infundibular hyperkeratosis extending into the opening of the secondary follicles and melanosis of the sebaceous glands. The follicles were narrowed at the base (Figure 3). No anagen follicles were present. There was no significant inflammatory infiltrate present. The overlying epidermis was hypermelanotic in dog A.

Figure 3. Histologic image of lesional canine skin of dog A on day 0. Note the follicular hyperkeratosis and absence of anagen follicles. H&E. Bar = 200 µm.
Histological examination of the xenografts of lesional and non lesional skin at day 30 revealed fully fused borders of canine and murine skin in all but one xenograft (mouse 5, the above mentioned extruded xenograft) (Figures 4 & 5). The epidermis showed a mild hyperkeratotic acanthosis. Neovascularisation was seen in the dermis of all grafts.

A small amount of oedema was present in all skin samples in the dermis and slightly more pronounced in the perifollicular regions. Six of the ten xenografts had occasional minimal lymphocytic perivascular infiltrate. The hair follicles in the xenografts of lesional skin demonstrated some remaining follicular hyperkeratosis (Fig 5). Anagen follicles were present in all samples.

Figure 4. Histologic image of graft of non lesional canine skin to athymic mouse 3 on day 30 demonstrating presence of anagen follicles. Note the fully fused border of canine (C) and murine (M) skin (arrows). Canine anagen follicles are marked with an asterisk. H&E. Bar = 200 µm.
Figure 5. Histologic image of graft of lesional canine skin to athymic mouse 3 on day 30 demonstrating presence of anagen follicles and some remaining follicular hyperkeratosis. Note the fully fused border of canine (C) and murine (M) skin (arrows). Canine anagen follicles are marked with an asterisk. H&E. Bar = 200 µm.

Four of the ten skin grafts demonstrated histological evidence of a graft versus host reaction (GVH). It should be noted that these were both from the transplants of two mice (mouse 1 with grafts of donor dog A and again mouse 5 with grafts of donor dog B). Histological findings in these latter mice consisted of an interface dermatitis with a mononuclear cell infiltrate in the graft epidermis and follicular epithelium associated with scattered basilar and suprabasilar keratinocyte apoptosis. A mild multifocal to interstitial lymphohistiocytic infiltrate was present. This inflammatory infiltrate was most pronounced at the borders of canine and murine skin. Some pigmentary incontinence was present in the superficial dermis and perifollicular regions.
To our knowledge this is the first study reporting on the behaviour of lesional and non lesional skin from CRFA dogs in athymic mice. This study shows an equal regrowth of hair in grafts from lesional and non lesional canine skin within 30 days of transplantation onto athymic mice. In contrast, lesional skin of canine donors had no visible regrowth at that time. In one study examining the influence of the hair follicle cycle on skin allografts in athymic nude mice allografts with telogen skin demonstrated a faster and better hair regrowth compared to skin in the anagen hair follicle cycle (Ramselaar and Ruitenberg, 1981). Presumably we did not observe a difference in the amount of hair regrowth in lesional versus non lesional skin transplants because the non lesional skin of the canine donors also had a lot of hair follicles in the telogen phase. Indeed, in shorthaired dogs up to 90% of hair follicles may be in telogen phase during winter (Miller et al., 2013). The observed hair regrowth again proves that affected skin responds in an identical manner as normal skin once it is removed from the donor dog. Hair regrowth was seen starting from the periphery of the xenograft. This is most likely due to perfusion differences in the graft tissue, where tissue closest to the recipient skin is better perfused. Indeed, the presence of some dermal and perifollicular oedema in the xenograft tissue on day 30 is most likely the result of insufficient vascularization and resultant low grade ischemia.

The fading of the lesional hyperpigmentation within 30 days was an unexpected and very intriguing finding. In CRFA patients, this hyperpigmentation is often the first clinical change seen by the owner. Most but not all dogs with CRFA develop lesional hyperpigmentation and only certain dog breeds such as the shorthaired pointer do not develop lesional hyperpigmentation (Paradis, 2009). If present, this hyperpigmentation persists once hair regrowth is started. In this study depigmentation of hyperpigmented lesional skin was seen within 30 days. At that time the donor dog still demonstrated hyperpigmentation. When human skin is transplanted to athymic mice the pigment is retained up to six months post grafting.
Moreover, human skin grafted to athymic mice is known to hyperpigment spontaneously as a result of melanocyte proliferation and an increase in the number of melanosomes per cell with time (Boyce et al., 1993). The histologically confirmed graft versus host reaction seen in mouse 1 could explain the depigmentation of the skin. However, a GVH reaction was not seen in mice 2 and 3.

It is therefore more likely that our findings in mice suggest that the hyperpigmentation in dogs with CRFA is caused by the same systemic factor that causes the follicular arrest. We assume that transplanting the skin to athymic mice caused a change in this systemic factor enabling hair regrowth and restoration of normal skin pigmentation. Xenograft experiments in athymic mice allow investigators to differentiate if a follicular pathology is due to either a systemic or an intrinsic factor (Gilhar and Etzioni, 1994).

Our results strongly indicate that a systemic factor is involved in the initiation of the alopecia in the pathogenesis of CRFA.

Athymic mice accept both normal tissues and tumours. The rate of acceptance varies greatly. In one study the acceptance rate of guinea pig nevi to athymic mice was 58% (Stubbs et al., 1983). In our study a graft survival of 60% was achieved while rejection of the grafts occurred in two mice. Histologically the rejected grafts showed an interface dermatitis with vacuolar degeneration of the basal layer of the epidermis and follicular epithelium with exocytosis of lymphocytes as well as the presence of apoptotic keratinocytes. This observation was not unexpected as it is now generally accepted that nude mice are not immunologically inert (Croy et al., 2001). Most graft failures in the nude mice are attributed to unusual high natural killer cell activity (Croy et al., 2001).

During the study one graft of non lesional skin was partially extruded from the mouse skin. It is unclear if this was due to the GVH reaction or due to inadequate adherence. The principal consideration in ensuring success of grafting is adequate protection from chewing or scratching by the recipient.
By placing the grafts in the intrascapular area, scratching and biting to the area is limited. Some studies describe the use of Elizabethan collars, bandages or even plaster body casts. In this study no such measures were taken to protect the grafts (Krajcik et al., 2003; Manning et al., 1973). Single housing and placement of the grafts in a difficult to reach area were deemed enough as precautions to prevent damage to the grafts. Moreover, not bandaging the grafts enabled a good macroscopical evaluation throughout the study.

This study also demonstrated the formation of thick adherent crusts on the graft surface. These crusts were formed within the first week of grafting. They shrunk peripherally and eventually shed after three weeks.

