UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

TOPICAL TREATMENT WITH A SEROTONINERGIC DRUG FOR CANINE ATOPIC DERMATITIS

ANA RAQUEL BARRADAS REIS

ORIENTADORA:
Doutora Ana Mafalda Gonçalves Xavier Félix Lourenço

COORIENTADORA:
Doutora Joana Marques Marto

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TRATAMENTO TÓPICO COM FÁRMACO SEROTONINÉRGICO PARA A DERMATITE ATÓPICA CANINA

Resumo

Na pele, o sistema serotoninérgico desempenha um papel importante na inflamação crónica, estando envolvido na vasodilatação, inflamação e imunomodulação. Assim, os fármacos serotoninérgicos, como os inibidores de recaptação da serotonina (SSRIs), podem ser potencialmente benéficos no tratamento de doenças dermatológicas, nomeadamente através da administração tópica. Como tal, colocou-se a seguinte hipótese: poderá uma formulação tópica de um inibidor de recaptação da serotonina (SSRI) ser uma opção terapêutica para o tratamento da dermatite atópica canina (cAD)?

Este é o primeiro estudo que avalia o uso de um SSRI tópico em cães com cAD. Procurou-se avaliar se a aplicação tópica de um fármaco serotoninérgico beneficiaria cães com dermatite atópica, melhorando o prurido, o eritema e a barreira cutânea. Os cães atópicos que participaram no estudo serviram como seu próprio grupo controlo e grupo experimental. A cada um deles foi administrado o creme com o fármaco serotoninérgico (creme A) e o creme controlo (creme B), cada um numa pata diferente, durante duas semanas. Durante o estudo, desde o dia 0 (D0) ao dia 14 (D14), foram quantificadas várias variáveis: prurido, eritema, perda de água transepidérmica (TEWL), pH e hidratação. No D0, foi também avaliada a qualidade de vida (QoL) e, no D14, os tutores responderam a um questionário de análise sensorial. Também se avaliou o grau de correlação entre o prurido e eritema e as medidas de avaliação da barreira cutânea (TEWL, hidratação e pH).

Foi encontrada uma diferença estatisticamente significativa nos valores de TEWL ($p = 0,008$) entre D0 e D14. As diferenças observadas para o prurido ($p = 0,50$), eritema ($p = 0,42$), pH ($p = 0,09$) e hidratação ($p = 0,094$) entre o D0 e o D14 não foram estatisticamente significativas. Entre o TEWL e o eritema ($p = 0,04$; $rho = 0,404$) encontrou-se uma correlação positiva significativa, entre a hidratação e o prurido e o pH e o prurido a correlação foi significativa, embora negativa ($p = 0,048$; $rho = -0,390$ e $p = 0,008$; $rho = -0,51$, respetivamente). Entre a QoL e o prurido ($p = 0,08$; $rho = 0,49$), a correlação encontrada não foi significativa. No parâmetro TEWL, que avalia a barreira cutânea, verificou-se uma melhoria estatisticamente significativa no grupo que recebeu a intervenção terapêutica.

Palavras-chave: Serotonina, Dermatite Atópica, Cães, Prurido

TOPICAL TREATMENT WITH A SEROTONINERGIC DRUG FOR CANINE ATOPIC DERMATITIS

Abstract

In the skin, the serotoninergic system has an important role in chronic inflammation since it is involved in vasodilation, inflammation, and immunomodulation. Thus, serotoninergic drugs, like selective serotonin reuptake inhibitors (SSRIs), may be useful in treating dermatological disorders, namely through topical administration. Therefore, the following question was raised: Could topical SSRIs be a new and safe pharmacological option in canine atopic dermatitis (cAD)?

This is the first study evaluating the use of a topical SSRI for cAD. The investigators sought to evaluate whether topical application of a serotoninergic drug would benefit dogs with atopic dermatitis, improving pruritus, erythema, and the skin barrier. The enrolled atopic dogs served as their own control and active control group. Each of them received the serotoninergic cream (cream A) and the control cream (cream B) in two different paws for two weeks. During the study, from D0 to D14, several variables were measured: pruritus, erythema, transepidermal water loss (TEWL), pH, and hydration. On D0, the QoL was also assessed, and on D14, a sensory analysis questionnaire was also answered by the owners. It was also assessed whether there was a correlation between the cAD clinical outcomes (pruritus and erythema) and the skin barrier assessment measurements (TEWL, hydration, and pH).

Regarding the study's results, it was found a statistically significant difference in TEWL (p=0,008) values between the serotoninergic cream and the control cream between D0 and D14. A statistically significant difference was not found for pruritus (p=0,50), erythema (p=0,42), pH (p=0,09) and hydration (p=0,094). Regarding the possible correlations, a significant positive correlation was found between TEWL and erythema (p=0,04; rho=0,404) and a significant negative correlation was found between hydration and pruritus (p=0,048; rho=-0,390) and between pH and pruritus (p=0,008; rho= -0,51). No significant correlation was found between QoL and pruritus (p=0,08; rho= 0,49).

The study's results suggest that the daily topical application of a serotoninergic drug for two weeks did not appear to have a superior effect compared to the control cream, except for the TEWL parameter, which evaluates the integrity of the cutaneous barrier, and showed a statistically significant improvement in the group that received the serotoninergic druf.

Keywords: Serotonin, Atopic Dermatitis, Dogs, Pruritus

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5-HIAL – hydroxy-3-indoloacetaldehyde

5-HT – 5-hydroxytryptamine or serotonin

5-HTR – 5-hydroxytryptamine receptors

5-HTT – 5-hydroxytryptamine transporter

6BH4 – 6-tetrahydrobiopterin

AAD – amino acid decarboxylase

ACVD – American College of Veterinary Dermatology

AD – atopic dermatitis

Ag – antigen

AIS – adaptive immune system

AMPs – antimicrobial peptides

ANS – autonomous nervous system

APCs – antigen-presenting cells

ASIS – allergen-Specific IgE Serology

cAD – canine atopic dermatitis

CADESI-04 - Canine Atopic Dermatitis Extent and Severity Index, 4th iteration

CNS – central nervous system

CT – computed tomography

CTLs – cytotoxic T cells

DCs – dendritic cells

HPA – hypothalamic-pituitary-adrenal axis

ICADA – International Committee on Allergic Diseases of Animals

IDT – Intradermal testing

IL – interleukine

IIS – innate immune system

IFN- γ – interferon γ

KCs – keratinocytes

LCs – langerhans cells

MAO – monoamine oxidase

NK – natural killer

PAMPs – pathogen-associated molecular patterns

PRRs – pattern recognition receptors

PVAS – Pruritus Visual Analogous Score

QoL – quality of life

SERT – serotonin transporter

SIS – skin immune system

SSRIs – specific serotonin reuptake inhibitors

SUB – subcutaneous ureteral bypass

TEWL – transepidermal water loss

Th cells – helper T cells

TNF-α – tumor necrosis factor alfa

TPH – tryptophan hydroxylase

Treg – regulatory T cells

TSLP – thymic stromal lymphopoietin

VMAT – vesicular monoamine transporter

1. TRAINEESHIP REPORT

This report concerns the activities performed during the curricular internship that occurred between 14th September 2020 and 28th February 2021 at the Veterinary Hospital of the *Faculty of Veterinary Medicine - University of Lisbon*. During this period, several activities were performed on a rotation basis in the hospital services: dermatology (200 hours), surgery (200 hours), inpatient care (168 hours), general practice (160 hours), imagiology (160 hours), internal medicine (80 hours), oncology (80 hours), ophthalmology (40 hours), and infectious diseases (28 hours). The internship had a duration of 5 months and 2 weeks, with a total of 1116 hours.

In the weeks spent at the dermatology service, under the supervision of Professor Ana Mafalda Lourenço, who also supervised the master thesis project, and Dr. Hugo Pereira, it was possible to attend first-opinion, re-evaluation, and second-opinion consultations. There was also the opportunity to practice several clinical procedures, such as performing diagnostic tests for skin conditions like cytology, trichography and wood lamp exam.

The two week-period at the internal medicine service allowed the attendance of several consultations, and it was also possible to attend endocrinology lectures and complementary exams, such as rhinoscopy, bronchoscopy, gastrointestinal endoscopy, and bone marrow aspiration.

The two weeks spent at the oncology service, allowed the attendance of oncology consultations and chemotherapy sessions.

In the service of general practice, it was possible to attend mainly first-opinion consultations and preventive medicine consultations. There was the possibility of performing several supervised activities, such as performing anamnesis and physical examinations, collecting blood samples, performing blood pressure measurements, and cystocentesis. The time spent in the inpatient care service offered the possibility to observe and help perform emergency procedures, such as cardiorespiratory resuscitation.

In the ophthalmology service, there was the possibility of participating in first-opinion, second-opinion, and referral consultations. It was also possible to perform supervised ophthalmological evaluations.

In the time spent assisting the ultrasonography exams, it was possible to attend abdominal and cardiac ultrasounds, ultrasound-guided percutaneous needle biopsies, subcutaneous ureteral bypass (SUB) flush, and echo-guided abdominocentesis. In the time spent assisting the radiography and computed tomography (CT) exams, there was the possibility of assisting myelography and contrast-CT exams and performing supervised anesthetic induction in the exams that required it.

In the service of infectious diseases, it was possible to perform the patient's clinical monitorization, drug preparation, and subsequent administration. It was also possible to attend follow-up consultations of animals hospitalized and assist the performance of wound dressing in animals with multi-drug resistant bacteria.

In the surgery service it was possible to attend and participate in several surgeries, such as ovariohysterectomies, orchiectomies, cystotomies, removal of mammary chains, cesarean sections, correction of brachycephalic syndrome, dog's mandibulectomy, dog's maxillectomy, among others.

2. LITERATURE REVIEW

2.1. The skin: an essential organ

The skin is an essential organ and has several vital functions, namely, it acts as a physical and anatomical barrier, protecting the organism from the environment; is essential for thermoregulation and storage of diverse materials, such as lipids, carbohydrates, proteins, vitamins, electrolytes, water, and others. Additionally, it has immunoregulatory functions; provides sensory perception; has antimicrobial actions and exocrine functions since it is responsible for the secretion of apocrine, eccrine, and sebaceous glands. It is also responsible for the production of hormones (Muller and Kirk 2013). These functions are integrated into the skin's immune, pigmentary, epidermal, and adnexal systems, all of which are connected by an open communication lane with the body's immune, nervous and endocrine systems. There is also a complex network of immune, nervous and endocrine systems present in the skin, responsible for the rapid responses to trauma, pathogenic agents, temperature, radiation, allergens, and toxins (Slominski et al. 2012; Muller and Kirk 2013).

Albeit it is an essential organ, the skin is often overlooked. Only when its homeostasis is corrupted, either by diseases or simply aging, its value is genuinely appreciated (Wong et al. 2016). It is the largest organ of the body, representing 12% of the dog’s total body weight (Muller and Kirk 2013).

2.1.1. The skin as an immune organ

One of the major skin functions is the protection against environmental insults, which is accomplished through physical barriers (the stratum corneum), biomolecules (e.g. antimicrobial peptides (AMPs) and lipids), the skin's pH, and a network of immune and nonimmune cells (Nguyen and Soulika 2019).

In 1986, Bos and Kapsenberg described, for the first time, the skin immune system (SIS), which is defined as a network of resident and attracted immune cells in different skin locations and the interface between innate and adaptive immune responses (Bos and

Kapsenberg 1986; Abdallah et al. 2017). The innate immune response is the organism's first line of defense. It is characterized by a rapid, nonspecific response to pathogen's invasion through complement activation, recruitment and activation of phagocytic cells (macrophages and neutrophils), recruitment of eosinophils, activation of mast cells and natural killer (NK) cells, and production of chemokines and cytokines (Muller and Kirk 2013; Yazdi et al. 2016).

The adaptive immune response is the organism's second line of defense and is defined as a humoral and cellular response provided by B and T cells, respectively. It is an antigen-specific immune response characterized by the production of cells with immune memory (memory T cells), which provide a quicker immune response in the case of future antigen (Ag) exposure. The adaptive immune response is more specific and prolonged than the innate immune response (Bos and Luiten 2009; Abdallah et al. 2017; Hugh and Weinberg 2018).

2.1.1.1. Skin's innate immune system

In the skin, neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, and keratinocytes (KCs) are the primary immune innate cells (Yazdi et al. 2016; Abdallah et al. 2017). These cells carry pattern recognition receptors (PRRs) on their surface that can detect pathogen-associated molecular patterns (PAMPs) and consequently initiate innate immune responses (Richmond and Harris 2014; Yazdi et al. 2016; Abdallah et al. 2017). Once there is a skin barrier dysfunction, the skin's Langerhans cells (LCs), which are epidermal DCs, are attracted to the epidermis by the chemokines produced by the KCs and elongate their dendrites to capture the Ags (Mueller et al. 2014; Matejuk 2017; Martins et al. 2020a)

KCs are the immune response initiators since they are the first cells to sense pathogen invasion. They carry a PRR and, once they contact with a PAMP, they become activated and are able to produce cytokines, chemokines, growth factors, and AMPs (Abdallah et al. 2017). LCs are in close contact with KCs and are the first immune cells to contact with the pathogen. Once activated, they migrate to lymph nodes and stimulate the naïve T cells, priming and differentiating them into different T cell subtypes (Abdallah et al. 2017). KCs and LCs, through the production of inflammatory mediators, attract more innate immune cells - neutrophils, monocytes, and NK cells - to the inflammatory area, promoting cell phagocytosis and cell lysis (Abdallah et al. 2017). The innate immune response's ultimate objective is the elimination of the pathogenic agent, and most of the time, it can overcome the insult without triggering an adaptive immune response (Yazdi et al. 2016).

2.1.1.2. Skin's adaptive immune system

An effective immune response is sustained by the robust interconnection between the adaptive immune system (AIS) and the innate immune system (IIS), provided by antigen-presenting cells (APCs). In the skin, this group of cells is highly represented by LCs and

macrophages. Their principal functions are Ag presentation to T cells and priming of naïve and memory T cells (Yazdi et al. 2016).

When the innate immune response is ineffective in pathogenic agent elimination, the APCs migrate to the lymph nodes, interact with antigen-specific T cells, and subsequently initiate the adaptive immune response (Yazdi et al. 2016; Abdallah et al. 2017). Once antigen-specific T cells are activated, they proliferate, exit the lymph node and enter the bloodstream in direction to the skin's inflammation site, where they induce cellular and humoral mediated responses that lead to the release of mediators by immune cells (Richmond and Harris 2014; Abdallah et al. 2017). The created inflammatory environment stimulates the production of mediators by epidermal cells (mainly KCs) that continuously activate and maintain the immune response in the dermis (Abdallah et al. 2017).

T cells are the primary immune effector cells in the skin and can be divided into resident and circulatory. T cells can be further classified into CD4+ and CD8+ T cells, according to their cell surface proteins (CD4 and CD8, respectively) (Abdallah et al. 2017). The CD8+ T cells are cytotoxic T cells (CTLs) that produce tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) and cause cellular lysis upon Ag's recognition. Epidermal CD8+ T cells remain there for long periods and are recognized as tissue-resident memory T cells with a crucial role in immune surveillance and protective actions against reinfection (Richmond and Harris 2014; Kabashima et al. 2019). CD4+ T cells, also called helper T cells (Th cells), have a fundamental role in the T- and B-cell mediated responses (Abdallah et al. 2017).

The skin's AIS is essentially represented by Th cells, which can adopt different phenotypes (e.g. Th1, Th2, Th17, Th22, and regulatory T cells (Treg)) according to the cytokines produced by the APCs that activate them. According to the respective phenotype, T cells produce different cytokines and have different functions. The Th1 is responsible for the recruitment of CTLs. The Th2 produces interleukin (IL) IL-4, IL-5, and IL-13, leading to the recruitment of basophils, eosinophils, and mast cells. The Th17 produces IL-17, IL-21, and IL-22, whereas the Th22 produces mainly IL-22, but not IL-17. The Treg controls the T cell-dependent inflammatory responses in the skin (Richmond and Harris 2014; Abdallah et al. 2017).

Regarding the humoral response, there is not much information about the role of B cells in the skin's immunity. However, it is known that B cells can be activated and recruited to the skin’s immune response. They can present antigens to Th cells and prime them into Th2 cells. They can also produce antibodies that are then carried to the skin. Despite their functions, they usually remain in the lymph nodes and are rare in healthy skin. In skin with chronic inflammation, such as atopic skin, they can be found in the dermis (Richmond and Harris 2014; Abdallah et al. 2017; Martins et al. 2020a).

2.1.2. The skin as a neuroendocrine organ

In 1964, Günter Stüttgen, a dermatologist, proposed the existence of a connection between the central nervous system (CNS), the autonomous nervous system (ANS), the endocrine glands and the skin (Stüttgen 1964; Theoharides et al. 2016).

For a long time, the skin was seen as a "passive" neurohormone target, only responding to extracutaneous endocrine stimuli. However, in the last decades, this idea has evolved, and this organ started to be seen as an active neuroendocrine organ with multidirectional connections between itself, the endocrine system, the immune system, the CNS, and other internal organs (Slominski 2005; Ramot et al. 2021).

The skin depends on its vital properties to maintain and reestablish homeostasis and, whenever its homeostasis is compromised, this organ can produce rapid (neural) and slow (immune) responses locally and systemically to restore it (Slominski et al. 2012). The coordination between these skin responses is mediated by skin cells' capacity to produce and/or release several hormones, neuropeptides, neurotransmitters, and biological regulators that communicate with the central neuroendocrine system through the skin's vascular system and nerve endings (Zmijewski and Slominski 2011; Slominski et al. 2012; Martins et al. 2020a). These substances can exert their actions in a paracrine or autocrine way, having local effects. Nonetheless, there is also growing evidence that they can diffuse to the dermis' blood vessels and nerve endings and exert their actions on other organs such as the brain (Zmijewski and Slominski 2011).

Several facts sustain the existence of a cutaneous neuroendocrine system. The first is the fact that skin has neuroendocrine activities equivalent to the functions of several systems, such as the thyroid, the hypothalamic-pituitary-adrenal axis (HPA), the opioid, the endocannabinoid, the cholinergic, the secosteroidogenic, the melatonergic, and the serotoninergic system (Slominski et al. 2012; Soeberdt et al. 2020). The second is the presence of biogenic amines (like serotonin), catecholamines, melatonin, proopiomelanocortin-derived ACTH, β-endorphin, MSH peptides, CRH, and related peptides activity on the skin (Theoharides et al. 2016). The third is the common embryologic origin of the skin and the brain. They are both derived from ectoblast differentiation, which is reflected by the skin's sensory function, the dense innervation on its constitution, and its role as target and source of neurotransmitters, neuropeptides, neurohormones and neurotrophins, all associated with the central neuroendocrine system (Paus et al. 2006; Nordlind et al. 2008; Martins et al. 2020a).

There is strong evidence of a correlation between the cutaneous neuroendocrine system and its involvement in skin physiology and pathology. Even in ancient times, a perplexing interconnection between stress and skin disorders was suspected (Theoharides et al. 2016). Nowadays, it is known that physiological stress plays a part in the aggravation and

propagation of cutaneous conditions through the HPA stimulation and consequent glucocorticoid production that leads to a reduction in KCs proliferation, a decrease in the AMPs content in the stratum corneum, and a defective stratum corneum integrity. It became clear that the brain-skin connection plays a role in the pathogenesis of skin disorders, including allergic and inflammatory diseases, like atopic dermatitis (AD) (Slominski et al. 2008). Recently, more emphasis has been given to the presence of a serotoninergic system in the skin and several possible roles for serotonin in inflammatory skin diseases were discovered (Martins et al. 2020b). Due to the strong similarities between human and canine AD (Marsella and De Benedetto 2017), it was hypothesized that the same could be presumed for canine atopic dermatitis (cAD).