Once the crusts disappeared healthy looking skin was evident. Similar findings were noted in a study describing feline grafting onto athymic mice and an allograft study in athymic mice (Ramselaar and Ruitenberg, 1981; Manning et al., 1973). It was found that the thickness of the crusts is related to the thickness of the transplanted skin (Manning et al., 1973). Caution should be taken not to misinterpret the formation of crusts as immunological rejection.

Because of the limited number of grafts a statistically valid conclusion cannot be made, however, a number of interesting observations have been described in this article.

Our results indicate that a systemic factor might be involved, at least partially, in the pathogenesis of CRFA. This would imply that transplants on immunodeficient mice cannot be used as a direct model for CRFA. Further studies are needed to define the systemic factor causing this unique canine skin disease.
References


GENERAL DISCUSSION
Canine recurrent flank alopecia is a frequently encountered dermatological problem in practices in higher latitudes such as Belgium (around or north of the 45° parallel in the northern hemisphere) (Paradis, 2009; Miller et al., 2013a). However, the pathogenesis is still unclear. The diagnosis of this disease is based on history, clinical findings, histopathology of skin biopsies and laboratory findings (Paradis, 2009; Miller et al., 2013a). Histologic changes indicate that there is a hair cycle arrest or impaired promotion of the hair cycle (Müntener et al., 2012). Why this hair cycle arrest, resulting in alopecia, is only seen in certain anatomical regions such as the thoracolumbar area is unknown.

The classical distribution of the lesions in CRFA is the lateral to dorsolateral thorax and lumbar region. Lesions consist of well circumscribed patches of alopecia exhibiting a geographic and irregular pattern (Paradis, 2009; Miller et al., 2013a). Several atypical presentations have occasionally been described in the literature and are recognized in practice: a generalized presentation, flank alopecia without an episode of visual alopecia and flank alopecia with interface dermatitis (Miller et al., 2013a; Miller et al., 2013b; Paradis, 2009; Declercq, 2008; Mauldin, 2005; Rachid et al., 2003). Other atypical clinical presentations of CRFA are recognized in practice, but are not documented in the literature. Good descriptions of these atypical presentations are important in understanding and characterising CRFA. Those atypical presentations may represent a breed specific presentation of CRFA.

In the first chapter, we documented an atypical presentation of CRFA were the lesions were limited to the face (facial folds and pinnae). Previously, lesions on the dorsal muzzle consisting of alopecia and hyperpigmentation have always been described in conjunction with lesions on the flanks (Miller et al., 2013a). Alopecia of the dorsal muzzle and pinnae in combination with alopecia of the flanks have been described by Paradis (Paradis, 2012). These presentations have been documented as generalized forms of CRFA (Miller et al., 2013a; Declercq, 2008).
This generalized form has been described in certain breeds such as Airedales, Golden Retrievers, Griffon Korthals, Dobermans, Wirehaired Pointers and Giant Schnauzers (Paradis, 2012; Miller et al., 2013a; Fontaine et al., 1998). The patient we reported on had lesions restricted to the facial folds and pinnae. This distribution of lesions is in contrast with the content of the latest edition of “small animal dermatology”, that states that the flanks are always affected in this disease (Miller et al., 2013a). The dog we described had three recurrent episodes of alopecia and spontaneous regrowth in three consecutive years. Alopecia started in the month of March or April and regrowth was seen in July. The diagnosis of CRFA was based on history (recurrent episodes of alopecia with spontaneous regrowth), histopathology and exclusion of hypothyroidism. However, until a good diagnostic test is available for the detection of CRFA, we cannot completely exclude that some of those atypical presentations could also potentially represent another disease or a concurrent disease. The characteristic feature of CRFA is a spontaneous regrowth, caused by a spontaneous re-entry of follicles in the anagen phase. This is not seen in dogs with hypothyroidism, because in hypothyroidism the follicles experience a continuous hair cycle arrest (Credille et al., 2001). Indeed, dogs with hypothyroidism can also present with alopecia and hyperpigmentation of the dorsal muzzle (Paradis, 2009; Frank, 2006; Rosychuk, 1998). It is interesting that both diseases can present with a similar distribution of alopecia. The reason why certain anatomical regions develop alopecia in CRFA and endocrine diseases such as hypothyroidism is not clear. It is speculated that patterns of alopecia may be explained by regionalization of hormone receptor numbers or different receptivity of these receptors (Bratka-Robia et al., 2002; Rosychuk, 1998). Indeed, certain areas of the body are always spared (for example distal extremities, dorsal head). This might be because a smaller amount of hormone is necessary to maintain follicular activity in these areas of the body (Rosychuk, 1998; Miller et al., 2013a). Furthermore, this regional difference in follicular receptivity, might also be breed specific (Rosychuk, 1998), explaining why there is a breed predisposition to develop CRFA, a generalized form of CRFA, or only the alopecia restricted to the face, as seen in our Cane Corso patient. Our case report also reemphasises that the current terminology used to describe this disease is not ideal. Indeed, our patient presented without any lesions on the flanks.
A better terminology would perhaps be “canine seasonally influenced temporary hair cycle arrest”, indicating the unknown etiology of this disease and the spontaneous hair regrowth, unique for this disease entity.

Because of the absence of systemic abnormalities in studies evaluating thyroid function, reproductive hormones and growth hormones in CRFA affected dogs (Curtis et al., 1996; Daminet and Paradis, 2000) and the unexplained pattern of alopecia, studies focusing on the local follicular level of CRFA are indicated. Indeed, with the increased knowledge and emphasis on molecular changes in the normal hair cycle, hair cycling abnormalities can now be studied at a follicular level too. Many growth factors have been evaluated and found to play a role in hair cycling. Previously, an impaired catagen propagation was reported to cause the hair cycle arrest in CRFA (Cerundolo and Mecklenburg, 2007; Mecklenburg, 2006), but the most recent research demonstrates that the anagen induction is impaired in this hair cycle disorder (Müntener et al., 2012). FGF18 acts in a paracrine manner, is mainly present at the end of the telogen phase and was able to induce anagen when injected in uniform telogen mice (Haque et al., 2007; Kawano et al., 2005). The knowledge of the presence and the localization of FGF18 in the canine hair follicle could be crucial to a better understanding of the molecular regulation of the normal cycling dog follicle and its function in canine alopecic diseases caused by hair cycling arrest, such as canine recurrent flank alopecia.