2.2. Serotonin and its role in the skin

2.2.1. Serotonin: the molecule

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine diversely present in plants and animals (Jonnakuty and Gragnoli 2008). Indeed, in the animal kingdom, it can be found from the most simple organisms (like nematodes) to the most complex ones (humans) (Marston et al. 2011; Fidalgo et al. 2013).

Its broad distribution in living organisms and the ubiquitous presence of its receptors confirms serotonin's fundamental role in homeostasis (Mohammad-Zadeh et al. 2008). It can act as a hormone, a mitogen, and is considered one of the oldest neurotransmitters (Mohammad-Zadeh et al. 2008; Martins et al. 2020a). It was discovered by Rapport et al. in 1948, and its name characteristics identified at the time, as it both was present in the serum (sero) and had vasoactive properties (tonin) (Rapport et al. 1948; Jonnakuty and Gragnoli 2008).

Despite its ubiquitousness, its role in health and disease was under-recognized and poorly studied in veterinary medicine even though most of the studies investigating this molecule were carried out in animal models, including dogs (Mohammad-Zadeh et al. 2008).

Its presence in the plasm, brain, and other organs (including the skin) allows the cells to integrate signal effects from sensory and motor systems and from endocrine, digestive, immunological, and vascular signals. Consequently, modifications in 5-HT concentrations can modify the maturation, metabolism, migration, and mitosis of cells bearing serotonin receptors, including those in the skin (Nordlind et al. 2008).

It has several behavioral and biological functions (appetite, thermoregulation, sleep, and pain perception) that regulate physiological processes, both in the CNS and peripheral tissues, such as the skin, the lungs, the kidneys, and the gastrointestinal tract (Mohammad-Zadeh et al. 2008; Thorslund 2012).

As elucidated by Mohammad-Zadeh et al., much of what is known about serotonin's role in health and disease is based on studies performed on animal models. In the review performed by the authors aforementioned, they demonstrated that serotonin plays a part in several diseases in humans, like migraine, irritable bowel syndrome, systemic and pulmonary hypertension. More recently, its role was also questioned in the etiology of inflammatory skin diseases (Mohammad-Zadeh et al. 2008; Martins et al. 2020a).

2.2.2. Serotonin synthesis

Serotonin, just like epinephrine, norepinephrine, dopamine, and histamine, is a biogenic monoamine (Mohammad-Zadeh et al. 2008). It is synthesized from L-tryptophan, a naturally occurring essential amino acid. First, L-tryptophan is converted to 5-OH-tryptophan by the enzyme tryptophan hydroxylase (TPH), an enzyme encoded by the TPH1 gene (expressed ubiquitously) and the TPH2 gene (expressed principally in the brain). In order this first step reaction to occur, the presence of molecular oxygen and the reducing cofactor 6-tetrahydrobiopterin (6BH4) are required (Walther et al. 2003; Zhang et al. 2004; Jonnakuty and Gragnoli 2008). After that, the 5-OH-tryptophan is decarboxylated to form 5-HT by the L-aromatic amino acid decarboxylase (AAD), in a reaction that requires vitamin B6, vitamin B3, and magnesium as cofactors (Boadle-Biber 1993; Jonnakuty and Gragnoli 2008). Although both the enzymes mentioned above are vital for the tryptophan conversion in serotonin, the TPH is considered the rate-limiting enzyme. The TPH is expressed in several locations, like brainstem neurons, pineal gland, lungs, gut, and skin (Slominski et al. 2005; Nordlind et al. 2008).

2.2.3. Serotonin location, storage, release and reuptake

Serotonin occurs at higher concentrations in three principal locations: in the intestinal mucosa' enterochromaffin cells, where 95% of the body's 5-HT is synthesized, stored, and released; in the blood, stored in platelets; and in the CNS (Thorslund 2012; Wu et al. 2019). Smaller quantities are produced by several other tissues, including the skin (Slominski et al. 2005).

As mentioned above, the body's serotonin's major portion is located in the periphery (blood and intestinal mucosa), and approximately 99% is located intracellularly (Mohammad-Zadeh et al. 2008). The extracellular serotonin, free in circulation, is soluble in plasma, but platelets rapidly take the majority of it. Once within, it is stored in granules via the vesicular monoamine transporter (VMAT). Almost all total body circulating 5-HT is stored in platelet vesicles and taken up by the 5-HT transporter (5-HTT), also called serotonin transporter (SERT) (Jonnakuty and Gragnoli 2008; Fidalgo et al. 2013; Yabut et al. 2019).

2.2.4. Serotonin metabolism

Serotonin degradation occurs mainly in the liver through oxidate deamination. However, the molecules that escape liver metabolism are usually metabolized in lung epithelial cells (Jonnakuty and Gragnoli 2008; Thorslund 2012).

There are two major serotonin metabolism pathways in the organism. The main metabolic pathway is through the monoamine oxidase (MAO) enzyme, which converts serotonin into hydroxy-3-indoloacetaldehyde (5-HIAL), a molecule excreted primarily in the urine (Jonnakuty and Gragnoli 2008; Mohammad-Zadeh et al. 2008; Nordlind et al. 2008). Alternatively, 5-HT can be metabolized by arylalkylamine N-acetyltransferase into N-acetyl-serotonin, and then into melatonin through the hydroxyindole O-methyltransferase (Ganguly et al. 2002; Yabut et al. 2019). Although the liver and the lungs are the primary organs for serotonin's metabolization, the MAO enzyme is a ubiquitous enzyme present in several active sites that include the brain, gastrointestinal tract, lungs, liver, platelets, and also the skin (Tyce 1990; Mohammad-Zadeh et al. 2008; Nordlind et al. 2008).

2.2.5. Serotonin receptors

The various actions of serotonin are mediated through membrane-bound receptors, the 5-HT receptors (5-HTR). Since the 5-HTR discovery and later classification, in 1957, by Gaddum and Picarelli, much of what was known at the time evolved, and nowadays, an extensive and unexpected receptor diversity is recognized (Gaddum and Picarelli 1957; Barnes and Sharp 1999; Marin et al. 2020).

The current classification distinguishes seven families of receptors (5-HT1 to 5-HT7), the 5-HT1, 5-HT2, and 5-HT3 being the major ones (Mohammad-Zadeh et al. 2008). Due to the heterogeneity presented by the receptors, most of them are further classified into subtypes: the 5-HT1R is classified into 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F; the 5-HT2R into 5-HT2A, 5-HT2B, and 5-HT2C; the 5-HT3R into 5-HT3A, 5-HT3B, and 5-HT3C,D,E; the 5-HT5R into 5-HT5A and 5-HT5B (Mohammad-Zadeh et al. 2008; Marin et al. 2020). All of them can be found both in the CNS and in the peripheral tissues (Barnes and Sharp 1999). The 5-HT receptors can act as metabotropic or ionotropic receptors, the 5-HT3R being the only ionotropic receptor. While the ionotropic receptors act as ion channels, the metabotropic receptors are coupled to G proteins and act through the G protein activation and second messenger production) (Martins et al. 2020a).

2.2.6. Serotonin transporter

The serotonin transporter (5-HTT or SERT) is a transmembrane monoamine transporter whose mechanism of action is based on the Na+ and Cl- transportation to the cell

in exchange for K+ (Mohammad-Zadeh et al. 2008). It influences the extent and magnitude of serotonin actions through serotonin release and reuptake and prevents the 5-HTR desensitization (Fidalgo et al. 2013; Martins et al. 2020a). It is mainly expressed in the platelets, but it can also be found in the CNS, gastrointestinal tract, pulmonary system, peripheral vasculature, and skin (Mohammad-Zadeh et al. 2008; Nordlind et al. 2008; Martins et al. 2020a).

2.2.7. Selective serotonin reuptake inhibitors (SSRIs)

The selective serotonin reuptake inhibitors (SSRIs) are a class of antidepressant drugs that bind specifically to the serotonin transporter. They block the serotonin reuptake pump and consequently inhibit the reuptake process, increasing the extracellular serotonin availability and the binding of serotonin to 5-HTR (Mohammad-Zadeh et al. 2008; Fidalgo et al. 2013).

Although these antidepressants are some of the most commonly used drugs in the treatment of depressive disorders, they have also been described as a new class of immunosuppressants since their actions in suppressing immune reactions in several immune cells have already been demonstrated in *in vitro* studies and in animal models (Wu et al. 2020). Besides their anti-inflammatory and immunomodulatory effects, some studies recently brought to light their possible anti-pruriginous effects by demonstrating that SSRIs could have a beneficial effect on humans with dermatological conditions (Eskeland et al. 2017; Boozalis et al. 2018).

2.2.8. Serotonin actions in the skin

Several studies have already demonstrated serotonin actions in skin cells. *In vitro* studies showed that serotonin added to the medium of skin cell cultures has different results according to the cell type and the conditions of the culture (Slominski et al. 2003). A study from 1997 had already proved that 5-HT modulates the proliferation of murine KC (Maurer et al. 1997; Slominski et al. 2012). 5-HT also stimulates, in a dose-dependent effect, the growth of dermal fibroblasts and stimulates the growth of epidermal melanocytes in the absence of growth factors (Nordlind et al. 2008). More recently, a study whose hypothesis was that 5-HT might play a role in skin healing in post thermal injury proved that 5-HT enhanced fibroblasts and KC survival, proliferation, and migration (Sadiq et al. 2018).

Nowadays, 5-HT has several important functions in the skin, being involved in vasodilatation, inflammation, immunomodulation, and in pruritus. It is considered a mediator between the skin and the central neuroendocrine system, playing a crucial role in the skin's homeostasis (Martins et al. 2020a).

Beyond the functional roles of serotonin in the skin, this organ also can synthesize and metabolize this molecule, which means that it has its own serotoninergic system. The

cutaneous serotoninergic system may be considered one of the critical points of the skin's first line of defense since it acts to maintain homeostasis, preserving the skin's physical and functional integrity. Serotonin can influence skin's immune system responses, KC differentiation, fibroblast activity, pigmentary system activity, and hair follicle activity, playing an important role in maintaining an efficient epidermal barrier (Slominski et al. 2005).

2.2.8.1. The skin serotoninergic system

As a neuroendocrine organ, the skin possesses a fully operative serotoninergic system. Mammalian skin cells have the needed enzymes for the serotonin biosynthesis and metabolization pathways (Zmijewski and Slominski 2011). They can convert L-tryptophan into serotonin since they can produce and regenerate the 6BH4 cofactor and possess THP. In the skin, THP is present in blood vessels, mast cells, melanocytes, keratinocytes and fibroblasts, functioning as an antioxidant and influencing cellular metabolism (Slominski et al. 2005; Nordlind et al. 2008). Activated T cells in the skin also express TPH1 (O'Connell et al. 2006; Nordlind et al. 2008). Regarding serotonin's degradation, the mammalian skin expresses MAO, which deaminates 5-HT and leads to the formation of 5-HIAL (Slominski et al. 2005; Nordlind et al. 2008). Moreover, some findings suggest that mammalian skin converts 5-HT into melatonin (Nordlind et al. 2008; Zmijewski and Slominski 2011).

The SERT is an important component of the skin's serotoninergic system. It can be expressed in skin cells and also in immunocyte cells. In human skin, it is present in KCs, mast cells, melanocytes, LCs, dermal fibroblasts, T cells, NK cells, and sensory nerve endings (Kim 2012; Zabolinejad et al. 2019). There is not much information about the SERT presence and localization in dogs' skin cells.

The 5-HT receptors are a fundamental element in the serotoninergic system. Nevertheless, information about the 5-HTR in the dog's skin is still scarce. In contrast, several studies have been done with human skin and concluded that skin cells express different 5-HTR according to each cell type (Martins et al. 2020a). Although there is a lack of data about the serotonin receptors in canine skin, Fröberg et al. have already demonstrated the presence of 5-HT1AR in canine mast cells (Fröberg et al. 2009). More studies should be performed to identify 5-HTR in the dog's skin cells and elucidate the different functions of serotonin receptors in skin inflammation and immunomodulation.

2.2.9. Role of serotonin in the skin's immune system

Serotonin was recognized as an immunomodulator around the 1980s, when its capacity to suppress or stimulate inflammation was demonstrated (Arreola et al. 2015). Nowadays, the immunomodulatory effects of serotonin are well proved, and 5-HT and its receptors are

recognized as having critical roles in immune signaling regulation. However, the mechanisms of action by which serotonin regulates a wide variety of immune responses have not been fully elucidated (Arreola et al. 2015; Martins et al. 2020a). There is evidence, both in humans and rodents, that serotonin is able to regulate inflammation and immunomodulation through 5-HTR differentially expressed on immune cells. 5-HT plays a role in cell proliferation and migration, recruitment of innate immune cells, production of cytokines and chemokines, and in the connection between innate and adaptive immune responses (Martins et al. 2020a).

Several immune cells resident in peripheral tissues, including T cells, DCs, and macrophages, can synthesize, store, respond to, and transport serotonin (Wu et al. 2019). So, it ís presumed that the exact mechanisms that occur in the central immune system also occur in the peripheral tissues, like the skin. Indeed, serotonin has powerful immunomodulatory effects on the skin (Martins et al. 2020a).

2.2.9.1. Serotonin actions in skin's innate immune system

Several studies have already investigated serotonin's actions in innate immune cells, like mast cells, neutrophils, eosinophils, macrophages, NK cells, and DCs (Martins et al. 2020a). Although some of the skin's immune cells are capable of serotonin synthesis, in the skin, the major source of 5-HT is the platelets, which release this molecule upon aggregation (Nordlind et al. 2008). Upon its release in a skin injury, 5-HT can exert its actions in several skin cells.

In an inflammatory reaction, 5-HT exerts a chemoattractant effect in neutrophils, recruiting these cells to sites of acute inflammation, as was proved by a study performed on mice by Duerschmied et al. (Duerschmied et al. 2013; Roumier et al. 2018).

Murine and human mast cells have the ability to produce, store, transport, and respond to 5-HT (Kushnir-Sukhov et al. 2007; Martins et al. 2020a). It was also possible to find 5-HT in dog's mast cells (Fröberg et al. 2009). Serotonin exerts several actions in mast cells. It promotes their adhesion and migration and attracts them to the inflammatory sites through 5-HT1AR (Kushnir-Sukhov et al. 2006). By expressing SERT, they can store 5-HT that was sequestered from the environment and then release it upon mast cell IgE linking (Roumier et al. 2018).

Serotonin also has an important role in eosinophils migration since it is a potent chemoattractant for these cells. An *in vitro* study demonstrated that 5-HT can stimulate the migration of murine and human eosinophils and that his action is mediated by the 5-HT2AR (Boehme et al. 2004; Roumier et al. 2018).

Evidence suggests that macrophages can express 5-HTR and SERT, although they may not be capable of synthesizing 5-HT. Serotonin is involved in macrophage function and may have an immunomodulatory role in these cells since it upregulates the phagocytosis

activity of macrophages through 5-HT1AR (Garabal et al. 2003; Wu et al. 2019). Moreover, when in contact with 5-HT, human and murine macrophages decrease inflammation via 5-HT7R (Quintero-Villegas and Valdés-Ferrer 2019). This molecule may also be involved in the NK cell upregulation since it protects these cells against functional inhibition and apoptosis (Betten et al. 2001; Wu et al. 2019).

5-HT plays an immunomodulatory role in DCs that is influenced by the maturity status of these cells, which means that the 5-HTR are expressed differently in mature and immature DCs. For example, in humans, while immature DCs express 5-HT1BR, 5-HT1ER, 5-HT2AR, and 5-HT2BR, mature ones express 5-HT4R and 5-HT7R (Idzko et al. 2004; Roumier et al. 2018; Martins et al. 2020a). 5-HT is a potent chemoattractant for immature but not mature DCs and influences both the mature and immature DCs cytokine profile (Müller et al. 2009). In mature DCs, 5-HT enhances IL-1*β* and IL-8 production and reduces IL-12 and TNF-α production via 5-HT4R and 5-HT7R, and also enhances IL-6 and IL-10 production through 5-HT3R, 5-HT4R, and 5-HT7R (Idzko et al. 2004; Roumier et al. 2018). Overall, it's clear that 5-HT modulates the cytokine profile production by DCs and can consequently influence other cell types, like T cells. Indeed, it modulates the T cell printing capacity. DCs can also modulate the 5-HT concentration in the microenvironment by sequestering 5-HT, via SERT, released from mast cells and platelets and then releasing it during interaction with T cells. They can function as a "5-HT bridging mechanism" between T cells, as was demonstrated by O'Connel, by sequestering 5-HT from activated T cells and subsequently releasing it to naïve T cells, modulating T cells functions (O'Connell et al. 2006).

All these actions regulate the interaction between DCs and T cells, playing a part in the connection between IIS and AIS. Serotonin could be considered an efficient and rapid form of immune communication that may affect early T cell signaling (O'Connell et al. 2006; Martins et al. 2020a).

2.2.9.2. Serotonin actions in skin's adaptive immune system

Although 5-HT influences the innate immune response significantly, it also plays a prominent role in modulating the adaptive immune response. Indeed, 5-HT has been considered a T cell immunomodulator (Ahern 2011).

Some studies have already proved that human and mouse T cells have the needed cellular machinery to synthesize, store, degrade and release 5-HT (Chen et al. 2015). However, studies in canine T cells were not found.

In contrast to active T cells, naïve T cells do not express TPH1. Therefore, while active T cells can produce 5-HT, naïve T cells cannot (O'Connell et al. 2006). Both CD8+ and CD4+ T cell subtypes have the needed enzymes for 5-HT production and degradation. CD8+ T cells have higher TPH1, MAO, and serotonin levels than CD4+ T cells, which could emphasize a

more specific role for CD8+ T cells. More recently, in 2020, Wu et al. brought attention to CD4+ T cells by studying the cellular machinery of their subsets (T naïve, T effector cells, and Treg). They concluded that effector T cells and Treg express different receptors and have different serotonin and enzyme levels, which points out a specific serotonin role in these cell subsets (Wu et al. 2020).

The precise signaling mechanisms of serotonin produced by T cells are not fully elucidated. However, it was hypothesized that it acts in an autocrine or paracrine way to promote T cell activation (O'Connell et al. 2006).

Like DCs, T cells also express 5-HTR differently according to their maturity status (Martins et al. 2020a). Naïve T cells express 5-HT7R while activated T cells enhance the expression of 5-HT7R and also express 5-HT1BR and 5-HT2AR (Quintero-Villegas and Valdés-Ferrer 2019). While 5-HT1BR has been shown to be involved in human and murine CD4+ T cell proliferation (Yin et al. 2006), 5-HT2AR enhances the IL-2 and INF- γ production by T cells (Inoue et al. 2011).

The balance of T cells subsets is affected by the presence of serotonin. By altering cytokine production, serotonin alters the T cell printing capacity and consequently the ratio between the T cell phenotypes. Indeed, treatment with SSRIs alters the Th17/Th reg ratio, reducing it and therefore having an anti-inflammatory effect. It also influences the Th1/Th2 ratio as DCs, in the presence of 5-HT, induce Th-2 priming in naïve T cells, promoting a Th2 response (Müller et al. 2009; Wu et al. 2019).