In the second and third chapter we first identified, located and quantified FGF18 in the normal canine hair follicle in association with different parameters such as location on the body, sex, season and phase in the growth cycle. Next these results were compared with location and quantity of FGF18 in canine follicles affected by CRFA. FGF18 protein was visualized in the normal canine skin and CRFA affected skin with an immunohistochemical technique using an anti-FGF18 antibody. In both studies, FGF18 was detected immunohistochemically in the apocrine glands, in arrector pilli muscles and in vascular endothelial cells.
In follicles, FGF18 was detected as granular cytoplasmatic staining in follicles at the level of the inner root sheath, rarely the outer root sheath and the dermal papilla. Most of the follicles with positive staining were anagen follicles. These findings are in accordance with a study conducted in mice by Kawano et al., who demonstrated with in situ hybridization that FGF18 mRNA is mainly expressed in the anagen inner root sheath and found that the site of mRNA expression in mouse skin moved to the transient portion of the inner root sheath with progression of hair follicle growth (Kawano et al., 2005). Kawano also described a peak in mRNA expression in the telogen bulge region of mice follicles, just prior to anagen initiation. However FGF18 mRNA expression was difficult to detect in the telogen bulge area as the signals there were rather weak (Kawano et al., 2005). More recent research suggested that FGF18 controls the telogen phase of the hair follicle: FGF18 mRNA was found to be expressed in telogen bulge cells in vitro and it was hypothesized that FGF18 places the bulge stem cells in a quiescent stage, suggesting that FGF18 regulates the telogen hair cycle phase (Greco et al., 2009).

The Kimura-Ueki research group demonstrated that FGF18 conditional knockout mice display a shortened telogen phase, providing further support for the role of FGF18 in the telogen follicles (Kimura-Ueki et al., 2012). Indeed, a conditional knockout defines a biological model in which a target gene can specifically be inactivated in specific tissues such as the skin while the target gene exhibits normal functions in the other tissues. This strategy bypasses the limits that can be observed with conventional knockout models, the biggest limit being embryonic lethality (Bockamp et al., 2008). Indeed, conventional FGF18 knockout mice could not be studied, because conventional FGF18 knockout in mice is perinatally lethal (Ohbayashi et al., 2002).

In contrast, our studies did not demonstrate marked presence of FGF18 in the telogen follicles in normal hair follicles and hair follicles of CRFA regions. This can be explained with several hypotheses:

First, as our studies used immunohistochemistry to determine the presence of FGF18, a discrepancy between the FGF18 mRNA expression site and the localization of FGF18 protein is possible.
Indeed, gene transcription during the telogen phase in mice, as detected by PCR might proceed the hair follicle stage in which FGF18 protein can be found by immunohistochemistry (anagen phase in the dog). In addition incomplete recognition of the canine FGF18 by the human FGF18 antibody might strengthen this effect. In this case the concentration of FGF18 needs to be even higher in order to be visualized by the antibody which might explain the overlap of hair cycle phases.

Second, both studies in mice reported on the presence of mRNA expression at the bulge area of the murine telogen follicle (Kawano et al., 2005; Kimura-Ueki et al., 2012). The bulge area is a well-defined area, seen as a discrete projection of the outer root sheath in the murine and human hair follicle, found at the insertion of the arrector pili muscle, and is a specific niche of stem cells responsible for hair follicle regeneration (Liu et al., 2003). Dogs however, do not have an anatomic structure analogous to the human and murine follicular bulge (Miller et al., 2013c; Kobayashi et al., 2009). Studies in dogs suggest that stem cells are distributed in the outer root sheath between the insertion point of the arrector pili muscle and the sebaceous gland (Kobayashi et al., 2009). The rare positive FGF18 staining that was seen in our studies in the outer root sheath correlates with these findings.

The antibody used in both our studies was a polyclonal human antibody. The advantage of a polyclonal antibody is that a broader spectrum of epitopes can be recognized, which is preferable in pilot investigations (Taylor et al., 2002). A commercial canine anti-FGF18 antibody was not available at the time of our studies. However, as there is a high degree of cross-reactivity between antibodies from different species, we think that the positive staining is a true reflection of canine FGF18. Moreover, the specific antibody that was used was reported to also detect canine FGF18 (Santa Cruz data sheet).

Exact numbers on the amino acid homology between human and dog were not found searching the literature.

In general, FGF’s are highly conserved in gene structure and amino acid sequence between vertebrate species (http://genomebiology.com/2001/2/3/reviews/3005.1). Indeed, there is a reported 99% identical amino acid sequence between murine and human FGF18 and the nucleotide sequence is conserved for 90% (Hu et al., 1998).
Despite this small variation in nucleotide sequence, a similar tertiary (three-dimensional) structure of the protein can be expected (Eklund et al., 1991). We compared the nucleotide sequence of canine and human FGF18 and found that it is conserved for 93% (http://www.ncbi.nlm.nih.gov/BLAST/). Furthermore, comparison of the amino acid sequence between canine and human FGF18 revealed a 99% identical amino acid sequence (http://www.ncbi.nlm.nih.gov/BLAST/). Moreover, when comparing the tertiary structure of canine and human FGF18, 100% analogy was found (http://swissmodel.expasy.org).

The polyclonal human antibody that was used is reported to cross react with FGF8. FGF8 is mainly an early fetal protein and so far only reported to be expressed in the gonads in adults (Gemel et al., 1996; Heikinheimo et al., 1994). In addition, Kawano et al did not detect any FGF8 mRNA activity in the murine follicle (Kawano et al., 2005). Therefore, we do not expect cross reactivity with FGF8 in our studies.

In order to check the reliability of the detected immunohistochemical signal positive and negative controls were added in both studies. The choice of lung tissue as positive control was based on results of a search in http://www.proteinatlas.org/ where is stated that lung tissue stains positively for FGF18. Staining pattern in mouse lung tissue (recommended species for use with this antibody, Santa Cruz data sheet), was compared to staining pattern in dog lung and an identical staining pattern was detected. Similarly negative mouse and dog control tissue composed of kidney tissue was tested (Ohbayashi et al., 1998). Both studies demonstrated the presence of FGF18 in identical locations with similar tissue distribution.

Based on the highly conserved nature of FGF18 in different species, similar immunohistochemical tissue expression and recommendation by the company we feel very confident that the detected signal corresponds to canine FGF18.

In addition in each run a negative control was added by omission of the primary antibody and substitution with the antibody diluent. This technique has been described by Taylor et al (Taylor et al., 2002).
Stenn describes that many molecules of apparently disparate function are found within the inner root sheath, and states that the inner root sheath could take up marker antibodies non-specifically in many instances (Stenn and Paus, 2001). However, the arrector pili muscles, myoepithelial cells of the apocrine glands and vascular endothelial cells also consistently demonstrated positive FGF18 staining in our studies. This corresponds with the studies that demonstrated that FGF18 is also expressed in smooth vascular muscle tissue and endothelial cells (Antoine et al., 2005; Haque et al., 2007) and indicate specific positive FGF18 staining in our studies.