Although less studied in the context of their serotonin interactions, B cells are also influenced by it. Indeed, mediated by 5-HT1AR, 5-HT increases B cell proliferation. These cells also express SERT, and its expression is markedly enhanced in activated B cells comparing to inactivated ones (Roumier et al. 2018; Martins et al. 2020a).

2.3. Canine Atopic Dermatitis

Canine atopic dermatitis (cAD) is a complex and challenging disease for both the veterinarians and the owners of atopic dogs, significantly affecting the quality of life (QoL) of both dogs and their owners (Noli, Minafò, et al. 2011). Its pathogenesis is complex, its diagnosis is not straightforward, and neither is the treatment, having multiple possible options with variable efficacy and safety (Vogelnest 2021).

Nonetheless, it is very common, having a prevalence that ranges from 10% to 15%, according to the *American College of Veterinary Dermatology (*ACVD) task force on cAD, on a paper from 2001 (Hillier and Griffin 2001). However, this number may have changed most likely because dogs have spent more time at home, having more close contact with indoor allergens (Hillier and Griffin 2001; Marsella and De Benedetto 2017).

Due to its complexity, our understanding of the disease has changed over the years. Much progress in comprehending the diseases has been made since it was considered a type I hypersensitivity reaction to inhaled allergens. In 2006, the *International Committee on Allergic Diseases of Animals* (ICADA), which was the former ACVD task force on cAD, defined it as "a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens" (Halliwell 2006). Nowadays, it is no longer seen as a single entity, but instead, it is considered "a multifactorial and complex inflammatory syndrome that may or may not be associated with a demonstrable allergic response, and in which the skin is the main avenue of allergen exposure" (Marsella 2021).

2.3.1. Pathophysiology

Although its pathophysiology is not fully understood yet, it's clear that cAD occurs due to complex interactions between genetics and environmental factors (e.g. allergen exposure and urban vs. rural life) that modulate the skin barrier and the immune responses (Marsella 2021).

CAD's strong breed association, with the disease being more common in some breeds like the French bulldog, German shepherd dog, Shar-pei, and West Highland white terrier, enforces cAD’s genetic bases (Wilhem et al. 2011). Indeed, its heritability has long been proved, and several studies had already identified a large number of genes associated with cAD features, like IgE function, inflammatory and immunity mediators, epidermal barrier function, among others, that are differently expressed in atopic dogs (Nuttall et al. 2013). However, no consistent genetic factors have been identified yet (Vogelnest 2021).

The skin barrier dysfunction present in cAD could either be a primary skin defect, secondary to the characteristic inflammation of the disease, or a combination of both (Marsella 2021). Whatever the cause, atopic dogs usually have an abnormal stratum corneum morphology and an abnormal stratum corneum lipid lamellae organization with a decreased fatty acid and ceramides content in it. All of these differences contribute to an aberrant skin’s structure (Olivry 2011). Also, cultured KCs from atopic skin have a different form and grow differently from KCs from normal skin (Marsella 2021).

In humans, to assess the skin barrier function in a non-invasive manner, the measurement of transepidermal water loss is used, which represents the total cutaneous water loss, and is related to lower ceramide content and reduced skin integrity (Olivry 2011; Cobiella et al. 2019). A pilot study on atopic dogs was performed to evaluate the feasibility of five methods (skin hydration, TEWL, pH, skin absorbance, and erythema) in the assessment of skin barrier function. This study concluded that the measurement of TEWL in dogs has a lack of reliability, being variable and inconsistent, making it difficult to use as a skin barrier

assessment parameter. Regarding the assessment of skin hydration and skin pH, this study supports their use for skin barrier evaluation since the results showed high repeatability and low variation (Cobiella et al. 2019). Although decreased hydration is a common finding in human AD, there is no evidence that the same occurs in cAD since it was not possible to find significant differences between atopic and healthy dogs. The skin pH is the most repeatable and less variable of the methods assessed. In addition, skin pH reliably correlates with clinical signs. The dog's skin pH has a tendency towards alkalinity, and atopic dogs have a more alkaline pH value comparing to healthy ones (Cobiella et al. 2019; Marsella 2021).

For a long time, KCs were only seen as a structural component of the skin barrier. However, this cellular type is now recognized as an essential component of the skin's immune response, coordinating it and thus having a relevant role in the pathogenesis of cAD. Through the release of cytokines and chemokines that function as key signals, KCs modulate the immune response by influencing how LCs process Ags and the consequent type of lymphocytic response (Marsella 2021). Upon cutaneous exposure and absorption of Ags through a defective epidermal barrier, the Ags are then processed by LCs that posteriorly present them to naïve T cells in the regional lymph node, priming them to Th2 cells, that migrate to the skin and produce Th2 cytokines (e.g. IL-4 and IL-13). These cytokines lead to a B cell activation and consequent IgE production. Antigen-specific IgEs enter into circulation and bind to cells expressing specific IgE receptors, like mast cells. Besides these cells, neutrophils and eosinophils are also recruited to the skin's inflammatory reaction. Upon a subsequent reexposure to the same Ag, the epidermal LCs, with cell surface specific IgE, process the Ag and migrate to the dermis, presenting it again to naïve T cells, prolonging the Th2 response. With the chronicity of these pathological mechanisms, a polarization towards a Th1 response starts to exist with Th1 cytokines production (e.g. IL-2 and interferon γ (IFN- γ)) (Marsella et al. 2012). For a long time, much emphasis was given to the Th2 cellular response with IgE production (Marsella 2021). Nowadays, cAD is no longer seen as a Th2 disease, and instead, it is characterized by an imbalance in T cell phenotypes and inflammatory mediators, such as cytokines and chemokines (Pucheu-Haston et al. 2015). Indeed, an abnormal immune response in cAD is characterized by increased Th2, Th17, and Treg levels, with also increased levels of Th2 cytokines, like IL-4 and IL-13. In chronic cAD lesions, there is a strong presence of Th2, Th1, and Treg with high levels of IL-13, IL-22, and IFN-γ (Gedon and Mueller 2018; Rostaher et al. 2018; Marsella 2021). With further studies in the subject, new knowledge was acquired and more relevant roles for several cytokines are now recognized in cAD's pathogenesis. For example, IL-31 was described as being involved not only in pruritus mediation but also in epidermal differentiation and immune responses. IL-17 is a proinflammatory cytokine that is speculated to play a significant role in Th2 response and is

also increased in atopic dogs. Thymic stromal lymphopoietin (TSLP) and IL-33 are also elevated in the skin (Marsella 2021).

The skin barrier has not only a physical component, as was elucidated above, but it also has a chemical and microbiological one. The skin's microbiome, which comprehends the microorganisms and their genetic material, is crucial in modulating the immune response. It interacts with both the innate and the adaptive immune system, preventing microorganisms' overgrowth and infection and also educating it, promoting immune tolerance. A loss of biodiversity is correlated with the increase of inflammatory and allergic skin diseases, and atopic dogs have a decreased biodiversity and a larger number of coagulase-positive *Staphylococcus*. During AD flares, dysbiosis is usually seen and biodiversity is restored once the patient is treated (Marsella and De Benedetto 2017; Rodrigues Hoffmann 2017). In the past, *Staphylococcus* infection was only recognized as a consequence of the inflammation in atopic skin. Currently, it was suggested that *Staphylococcus* could promote typical atopic dermatitis inflammatory responses and affect the skin barrier integrity. The alkaline pH of dog's skin may be related to abnormal bacterial overgrowth (Marsella 2021).

2.3.2. Clinical Manifestations

Atopic dogs usually start manifesting clinical signs of cAD between six months and three years of age (Griffin and DeBoer 2001), pruritus being the main clinical manifestation. In fact, the absence of pruritus automatically rules out cAD (Favrot 2015). Dogs can demonstrate pruritus by several behaviors, such as licking, scratching, rubbing, and others. It can be seasonal if the dog does not contact with allergens all year, like pollens, or non-seasonal, if the dog is in constant contact with allergens, like dust mites (Hensel et al. 2015). The most common anatomical locations of pruritus are the paws, face, ears, and ventrum. However, severe cases can manifest with generalized pruritus (Marsella and Olivry 2001). Pruritus can be the only clinical sign present initially, and in such cases, the dog presents "*pruritus sine materia*", which means pruritus without skin lesions (Favrot 2015), or it can be associated with primary skin lesions like erythema and papules (Hensel et al. 2015). The main affected areas are the muzzle, periocular area, inner ear pinnae, distal member extremities, axillae, ventral abdomen, inguinal and perianal area (Griffin and DeBoer 2001). However, some breeds have different phenotypes, like West Highland white terrier, German shepherd dogs, and Shar-pei, as Willem et al. demonstrated (Wilhem et al. 2011). More recently, in 2017, Casimiro also reported a different phenotype for the Portuguese water dog breed (Casimiro 2017). Due to chronic pruritus, chronic inflammation, and secondary infections, dogs eventually present with secondary lesions characterized by excoriations, self-induced alopecia, lichenification, hyperpigmentation, crusts, and seborrhea (Hensel et al. 2015). Besides pruritus and primary and secondary skin lesions, dogs are often affected by secondary bacterial or yeast infections.

Less common cAD manifestations are hyperhidrosis, urticaria, hot-spot, interdigital fistulae, and seborrhoea oleosa (Bizikova et al. 2015). Atopic dogs can also present with nondermatological features, like otitis externa, ear canal pruritus, and conjunctivitis (Griffin and DeBoer 2001).

2.3.3. Diagnosis

The diagnosis of cAD can be challenging. As mentioned above, cAD can have multiple clinical presentations that vary with the genetic background, the extent of lesions, the presence of secondary infections, and stage of the disease (Hensel et al. 2015). Also, none of the clinical signs are pathognomic (Olivry 2010). Another feature that further complicates the diagnosis is the resembling and sometimes coexistence of cAD with other related skin diseases (Hensel et al. 2015).