Another possibility, although unlikely, is that we misinterpreted the stages of the follicles in which positive FGF18 staining was present. The different stages of mouse follicles are very well specified and multiple studies describe the morphological differences during the distinct hair cycle stages (Müller-Röver et al., 2001; Paus et al., 1999; Chase, 1954; Dry, 1926). In dogs similar studies were lacking and limited to the main categories anagen, telogen and catagen. We classified the hair follicle stage according to Al-Bagdadi et al. (Al-Bagdadi et al., 1979). Indeed, positive follicles were identified as anagen follicles based on the presence of an inner root sheath and the location of the dermal papilla close to the adipose tissue. One recently published study by Müntener et al. describes morphological and immunohistochemical criteria to distinguish eight substages each in anagen and catagen (Müntener et al., 2011). As our studies preceded the publication of this article, we were unable to use that specific classification scheme. Nevertheless, this most recent study also emphases that the most important feature of all criteria used for the classification of the cycle stages is the position of the dermal papilla in relation to the follicle and its absolute location in the dermis or the subcutaneous fat (Müntener et al., 2011) and this is the criterium that was used in our studies. Furthermore, Müntener et al demonstrate that not all follicles could be assigned to a specific cycle stage. Indeed, 35% of the hair follicles could not be assigned to one specific hair cycle phase (Müntener et al., 2011).

Ultimately, our two immunohistochemical studies of FGF18 only demonstrated the presence of FGF18 protein. We did not correlate the presence of the protein with FGF18 gene expression studies nor did we determine the presence of the FGF18 receptors in canine hair follicles.
The FGF receptor (FGFR) family consists of four distinct tyrosine kinase receptors (FGFR1-4) (Haque et al., 2007; Marie, 2003; Dailey et al., 2005). There are many conflicting reports on the cognate receptors for FGF18 in the hair follicle. The expression of FGFR1, FGFR2, FGFR3 and FGFR4 has been characterized and all these receptors have been found in the murine hair follicle in different stages of the hair growth cycle (Rosenquist and Martin, 1996). FGFR2, FGFR3 and FGFR4 have all been implicated as being receptors for FGF18 in the hair follicle (Rosenquist and Martin, 1996; Zhang et al., 2006; Kimura-Ueki et al., 2012). According to Zhang et al., FGFR3 and FGFR4 are cognate receptors for FGF18 (Zhang et al., 2006). FGFR3 mRNA was expressed at high levels in both telogen and anagen skin. Within bulge cells, FGFR3 protein was mainly detected in the nuclear/perinuclear region and to a lesser degree in the cytosol, whereas FGFR3 protein was dominantly detected in the cytosol in the outer root sheath cells (Kimura-Ueki et al., 2012). The biological significance of the perinuclear/nuclear localization of FGFR3 protein is not completely understood, as FGF18 protein is found in the cytosol. Interestingly, FGFR4 was found to also be expressed in the murine inner root sheath, the area where we detected positive FGF18 staining (Rosenquist and Martin, 1996).

Quantification of immunohistochemical FGF18 protein expression in both studies was performed by counting the number of positive cells in the hair follicles and number of positive follicles in seven follicular units per slide. A follicular unit consists of one to six compound follicles that are grouped together (Credille et al., 2000). In these follicular units a central primary hair and several lateral hairs can be distinguished. The primary hairs are accompanied by an arrector pili muscle, sebaceous and apocrine glands (Meyer, 2009). Initially quantification using a digital image analysis system was planned. As the positive staining was only seen as a fine cytoplasmatic stain, the use of a digital image analysis was impractical and it was decided to evaluate the seven follicular units using x400 magnification.

In the pilot study (second chapter), there was no statistical difference in the number of FGF18 positive cells or follicles of beagle dogs between different anatomical sites (shoulder and flank), winter and summer, sexes or consecutive days of sampling.
As stated previously, there is a pattern of alopecia with predilection sites of flanks, perineum and neck in canine endocrine diseases. The reason why only certain areas are affected remains unknown. As CRFA affects the flanks most commonly and never the shoulder area, the two anatomical sites that were selected for this study were the shoulder and flank. Moreover, the chosen sites had to be large enough to have 10 samples taken with 2 cm margins. In normal canine hair growth studies, hair growth on the flanks was slightly slower than on the shoulder. It was therefore suggested that the hair growth is faster in those areas where the hairs are longer (Al-Bagdadi, 1977; Gunaratnam and Wilkinson, 1983). In our study, subjective visualization of the hair regrowth also demonstrated that all beagle dogs regrew faster in the shoulder area. There was no significant statistical difference in the number of FGF18 positive cells or positive follicles in the shoulder and flank, indicating that hair growth rate is not influenced by FGF18.

There is a seasonal occurrence of CRFA, with dogs presented with alopecia from November till April in the northern hemisphere. Therefore, the dogs were biopsied at two different times: in February (winter) and August (summer). Studies of hair growth in normal dogs demonstrated a peak growth in summer in intact dogs (Butler and Wright, 1981). However, domestication and housing can influence the shedding. Indeed, adult Labradors housed indoors do not show any significant differences in hair growth rates in the different seasons, they shed throughout the year (Miller et al., 2013c). In our study, subjective visualization of hair regrowth did not show any differences in hair regrowth rate depending on the season. No statistical difference was found when examining the number of positive follicles and number of positive cells at those two different times of sampling. As the beagle dogs were also housed indoors, similar to privately owned dogs, the absence of a significant difference in immunohistochemical FGF18 expression in both seasons is thus not unusual.

There is no sex predisposition for CRFA. However, it has been suggested that there are seasonal effects on growth rates unrelated to those caused by seasonal variations in hormone levels in intact dogs. Indeed, spayed dogs demonstrated growth during most of the year whereas intact dogs showed virtually no growth in late winter.
Subjectively following the growth rate, one of the intact female beagle dogs seemed to have a faster growth rate in both seasons. We could not demonstrate a significant statistical difference when examining positive cells or positive follicles in the male, intact female and female spayed beagle dogs.

These results demonstrate that the presence and quantification of the FGF18 protein is not influenced by the different parameters such as location on the body, sex and season in the normal canine hair follicle. It was important to establish this prior to comparing the presence of FGF18 protein in normal canine hair follicles to CRFA affected hair follicles because CRFA has a specific distribution of lesions and is seasonally influenced.