The diagnosis is clinical (Marsella and De Benedetto 2017). In 2015, the ICADA established the guidelines for the diagnosis, with the diagnostic approach being based on three fundamental principles. First, it is necessary to exclude skin diseases with similar clinical manifestations that could overlap or resemble cAD (e.g. ectoparasitic skin diseases, microbial skin infections by *Staphylococcus* and *Malassezia*, allergic skin diseases like cutaneous adverse food reaction). Secondly, a careful interpretation of the clinical history and clinical signs must be performed. The ICADA guidelines recommend using “Favrot’s criteria”, which are described in table 1. Finally, upon clinical diagnosis, through allergy tests (Intradermal testing (IDT) or Allergen-Specific IgE Serology (ASIS) testing), it is possible to assess the skin reactivity and identify the allergens that trigger the disease in order to avoid them and/ or use it to produce allergen-specific immunotherapy, which is the only curative treatment option. All the others possible treatment options are only symptomatic (Hensel et al. 2015; Gedon and Mueller 2018).

Table 1 - Favrot's criteria (Olivry et al. 2015)

FAVROT’S CRITERIA FOR CANINE ATOPIC DERMATITIS DIAGNOSIS
• Onset of signs < 3 years
• Dog mostly living indoors
• Corticosteroid-responsive pruritus
• Chronic or recurrent yeast infection
• Affected site: front feet
• Affected site: ear pinnae
• Non-affected site: dorso-lumbar areas
• Non-affected site: ear margins
A combination of 5 criteria fulfilled yields a sensitivity of 85,4 % and a specificity of 79,1 % to diagnose cAD over another pruritic disease. Additionally, if 6 criteria are fulfilled, the specificity increases to 88,5 % but the sensitivity decreases to 58,2 %

2.3.4. Treatment

Canine atopic dermatitis is not seen as a single identity anymore. Instead, it is considered a syndrome characterized by different clinical and molecular phenotypes (Olivry and Banovic 2019). Atopic dogs may present a large diversity of clinical manifestations, and several different pathophysiological mechanisms are involved in cAD pathogenesis. Due to all this diversity, the treatment approach should be individualized for each dog, according to the severity of clinical signs, the extent of lesions, stage of the disease, responses to interventions, side effects, among others factors (Olivry et al. 2015; Nuttall et al. 2019; Santoro 2019). The goal is to find the right combination of pharmacological tools to achieve a tailored and effective lifelong therapy for each patient. Historically, the treatment was mainly focused on inflammation management glucocorticoids being the primary pharmacological tool used. With the more profound knowledge about the disease, it became clear that the therapeutic approach should be broader and multifaceted, with the following main goals: identification and elimination of allergens that evoke flares, improvement of the skin barrier, control of secondary infections, and reduction of pruritus and skin lesions (DeBoer 2013; Olivry et al. 2015).

In 2010, the ICADA designed the guidelines for cAD treatment, which were updated in 2015. The guidelines recommend the best treatment options according to whether the dog is suffering from an acute flare or is in a chronic stage of the disease (Olivry et al. 2015). More recently, in 2019, Olivry and Banovic reviewed the treatment strategy and came to a different point of view, proposing a new approach. They realized that atopic dogs can have both acute (e.g. erythematous macules) and chronic (e.g. lichenification) skin lesions at the same time in different body regions. This concept could bring some confusion since the previous guidelines separated the treatment approach in acute and chronic. So, they proposed a new treatment strategy based on the drug's breadth of action. This new treatment strategy, whose leading factor is the drug's "inflammation-targeting breadth" is divided into two phases: reactive treatment and proactive treatment (Olivry and Banovic 2019). Phase 1 is the reactive phase, in which the atopic dog that is going through an acute flare and can present pruritus and both acute and chronic lesions is treated with the main goal of inducing remission. During this phase, several mediators and cells are activated, sustaining a complex inflammatory reaction, so the dog would benefit from a drug that acts rapidly and has a broad target, the oral short-acting glucocorticoid being the first choice. Once the inflammation is reduced, it should be considered the replacement of oral glucocorticoids for drugs with fewer side effects, like oclacitinib, which is a drug with a semi-broad targeting capacity, alone or combined with topical glucocorticoids. This new treatment concept also sustains a more prolonged therapy to achieve the complete remission of clinical signs, which is when Phase 2 begins. Phase 2 is the proactive phase, whose main goal is the prevention of recurrences. In this phase, it is time to think about the possibility of allergen-specific immunotherapy and to avoid allergens that

could trigger flares. In case of need, there are some pharmacological resources that can be used, like the proactive use of topical glucocorticoids in previously affected locations for two days per week, either the dog presents lesions or not. A single target therapy can also be used, as the monoclonal antibody lokivetmab, since the single target intervention is more efficient when the inflammation is reduced, and there are fewer inflammatory mediators present. If these therapies are not enough to control the dog's clinical signs, in case of flare, a short course of systemic glucocorticoids should be administered to reduce inflammation. Then, the chronic use of broader targeting therapies like oclacitinib and ciclosporin should be considered (Olivry and Banovic 2019).

Besides this strategy, it is essential not to forget the importance of individualized treatment. Due to this syndrome's complexity and the several different pathways that lead to the disease's development, there is a consequent need for a multimodal and customized therapy for each patient since different mechanisms may be significant for some patients and irrelevant for others. As the knowledge about cAD pathogenesis is growing, more possible targets are found out, and more targeted therapies could be developed that could work very well for some patients and not be beneficial at all for others. So, although targeted therapy will not be 100% efficacious in all atopic dogs, it still is a new pharmacological tool that could benefit some atopic dogs.

2.4. Serotonin, human atopic dermatitis, and canine atopic dermatitis: the possible role of serotonin in canine atopic dermatitis

cAD is very similar to human AD, both in clinical and pathophysiological features. A comparative approach between both diseases is sometimes beneficial in understanding not only the pathophysiology but also the possibility of new treatments (Marsella and De Benedetto 2017; Vogelnest 2021).

Several studies have already reported the possibility of serotonin playing a significant role in human AD pathogenesis (El-nour et al. 2007). This idea was reinforced by several studies also reporting the positive effects of SSRIs in the treatment of inflammatory dermatological diseases, including AD, and also as an antipruritic drug (Kaur and Sinha 2018; Martins et al. 2020b). All these facts raise some questions: could serotonin also play a role in cAD? Could the skin serotoninergic system be a new therapeutic target for cAD?

2.4.1. Serotonin and Pruritus in humans

Pruritus is the main feature of both human and canine AD (Marsella and De Benedetto 2017), and serotonin seems to be involved in this quality of life sequestering sensation. Pruritus or itch was first described in 1660, by Haffenrender, as "an unpleasant sensation associated with the desire to scratch" (Haffenreffe 1660; Pongcharoen and Fleischer 2016). It is a

multifactorial phenomenon that functions as an alarm system, protecting the skin against pathogenic stimuli (Miyahara et al. 2019).

Although its pathophysiology has not been fully understood, it is defined as a network of cutaneous and neuronal cells that act together to originate the itch sensation (Pongcharoen and Fleischer 2016). It arises from the stimulation of unmyelinated C neurons, which are in the dermoepidermal junction, by pruritogenic mediators released from epidermal and dermal cells. The impulses are sent to the dorsal root of the spinal cord and then through the spinothalamic tract to the thalamus and finally to the cortex (Kaur and Sinha 2018).

At the moment, there are still a lot of questions to answer about the pruritus mechanism and the involved mediators. However, it is clear that serotonin is involved in the itch processing. The exact mechanisms are still unclear, but several studies already proved serotonin's contribution to pruriceptive transmission (Miyahara et al. 2020).

The results of the studies performed to evidence the serotonin role in pruritus are contradictory. Some studies demonstrate the pruritogenic actions of serotonin, presenting it as a pruriceptive mediator, while other studies show the antipruritic effects of SSRIs. Ancient studies showed that the administration of serotonin intradermally or subcutaneously in human and rodent skin evokes pruritus (Weisshaar et al. 1997; Yamaguchi et al. 1999). The exact mechanisms that explain this reaction are still unknown. However, pharmacological and genetic studies proved that serotonin receptors (5-HT1A, 5-HT2A, 5-HT2B, 5-HT3, and 5-HT7) are involved in itch sensation (Morita et al. 2015). Indeed, 5-HT7R is a key mediator in pruritus, 5-HT3R antagonists reduce pruritus in humans, and 5-HT2R is involved in human and rats itch sensation (Morita et al. 2015; Ostadhadi et al. 2015). There are also reported cases of adverse cutaneous reactions with pruritus to SSRIs in humans, with an estimated incidence of 2% to 4%. This could be explained by the fact that all SSRIs are 5-HT2BR agonists, and this receptor is responsible for severe itching reactions (Lee et al. 2018). Additionally, 5-HTA1R also prologue inflammation by contributing to mast cell degranulation and prolonging their lifespan (Nordlind et al. 2008). However, these cases of adverse reactions can also be related to individual genetic predisposition and SSRI overdose (Lee et al. 2018).

Although there is the concept of serotonin as a pruritic mediator, there are also studies reporting antipruritic effects of SSRIs, suggesting the use of SSRIs to treat chronic pruritus. An open-label, prospective, proof of concept study about the use of two SSRIs (Paroxetine and Fluvoxamine) in patients with chronic pruritus proved the efficacy of these two drugs, with no significant difference between them in the pruritus reduction, showing best results in patients with AD (Ständer et al. 2009). Recently, in a systematic review about the use of oral antidepressants in patients with chronic pruritus, performed in 2017 by Kouwenhoven et al., the majority of the studies included showed a pruritus reduction once the patients were on oral antidepressant medication, showing that oral antidepressants are a valued option for patients

with chronic pruritus. Indeed, the *European Guidelines for Chronic Pruritus* recommend SSRIs for refractory pruritus that doesn't respond to conventional therapy (Kouwenhoven et al. 2017). Another systematic review performed in 2018 about the effects of SSRIs on chronic pruritus also came to the conclusion that this class of antidepressants is a suitable treatment option for pruritus (Boozalis et al. 2018). A recent study from 2020 also demonstrated that intrathecal injection of fluvoxamine (SSRI) ameliorated scratching behavior in mice with chronic pruritus. The mechanism by which it occurs is still not understood (Boozalis et al. 2018).

The underlying antipruritic mechanism of SSRIs is not clear, but some hypotheses were proposed, such as cerebral suppression of pruritus, downregulation of 5-HT3R after continuous stimulation (Ständer et al. 2009), and dulling of the stimuli transmission (Miyahara et al. 2020).

Although SSRIs demonstrated antipruritic effects in patients with AD, there are no certainties about the pathophysiology behind it. It is known that 5-HT exerts its actions in skin cells and immune cells, and the SSRIs immunomodulatory role could explain the antipruritic effects of this class of antidepressants; more specifically, their immunosuppressive role in T cells (Kim 2012).

2.4.2. Serotonin and Pruritus in dogs

Just like in humans, pruritus in dogs can be classified according to its etiology in 6 categories: dermatological (regarding skin diseases, e.g. ectoparasites, infections, allergies, etc.); systemic (regarding diseases in other organs than the skin); neurological (regarding CNS and peripheral nervous system diseases, e.g. acral mutilation syndrome); psychogenic-psychosomatic (e.g. acral lick dermatitis); mixed (coexistence of pruritic diseases); other (cause not determined). Until now, there are no causes recognized in the systemic category (Olivry and Baeumer 2017). Most dog's pruritus causes belong to the dermatological category, cAD being one of the most common diagnoses of dermatological diseases in dogs that came to the veterinarian with pruritus (Hill et al. 2006).

Humans and dogs share many skin pruritic diseases, like AD, so in 2009, Carr et al. performed a study to evaluate if dogs also share the same pruritogenic mediators with humans. They investigated if histamine, serotonin, substance P, IL-2 (mediators known to cause pruritus in humans) and tryptase (mediator known to cause pruritus in rats), could evoke pruritus behavior in dogs. Although none of the substances elicit pruritus when administered intradermally, they did cause erythema and wheal, similar to what happens in humans (Carr et al. 2009). According to these results, serotonin seems to be involved in the pruritus pathways in dogs, being less pruritogenic in dogs compared to humans.

More recently, in 2019, Wheeler et al. investigated the etiology of pruritus in dogs by characterizing the receptors expressed in the dog dorsal root ganglia for several mediators.

They concluded that dogs express functional receptors for IL-31, histamine, and serotonin (Wheeler et al. 2019). However, the dog's pruritic response to IL-31 is much stronger than the pruritic response to serotonin, demonstrating that serotonin is involved in the modulation of itch in dogs but has a reduced pruritic effect compared to IL-31.

Regarding the possible antipruritic effect of antidepressants in dogs, there is not much information. There is a study about the nonsteroidal management of canine pruritus that showed positive results in dogs treated with an oral tricyclic antidepressant (amitriptyline) (Miller et al. 1992). Recently, Olivry and Baeumer, in a systematic review about the treatment of pruritus in dogs, wrote about the use of antidepressants (SSRIs) as a narrow target drug that could help treat dogs with pruritus (Olivry and Baeumer 2017).

2.4.3. Serotonin and Atopic Dermatitis in humans

Several human and rodent studies already demonstrated the possible role of serotonin in the pathophysiology of many skin conditions like psoriasis, urticaria, allergic contact dermatitis, and atopic dermatitis. There seems to be a correlation between serotonin and inflammatory skin disorders, and serotonin expression is altered in these conditions (Younes and Bakry 2016).

Some studies have already pointed out a strong interaction between atopic dermatitis and serotoninergic markers, indicating the involvement of the serotoninergic system in this disease pathophysiology. Indeed, there is a higher serotonin concentration in allergic contact skin (Lundeberg et al. 1999; Lundeberg et al. 2002) and in chronic eczematous skin (Huang et al. 2004) compared to control skin. There is also a differential expression of serotonin receptors and SERT in atopic skin (Rasul et al. 2016). There is a reduced number of 5-HT1AR positive cells and an increased number of 5-HT2AR positive cells and SERT positive cells compared to healthy skin (El-nour et al. 2007). Rasul et al., years later, also studied the expression of 5-HT1AR, 5-HT2AR, and SERT in lesional and nonlesional skin of patients with atopic dermatitis and concluded that the expression of 5-HT1A and SERT was higher in lesional skin, and the expression of 5-HT2A was higher in nonlesional skin and in dermal vessels. The 5-HT1A expression was more evident in the apical epidermis, suggesting that this receptor is involved in KC differentiation (Rasul et al. 2016). These receptors' differential expression is in agreement with their functions since 5-HT1AR plays an anti-inflammatory role, while 5-HT2R is proinflammatory and is involved in the CD4+ T-lymphocytes recruitment to the inflammation site (Martins et al. 2020a). The actions of pharmacological drugs like buspirone and tandospirone (5-HT1AR agonists) and ketanserin (5-HT2AR antagonist) support the functions of the 5-HTR. Buspirone and tandospirone have been reported to decrease allergic contact reactions in mice and improve skin symptoms in atopic patients, respectively (McAloon et al. 1995; Kawana et al. 2010). Ketanserin has also been reported to

be involved in the reduction of mice allergic reactions (El-nour et al. 2007). The 5-HT7R is implicated in AD since it is a key mediator of acute and chronic itch and 5-HT3R also seems to mediate the pruritus response (Morita et al. 2015; Weisshaar et al. 1997).

The role of SERT in the pathophysiology of AD has gained interest in the past years, and alterations in its expression may be implicated since the rate and intensity of this protein expression are higher in patients with chronic urticaria than in healthy patients (Zabolinejad et al. 2019). There is also a higher degree of SERT expression in lesional atopic skin compared to nonlesional atopic skin. Its expression is more evident in the epidermal basal layer, which may indicate that this protein modulates KC proliferation (Rasul et al. 2016).

There are no certainties about the mechanism by which 5-HT is implicated in the pathophysiology of AD. However, it is clear that 5-HT is involved in it through its interactions with skin immune and nonimmune cells, as was reviewed in the sections “serotonin actions in the skin” and “role of serotonin in the skin immune system”. Some studies have already proposed some hypotheses. Huang et al. proposed that serotonin's role in skin inflammation involves stimulating KC proliferation and consequent cytokine production. They also suggested that 5-HT induces B and T cells via 5-HT1AR (Huang et al. 2004). In addition, 5-HT promotes IL-16 secretion from CD8+ T cells, which is a proinflammatory cytokine and a chemoattractant for CD4+ T cells (Laberge et al. 1996). It also promotes the recruitment of CD4+ T cells to the inflammation site via 5-HT2AR, influencing the CD4+ T cells activity (Thorslund 2012).

A role for 5-HT in the pathogenesis of AD is strengthened by reports on the efficacy of SSRIs and 5-HTR agonists in inflammatory skin diseases. Indeed, several studies have already suggested that both SERT and 5-HTR could be therapeutic targets for AD (Jaworek et al. 2020). Although there are no certainties about the mechanism by which SSRIs and 5-HTR agonists/antagonists act in AD, several reports already demonstrated the positive effects of SSRIs in dermatological diseases (Eskeland et al. 2017). These pharmacological findings, together with the correlation between serotoninergic markers (5-HT, SERT, and the 5-HTR) and AD, contribute to the idea of the serotoninergic system being implicated in the pathogenesis of AD.

2.4.4. Serotonin and Canine Atopic Dermatitis

At the moment, there is no information about the serotoninergic system in dog's skin and the possible serotonin role in cAD. There are, however, some studies about the use of SSRIs for the treatment of cAD and acral lick dermatitis (Wynchank and Berk 1998; Fujimura et al. 2014). In 1998, Wynchank and Berk performed a randomized, double-blind, placebo-controlled trial investigating the effects of fluoxetine in treating acral lick dermatitis in dogs and found it effective in reducing the symptoms (Wynchank and Berk 1998; Fujimura et al. 2014). The ACVD task force in 2001 also mentioned the possibility of using systemic SSRIs as a

nonsteroidal anti-inflammatory therapy but didn't find enough evidence to support it. They consider it a controversial theme, and fluoxetine is referred to as a limited treatment for behavioral problems, like psychogenic dermatitis (Marsella and Olivry 2001). In 2014, Fujimura et al. proposed the use of oral fluoxetine for canine atopic dermatitis and performed a randomized, double-blind, placebo-controlled, crossover trial with no encouraging results (Fujimura et al. 2014). More recently, in 2016, Saridomichelakis and Olivry, in an update about cAD treatment, described oral SSRIs as a therapy with low or less proven efficacy (Saridomichelakis and Olivry 2016).

According to all the present data, systemic SSRIs do not seem to be a useful tool in the management of cAD. However, due to serotonin's effects on the skin, Martins et al. proposed the use of topical SSRIs as a new pharmacological tool for inflammatory skin disease (psoriasis) in humans and demonstrated an encouraging point of view, mentioning it as an "interesting possibility" (Martins et al. 2020b). Due to the fact that cAD is also an inflammatory skin disease and that serotonin may also be relevant for the pathogenesis of cAD, the following question was raised: Could topical SSRIs be a new treatment for cAD?

2.4.5. Could Topical SSRIs be a new treatment for Canine Atopic Dermatitis?

It was proposed that antidepressant drugs, including serotoninergic drugs, might have a potential therapeutic effect in patients with dermatological conditions, like AD, because of their anti-inflammatory and immunomodulatory effects, through T cell suppression, cytokine production, and apoptosis induction, described in investigations with humans and rodent models (Li et al. 2016; Martins et al. 2020a). The idea of using antidepressants as a new therapeutic drug for dermatological conditions gained more relevance when patients with dermatological diseases and mental health disorders on antidepressant medications for their mental illness also had their dermatological symptoms alleviated (Martins et al. 2020b).