Finally, no statistical difference was found in FGF18 positive cells or positive follicles in the different days of sampling (days 0, 1, 3, 7 and 17). As stated previously, dogs have compound follicles, in contrast to mice and humans. It is stated that the different hair follicles of one compound follicle cycle independently from each other (Miller et al., 2013c). Indeed, dogs mostly show a non-synchronized mosaic pattern of hair follicle cycling (Meyer, 2009). In mice, follicles can be synchronized by hair plucking. Moreover, the truncal epidermis of the mouse lacks melanocytes and truncal pigmentation is entirely dependent of follicular melanocytes. Because follicular pigment production is only active during the anagen phase, the murine skin is dark, only when the hairs are growing. This enables the investigator to visualize anagen hair growth just by looking at the skin, making mice a favourite subject for hair studies (Stenn and Paus, 2001). In our pilot study, we took consecutive biopsies on day 0 and then the beagle dogs where clipped and biopsied again on day 1, 3, 7 and 17. By doing this, we wanted to attempt to synchronize the canine hair follicles. Indeed, anagen can be induced under controlled conditions by using spontaneous induction of hair growth due to trauma/wounding (Ghadially, 1958). However, clipping without injury to the epithelium and follicles was not traumatizing enough to induce anagen hair growth and synchronize the follicles. Indeed, a minimal threshold needs to be exceeded to induce hair growth: plucking a single hair in the mouse does not initiate anagen hair growth in that follicle, at least 1,000 hair shafts need to be plucked (Chase and Eaton, 1959). Synchronizing the canine hair follicles would have enabled us to study the FGF18 protein during the dynamic changes in the hair follicle cycle.
As trauma can induce hair growth, we wanted to assure that taking the biopsies during one sampling day did not influence the other biopsy sites on the consecutive days. Therefore, a distance of 2 cm was kept in between the biopsies. Investigations of the skin samples with haematoxylin and eosin stains revealed that there was no inflammation present in the skin, therefore, inflammation could not have influenced the presence of FGF18.

In the third chapter, immunohistochemical FGF18 protein expression in normal canine hair follicles from flank skin was compared to immunohistochemical FGF18 protein expression of hair follicles of flank skin of dogs affected with CRFA. In the control group a statistically significant higher amount of FGF18 positive cells in the hair follicle epithelium and FGF18 positive follicles were seen. When immunohistochemical FGF18 protein expression was seen in the CRFA group, it was in anagen follicles, and it was especially prominently present in the skin sample of one dog. It could be assumed that this was because the dog was already in the resolution phase. However, exact times of hair regrowth in relation to the time of the skin biopsy procedure in the CRFA patients was not gathered, as this was a retrospective study. CRFA is typically characterized histologically by a telogenization and catagenization of follicles. Telogen and catagen follicles are characterized by the absence of the inner root sheath. As the first study demonstrated the presence of FGF18 mainly in the inner root sheath, these findings are not unexpected.

Multiple investigators describe that the histological changes in CRFA affected skin depend upon the time of biopsy (Gross et al., 2005; Paradis, 2009). When patients are biopsied early in the disease process most follicles will be in the telogen or catagen phase. However, more often patients are biopsied when the alopecia has been present for several months and is in the resolution phase. If biopsied then, the follicles will often already be in anagen phase and the infundibular orthokeratotic hyperkeratosis might not be prominently present (Fontaine et al., 1998).
When FGF18 was found in the CRFA affected hair follicles, it was indeed seen in anagen follicles, from biopsies taken at the margins of the alopecic area, or patients where the disease was probably already in the resolution phase with microscopic hair regrowth already present.

However, when reviewing the latest FGF18 studies in murine follicles, where FGF18 is reported to keep the follicles in a quiescent state, one would expect to see more FGF18 in the CRFA affected follicles (Greco et al., 2009; Kimura-Ueki et al., 2012). Indeed, a peak of mRNA was described by Kawano et al. in the late telogen phase, just prior to anagen initiation in the bulge region of the murine follicle. It would thus seem reasonable that in dogs with CRFA a peak of mRNA is also present just prior to hair regrowth. As discussed previously, the bulge area in the canine follicle is not an anatomical bulge that is visible on histology, as it is in mice, this might account for the lack of an increase in FGF18 in the CRFA affected skin. Moreover, we used an immunohistochemical staining technique to demonstrate the FGF18 protein presence. It is possible, that a peak in FGF18 mRNA in the late telogen phase results in the presence of FGF18 in the anagen inner root sheath of regrowing hair follicles seen in a few CRFA cases.

In humans with alopecia areata, a downregulation of FGF18 gene has been demonstrated (Subramanya et al., 2010). A similar downregulation of the canine FGF18 gene might also explain the decreased presence of FGF18 protein in CRFA affected dogs.

Further evidence for the role of FGF18 in the hair follicle cycle can be gathered by administering this FGF to an animal model (Danilenko et al., 1996). Indeed, as anagen induction is impaired in CRFA and FGF18 was able to induce anagen when injected in mice in uniform telogen stage the question arose whether FGF18 could be used as a potential therapeutic agent. Moreover, as the exact etiopathogenesis of CRFA remains unknown, and research into the pathogenesis and potential therapeutic options is complicated due to its seasonal nature and unpredictable duration of alopecic phases, there is a recognized need for a reliable animal model. In the **fourth chapter**, the behaviour of lesional and non lesional CRFA follicles was studied when transplanted to athymic nude mice.
Lesional and non lesional skin of dogs with CRFA regrew hair within 30 days, whereas the donor dogs were still alopecic on day 30. This indicates that the factor causing the alopecia in these dogs is more likely to be a systemic factor. Indeed, if the alopecia would be caused by a local factor, the alopecia would persist on the transplants, as all the local factors present in the dog are transplanted onto the mice too. Xenograft studies with athymic nude mice have been shown to be able to differentiate whether the pathogenesis of a human alopecic skin disorder is caused by localized factors or systemic/extrinsic factors (Gilhar and Etzioni, 1994). An unfortunate consequence of these findings is that we were unable to study the effect of FGF18 injections in these xenografts. Indeed, the study was originally designed so that the lesional transplanted skin would be injected with FGF18, in order to study the effects of FGF18 on the hair follicle affected by hair cycle arrest. Hair regrowth did start spontaneously, first at the periphery of the xenograft. This was explained due to perfusion changes in the xenograft, where tissue closest to the mouse skin is better perfused. Perfusion changes were furthermore demonstrated by the presence of oedema in the xenograft tissue. However, hair regrowth starting at the periphery due to paracrine factors produced by the murine follicles cannot completely be excluded.

One way to further study the systemic versus paracrine factors would be to transplant affected flank skin to the shoulder area of the same dog. However, such study is more difficult to design as privately owned dogs would have to be recruited.

Another interesting finding of this transplantation study was the fading of the lesional hyperpigmentation. Indeed, hyperpigmentation is commonly seen in dogs with CRFA (Miller et al., 2013a; Paradis, 2009). Moreover, if present, the hyperpigmentation persists once hair regrowth is started. The mechanism of this lesional cutaneous hyperpigmentation in CRFA affected skin is not clear. Direct hormonal effects on melanin production or contributory indirect effects through changes in cutaneous temperature and perfusion have been proposed (Rosychuk, 1998). Our findings in mice suggest that the hyperpigmentation is more likely to be caused by a direct systemic factor on the skin.
In conclusion, we described for the first time an atypical clinical presentation of CRFA with lesions restricted to the face. We did also demonstrate the presence of FGF18 protein in the canine hair follicle. Moreover, we found a significant decrease in the immunohistochemical FGF18 protein expression in CRFA affected follicles. As more molecules are discovered, the search for the key molecule that controls hair follicle cycling continues. Our studies indicate that FGF18 is not a key molecule in the canine hair follicle cycle, and that its role in the pathogenesis of CRFA is minimal, at most. A transplantation study to athymic nude mice demonstrated hair regrowth, indicating that CRFA is at least partially induced by a systemic factor. Indeed, a systemically produced factor with breed specific regionalized differences in susceptibility can also explain the different clinical presentations of CRFA. The xenograft study elucidated that this approach is not ideal as a direct model for CRFA, but could have a potential role in differentiating systemically induced hair cycle disorders from localized hair follicle disorders.
References


Rachid MA, Demaula CD, Scott DW, Miller WH, Senter DA, Myers S. Concurrent follicular dysplasia and interface dermatitis in Boxer dogs. Veterinary Dermatology 2003; 14: 159-166.

Rosenquist TA and Martin GR. Fibroblast growth factor signalling in the hair growth cycle: expression of the fibroblast growth factor receptor and ligand genes in the murine hair follicle. Developmental Dynamics 1996; 205: 379-386.


SUMMARY
Summary

CRFA is a non-inflammatory alopecic disease that is frequently encountered in general practice. The unique features of CRFA are the spontaneous regrowth and the often seasonal recurrent nature. The exact etiopathogenesis of CRFA is currently unknown and there are limited treatment options. Research into the exact underlying cause of CRFA is complicated due to its seasonal nature and unpredictable duration of alopecic phases. A genetically predisposed melatonin deficiency has been suggested. Currently, no studies were able to demonstrate systemic hormonal deficiencies in CRFA. Lately the importance of local factors in the hair follicle cycle has gained increased interest. These local factors have never been investigated in the canine hair cycle or in CRFA. Especially the role of FGF18 needs to be investigated because it has been reported to play a role in the murine hair follicle cycle, and has the potential to induce anagen hair growth.

This thesis is introduced with a review on the basic hair follicle biology and the current knowledge of CRFA. In view of our aims, emphasis was also placed on the current knowledge of FGF18 in the hair follicle. Several atypical presentations have occasionally been described in the literature and are reviewed in the introduction. Other atypical clinical presentations of CRFA are recognized in practice, but are not documented in the literature. Good descriptions of these atypical presentations are important in understanding and characterising CRFA.

In chapter 1 we described for the first time a patient with CRFA without involvement of the classical thoracolumbar area. The patient presented with complete alopecia on the dorsal muzzle, facial folds and ear pinnae. Spontaneous regrowth was noted. Lesions recurred annually for three consecutive years. The current literature has always described canine recurrent flank alopecia with a distribution of lesions on the thoracolumbar area, sometimes in conjunction with the dorsal muzzle, pinnae and perineum area, but never with lesions restricted to the face. Description of this atypical presentation might be of use in further determining breed specific presentations of this disease and thus aid in a better characterization of the disease.
Despite its frequent occurrence, the pathogenesis of CRFA remains poorly understood. CRFA is believed to be caused by hair cycling arrest, more specifically by impaired anagen promotion. Normal hair follicle cycling is influenced by systemic and local factors. To date, no single systemic factor disturbance has been demonstrated in this unique canine disease, but still cannot be excluded with certainty. More recently discovered local factors such as the FGF’s play an important role in hair cycling in other species. These have not been evaluated in normal and diseased hair growth in dogs. As FGF18 induced anagen hair growth when injected in mice, the presence and localisation of FGF18 in canine normal and CRFA affected follicles was studied.

In chapter 2 FGF18 protein was identified, located and quantified in the normal canine hair follicle in association with different parameters such as location on the body, sex, season and phase in the growth cycle. FGF18 protein was visualized in the normal canine skin with an immunohistochemical technique using an anti-FGF18 antibody. Positive FGF18 staining was detected in the apocrine glands, in arrector pilli muscles and in vascular endothelial cells. In follicles, FGF18 was detected as granular cytoplasmatic staining in follicles at the level of the inner root sheath, rarely the outer root sheath and the dermal papilla. Most of the follicles with positive staining were anagen follicles. There was no statistical difference in the number of FGF18 positive follicles or positive cells between the different parameters. The results of this study led to a solid basis for further research in comparing FGF18 of normal hair follicles with CRFA affected follicles.

In chapter 3 immunohistochemical FGF18 protein expression in normal canine hair follicles from flank skin was compared to immunohistochemical FGF18 protein expression of hair follicles of flank skin of dogs affected with CRFA. A statistically significant higher amount of FGF18 positive cells in the hair follicle epithelium of the control group was found. Also a significant higher amount of FGF18 positive follicles was seen in the control group. CRFA is typically characterized histologically by a telogenization and catagenization of follicles. Telogen and catagen follicles are characterized by the absence of the inner root sheath. As the first study demonstrated the presence of FGF18 mainly in the inner root sheath, these findings are not unexpected.
Finally, in chapter 4, a transplantation study was described in order to assess the feasibility of a reproducible experimental model and to investigate local or systemic causes of CRFA.

Six mm punch biopsies were taken from lesional and non lesional skin of 2 patients with CRFA and grafted onto five 14 week old athymic mice. The lesional canine skin and xenografted skin were evaluated histologically on day 0 and day 30 respectively. Transplanted lesional and non lesional skin regrew hair within 30 days, while the donor dogs were still alopecic in the lesional areas. Graft rejection was evidenced histologically in 2 mice. Lesional hyperpigmentation disappeared in the athymic mice. This study showed that hair follicles of affected canine skin of dogs with CRFA quickly regenerated once removed from the dog and regrow quicker on the mice than on the donor patient, indicating that the pathogenesis of CRFA is –at least partially- mediated by systemic factors. Indeed, a systemic factor disturbance coupled to a regional difference in sensitivity to this factor can explain the different clinical presentations of CRFA. As spontaneous hair regrowth was present, the potential anagen inducing effect of FGF18 injections could not be investigated. These findings demonstrate that this xenograft model is not usable as a direct model for CRFA. Nevertheless it could be used to further identify the canine systemic factor(s) involved in the induction of the alopecia.

The present work documented a previously unknown clinical presentation of CRFA. It also has led to the identification and localization of FGF18 protein in the normal canine hair follicle and CRFA affected hair follicle. With a significant difference of FGF18 protein in normal versus CRFA affected follicles. Finally, a transplantation study demonstrated that the xenograft approach is not valuable as a direct model for the study of CRFA. It did however provided proof that the pathogenesis of this disease is at least partially mediated by a systemic factor disturbance. The potential therapeutic use of FGF18 in hair cycle arrest diseases such as CRFA remains to be elucidated.
SAMENVATTING
Samenvatting

CRFA is een niet-inflammatoire alopecia die frequent wordt gezien in de dagelijkse praktijk. De unieke eigenschappen van deze aandoening zijn het spontane herstel van de haargroei en het cyclisch verloop. De exacte etiologie van CRFA is onbekend en de behandelingsmethoden zijn schaars. Verder onderzoek naar de onderliggende etiologie wordt bemoeilijkt door het cyclisch voorkomen en de onvoorspelbare duur van de alopecia. Er wordt gesuggereerd dat een genetische predispositie voor melatoninedeficiëntie een onderliggende oorzaak zou zijn. Tot op heden konden echter geen systemische hormonale afwijkingen bij CRFA aangetoond worden. Het belang van lokale factoren in de haargroeicyclus werd recent aangetoond. Dit heeft geleid tot grotere belangstelling voor de rol van deze factoren in haarfollikels, zowel bij gezonde honden als bij honden met CRFA. Vooral het effect van FGF18 dient onderzocht te worden, gezien van deze lokale factor werd aangetoond dat het een rol speelt in de haargroeicyclus van de muis en dat het potentieel een anageen inducerend effect zou hebben.

In de inleiding wordt een overzicht gegeven van de biologie van de haarfollikel en van de huidige kennis van CRFA. In het kader van de wetenschappelijke doelstelling van deze thesis dit wordt de nadruk gelegd op de kennis van FGF18 in de haarfollikel. Meerdere atypische klinische presentaties van CRFA zijn beschreven in de literatuur en worden aangehaald in de introductie. Andere atypische klinische uitingen van CRFA worden herkend in de praktijk, maar zijn nog niet beschreven in de wetenschappelijke literatuur. Goede klinische beschrijvingen van deze cases zijn echter belangrijk om CRFA correct te kunnen karakteriseren en bestuderen.

In **hoofdstuk 1** wordt een patiënt beschreven met CRFA waarbij de klassiek aangetaste thoraco-lumbale regio geen letslers vertoonde. De patiënt werd aangeboden met een volledige alopecia van de neusrug, gezichtsploen en oorschelpen. Spontaan herstel van de haargroei werd waargenomen. De laesies vertoonden een cyclisch verloop waarbij gedurende drie opeenvolgende jaren telkens letslers werden waargenomen in de lente en een spontaan herstel van de vacht werd gezien in de zomer.
In de huidige literatuur wordt het distributiepatroon van de letsels bij CRFA steeds beschreven ter hoogte van het thoraco-lumbale gebied, al dan niet gepaard gaand met letsels ter hoogte van de neusrug, oorschelpen en het perineale gebied. Volgens de auteur van de voorliggende thesis werd alopecia waarbij de distributie zich faciaal beperkt, niet eerder in de literatuur beschreven. De beschrijving van deze atypische klinische presentatie van CRFA kan een rol spelen in het bepalen van rasspecifieke uitingen van deze aandoening en zo leiden tot een betere definitie van CRFA.

Alhoewel CRFA frequent voorkomt, is de pathogenese ervan nog niet volledig bekend. CRFA wordt waarschijnlijk veroorzaakt door een haarcyclusarrest, meer specifiek door een verstoorde vooruitgang van de anagene fase. De normale haarfollikelcyclus wordt beïnvloed door systemische en lokale factoren. Tot op heden kon geen enkele systemische factor aangewezen worden als oorzaak van CRFA, maar dit sluit een systemische factor als onderliggende oorzaak echter niet uit. Recent aangetoonde lokale groefactoren spelen een belangrijke rol in de haarregrowicyclus bij meerdere diersoorten. Dergelijke lokale groefactoren werden bij haarfollikels van zowel gezonde honden als honden aangetast door CRFA nog niet onderzocht. Op basis van een studie waarin FGF18 bij muizen werd geïnjecteerd en haarregrowei induceerde, werd in de voorliggende thesis geopteerd om het FGF18-eiwit te bestuderen op follikels van gezonde honden en van honden met CRFA.

De onderzochte parameters toonden geen statistisch significant verschil tussen het aantal FGF18-positieve follikels of positieve cellen. De resultaten van deze studie leidden tot een goede basis om daaropvolgend FGF18 te bestuderen in door CRFA aangetaste follikels.

**In hoofdstuk 3** wordt de hoeveelheid FGF18-eiwit in haarfollikels van gezonde honden vergeleken met dat van honden aangetast door CRFA. In de controlegroep werden een statistisch significant hoger aantal FGF18-positieve cellen in het haarfollikelepithel aangetoond, evenals een statistisch significant hoger aantal FGF18-positieve follikels. CRFA wordt beschreven als een aandoening waarbij histologisch telogenisatie en catagenisatie van de follikels worden gezien. Dergelijke telogene en catagene follikels worden gekenmerkt door de afwezigheid van een inwendige haarschacht. Aangezien er in de studie beschreven in hoofdstuk 2 wordt aangegeven dat FGF18 hoofdzakelijk ter hoogte van de inwendige haarschacht werd waargenomen, zijn deze bevindingen niet onverwacht.

Tot slot wordt in **hoofdstuk 4** een transplantatiestudie beschreven. Deze studie had een tweeledig doel. Enerzijds werd nagegaan of dergelijke transplantatiestudies een muizenmodel zouden kunnen vormen voor het bestuderen van CRFA. Anderzijds werd door middel van deze transplantatiestudie onderzocht of CRFA veroorzaakt wordt door een lokale of systemische factor.

Samenvatting

Dit geeft aan dat de pathogenese van CRFA minstens deels gemedieerd wordt door systemische factoren. Een systemische factordisregulatie gekoppeld aan een regionale veranderde gevoeligheid voor deze factor kan de verschillende klinische uitingen van CRFA verklaren. Aangezien er bij de athymische muizen een spontaan herstel van de haargroei werd waargenomen, kon het anageen inducerend effect van FGF18-injecties niet onderzocht worden.

De bevindingen van de auteur van deze thesis tonen aan dat een xenogreffenmodel geen meerwaarde biedt als direct muizenmodel voor CRFA. Desalniettemin zou het verder kunnen gebruikt worden in studies die als doel hebben systemisch geïnduceerde alopecia te onderzoeken.

Het huidige werk documenteert een ongepubliceerde klinische presentatie van CRFA. Het heeft ook geleid tot de identificatie van het FGF18-eiwit in de haarfollikel van zowel gezonde honden als van honden aangetast door CRFA: een duidelijk grotere hoeveelheid FGF18-eiwit werd vastgesteld in de haarfollikels van gezonde honden. Tot slot toont een transplantatiestudie met athymische muizen aan dat xenotransplantatie geen direct model kan zijn voor CRFA. Desalniettemin wordt door middel van de xenotransplantatiestudie aangetoond dat de pathogenese van de aandoening minstens deels veroorzaakt wordt door een systemische factor. Het mogelijk therapeutische gebruik van FGF18 bij aandoeningen waarbij haarcyclusarrest wordt veroorzaakt, zoals CRFA, blijft onbeantwoord.
CURRICULUM VITAE
Sophie Vandenabeele werd geboren op 24 augustus 1974 te Brugge in België.


Kort na het afstuderen startte zij een internship in een dierenkliniek in Antwerpen, waarbij zij eveneens op stage ging in de dermatologie afdeling van de Universiteit van Cornell (onder leiding van Prof D Scott en W Miller) en de dermatopathologie afdeling van de Universiteit van Guelph (onder leiding van J Yager). In 2001 startte zij een residentie in diergeneeskundige dermatologie aan de Universiteit van Davis, Californië. Dit resulteerde in 2004 in een specialisten diploma van het Amerikaanse College voor Diergeneeskundige Dermatologie.


Sophie Vandenabeele is auteur of medeauteur van meerdere publicaties in internationale tijdschriften. Daarnaast nam ze actief deel aan internationale congressen. Tevens was zij in 2011 lid van het wetenschappelijk en lokaal organiserend comité voor het congres van het Europees College van Diergeneeskundige Dermatologie (ECVD) in Brussel.
BIBLIOGRAPHY
Publications in international journals


Vandenabeele S. Traitement de l'otite externe à Malassezia à l'aide de Malacetic Otic. L'Hebdo Vétérinaire 2007; 205: 8-10.

Vandenabeele S. L'EDTA tris dans le traitement de l'otite à Pseudomonas chez le chien. L'Hebdo Vétérinaire 2007; 204: 8-10.


**Book chapter publications**


**Proceedings and abstracts on international meetings**

ACKNOWLEDGEMENT
Years ago I started this thesis, unknowing of what would come, and determined to crack the hair follicle cycling code. Now, several years later, I have realized that the hair follicle is a truly unique structure, and that it is not ready to reveal all of its secrets, at least not to me... I can honestly understand DW Scott when he states: “hair follicles drive me crazy”.

Nevertheless, I have enjoyed the opportunity to complete this PhD, whilst making sure the dermatology service of the Small Animal Department kept running. It has given me the opportunity to see how thorough research is challenging and (sometimes) rewarding at the same time. It has also given me new insights in the emotions that go along with fulfilling the thesis and hence enhanced my understanding of what it is like for the colleagues all around me being in various stages of their own work.

I could never have done this without the help of so many people...

First of all, my promoters, who stood with me all along the way. Hilde, I met you in Davis, and was and still am so excited that you agreed to be my promoter. As our children grew, so did the form of the thesis... I loved the brain storming we did, and hope the ending of this PhD does not mean the end of the brainstorming, because it is so enriching! Sylvie, you pulled me through, took away the hesitations and gave me the strength to keep on going. Luc, thank you for allowing me to complete this PhD, and providing me with all the details I needed to make the work complete.

To the members of the exam committee: Prof Deprez, Prof Ducatelle, Prof Paradis, Prof Declercq thank you for the thorough review of the manuscript. Your comments made me rethink certain things and helped in producing a better end result. Special thanks go out to Prof Paradis, who accepted this task and travelled from Canada to take part of the exam committee. I really hope you enjoyed Bruges, and maybe next time, I will be able to guide you around the city, and admire the Béguinage during the daytime! Prof Declercq, your keen eyes always look for the best lesions and your view on morphology of skin lesions is truly unique. It is always a pleasure to work with you!

Special thanks go out to all the people in the lab who helped me to find my way in an unravelling world of molecular biology. Thank you to the laboratory people of the RUCA and AML! While we are at the subject of molecular biology... Dear Nicole, thank you so much for your help during the final stages! Raphael, you saved my day!

Prof Meyer and Bert Maddens, thank you for all the help with the athymic mice.

Many thanks also to Filip Clompen. Your help with all of the figures was invaluable!!!
Acknowledgement

For the people of the reception: Saar, Katty, Karine, Hans, Dominique and Cindy, thank you so much for taking care of client communication and the administrative parts of the thesis, arranging the agenda so that there was some time for just the PhD, time with less phone calls, emergencies and appointments. You rock!!!

To all of the colleagues, in the small and large animal departments, thank you, for asking for an update on the status of the PhD at just the right times, giving me some distractions in the form of canine, feline, equine and caprine skin patients, and making me smile, at times where things weren’t going so swiftly! Knowing that the people surrounding you understand what you are dealing with makes it all more bearable. I have learned little tricks from all of you, that are of tremendous value...For the colleagues of the Flemish Dermatology Study Group, thank you for all of your support and the feedback on patients with CRFA. It is always a pleasure to attend the Thursday night meetings!

To all of my friends and family who have seen me stress out in these last months, and offered to reduce my stress level in so many ways, thank you! I think I would have developed a stress induced alopecia areata without you!! Marc and Françoise, you probably don’t realize it, but it was thanks to your analogy comparing taxi’s to articles that I finally started to publish the long gathered results. You are definitely right: articles are meant to be taxi’s (or hop-on, hop-off busses), not a whole public transportation system.

Mum and dad, life has been a rough road for you at times, and I feel really fortunate that you are both here to witness this moment. I know it makes you proud.

Last but not least, to my husband Philippe and my children Simon and Nicolas: you mean the world to me. Thank you, for keeping me inspired with all of the questions you relentlessly ask, and then, giving me the time to go and search for the answers. You are truly remarkable, and I am blessed to have you in my life.