A lot of studies have been done in the last decades about the anti-inflammatory power of antidepressants and have demonstrated the potential efficacy of these drugs in dermatology, both in human and veterinary medicine (Martins et al. 2020b). Studies on rodent models of human AD have demonstrated the possible efficacy of SSRIs in AD. For example, an investigation performed in Nc/Nga mice, a mouse model for human AD, revealed that Paroxetine (an SSRI) has inhibitory effects on the development of typical lesions of AD. However, their effect was probably due to their sedative effect, reducing the scratching behavior in mice (Jiang et al. 2007). Li et al. also performed a study on BALB/c mice, which is another model of human AD, and found that fluoxetine (another SSRI) also had beneficial effects in AD-like lesions and that the improvement of the symptoms was due to the anti-inflammatory effects and the reduction of psychological stress. Although the anti-AD

mechanism is still unclear, it was hypothesized that it might be related to a downregulation of IgE and Th2 response. Fluoxetine, by reducing the total IgE and suppressing the Th2 response, reduces lymphocyte proliferation (Li et al. 2016).

More recently, in 2017, Eskeland et al., in a systematic review on the use of antidepressants in dermatology, found out that there was evidence in both rodents and humans that the SSRIs attenuate inflammatory processes. Overall, the authors demonstrated that the use of antidepressants has positive effects in the treatment of inflammatory skin disorders (Eskeland et al. 2017).

The systemic administration of SSRIs for dermatological diseases was already discussed in detail. However, the possibility of using SSRIs in a topical formulation only recently was brought to light (Martins et al. 2020b). There are a few studies describing the analgesic and antipruritic effects of topical cyclic antidepressant drugs with favorable results. For example, Doxepin, when applied topically, showed positive results in the amelioration of pruritus (Berberian et al. 1999; Martins et al. 2020b). But to the best of our knowledge, there are no published studies about the topical administration of SSRI for dermatological diseases. However, in 2008, Nordlind et al. have already suggested that topical administration of SSRI might have beneficial effects on the skin (Nordlind et al. 2008). In 2016, an investigation on photocarcinogenesis and its prevention by serotonin receptor agonists (5-HT1AR) and antagonists (5-HT2AR) demonstrated that the topical application of these molecules could be beneficial, representing a pioneering concept at the time (Menezes et al. 2016). More recently, in 2019, a clinical trial performed on diabetic mice with wounds proved that topical fluoxetine (an SSRI) ameliorated wound healing through fluoxetine's local effect in several cell types. This study demonstrates that the topical application of SSRIs is efficient and indeed has positive effects on the skin (Nguyen et al. 2019). All these studie's results proved that SSRIs could exert their actions when applied topically and can have beneficial effects on the skin. Thus, even though there is not much information about this topic, there is hope due to the skin serotoninergic system role in the pathogenesis of AD and the positive effects of systemic SSRIs in people affected by this disease as already proved in several studies. Therefore, the possibility of using a topical formulation of SSRIs as a new pharmacological weapon for cAD is a concept worthwhile to pursue in a pilot study.

3. TOPICAL TREATMENT WITH A SEROTONINERGIC DRUG FOR CANINE ATOPIC DERMATITIS

3.1. Introduction and objectives

Recently, strong evidence suggested that serotoninergic drugs, like SSRIs, may be useful in treating dermatological disorders, such as atopic dermatitis (Boozalis et al. 2018). Due to the high similarities between human AD and cAD, it could be presumed that SSRIs might also benefit atopic dogs. The topical use of SSRIs was also suggested to have beneficial effects on the skin (Nordlind et al. 2008). So, a cream of fluvoxamine, an SSRI, offers the promise to be a new and safe therapeutic option as a cAD topical treatment. Pedal pruritus and pododermatitis are very common cAD manifestations, affecting 62-81% of atopic dogs, and very often are refractory to systemic treatments, requiring topical adjuvant therapy for these affected regions (Bizikova et al. 2015). This study will estimate whether topical fluvoxamine might be effective and tolerable in client-owned atopic dogs with pododermatitis.

In this study, we will determine whether daily topical administration of fluvoxamine for a two-week period could affect pruritus level (primary outcome) and paws' skin lesions (secondary outcome) as evaluated subjectively by clinical assessment of erythema and objectively by measurement of transepidermal water loss (TEWL), skin's pH, and skin's hydration of dogs with cAD. It will also be assessed if exists a correlation between the cAD clinical outcomes of this study (pruritus and erythema) and the skin barrier assessments (TEWL, pH, and hydration) (secondary outcome).

3.2. Materials and Methods

3.2.1. Study design

This study was designed as a prospective, randomized, double-blinded, intraindividual, placebo-controlled, small-scale study pilot study. It was assessed and approved by the ethical committee *Comissão de Ética para a Investigação e o Ensino (CEIE)* - of the *Faculdade de Medicina Veterinária (FMV) da Universidade de Lisboa (ULisboa)* and was also authorized by the owners, who signed the written informed consent (Annex 6). Each patient served as its own control, which means that two identical paws of the patient were selected (right and left), and each one received either the control or the fluvoxamine cream.

3.2.2. Study subjects

3.2.2.1. Enrollment criteria

To be included in the study, dogs must have fulfilled the inclusion criteria list and had no exclusion criteria.

Inclusion criteria:

a) Confirmed diagnosis of cAD, according to the 2015 ICADA guidelines for the diagnosis of cAD: compatible history, clinical criteria strongly associated with the disease, and exclusion of skin conditions with clinical signs that could mimic or overlap cAD, through veterinarian-approved flea control regimen, skin scraping, cytology and elimination diet trial consisting of either home-cooked single protein or commercial hydrolyzed protein diet for a minimum of 8 weeks. Dogs with food-induced AD were not excluded if they remained on the diets known not to cause a flare of AD (Hensel et al. 2015).

b) Regular antiparasitic prophylaxis with the same drug before and during the study.

c) Moderate to severe symmetrical pedal pruritus on (at least) two paws with a pedal Pruritus Visual Analogous Score (PVAS) ≥ 3.6/10 evaluated by the owners. This pedal PVAS was derivated from the validated PVAS.

d) Enrolled patients were allowed to be on concurrent medications, but the level of pedal pruritus must not have changed for the two weeks before enrollment. The dosage of the concurrent drugs could not have been changed according to the following indications: eight weeks for ciclosporin, eight weeks for oclacitinib, and two weeks for other anti-inflammatory or anti-pruritic medications.

e) Owners' written informed consent.

Exclusion criteria:

a) Non-adherence to the study protocol.

b) Clinical evidence of superficial pyoderma or yeast infection on the paws requiring oral antimicrobial and antifungal medications at the time of the enrollment.

c) Administration of oral glucocorticoids.

d) Application of other topical medications on the paws.

e) Allergen-specific immunotherapy initiated less than 12 months before the study.

3.2.3. Treatment presentation

3.2.3.1. Topical cream formulation

Both creams were generously provided by Professor Joana Marto from the *Faculdade de Farmácia da Universidade de Lisboa (FFUL)*, for this study. Cream A was the active cream and contained 0,5-5% of fluvoxamine, whereas cream B was the control cream and contained the same constitution of cream A, except for fluvoxamine. The creams were independently allocated and labeled according to an online-generated randomization code with a 1:1 group ratio (randomizer.org). They were supplied in containers of identical color, size, texture, odor and packed identically, being only distinguished by the package label's letter (A or B).

3.2.3.2. Treatment allocation

Each paw (right and left) of the enrolled subjects was randomly allocated to the treatment groups (A or B), using a coin tossed by one of the investigators. Both the owners and the investigators were blinded, which means that neither the owners nor the investigators knew the treatment each paw was receiving until the end of the study. The creams containers are represented in figure 1.

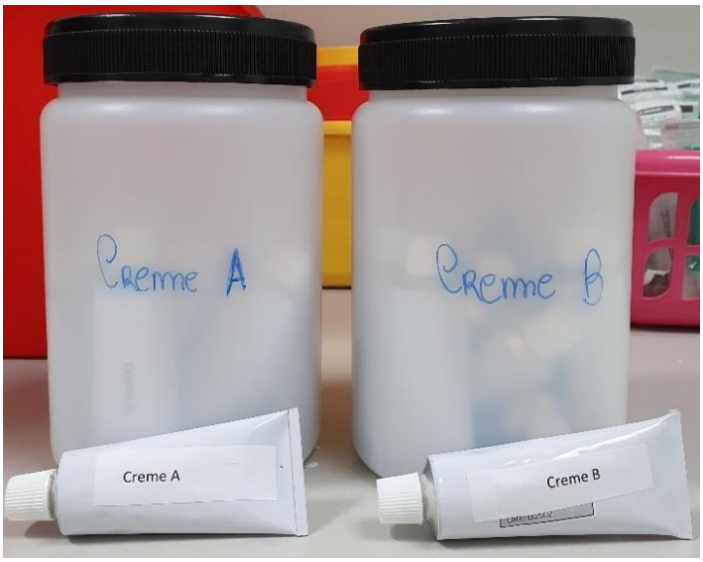


Figure 1 - Cream A and cream B containers (original picture)

3.2.3.3. Clinical Study

The owners of the enrolled atopic dogs were guided to apply each cream (A or B) on the selected paws (right or left) once a day for two weeks, according to the clinician's indications, in their home environment. After the cream application, the owners were instructed to monitor the dog for 15 minutes to prevent it from licking the paws.

3.2.4. Study schedule

After being selected for the study, three visits (on day 0 (D0), day 7 (D7), and day 15 (D15)) were programmed for each enrolled patient.

In the first appointment (D0), the clinician examined the animal and chose two paws with no significant visible inflammation variation. The clinician also rated the erythema of the two paws selected to receive the treatment and measured the TEWL, the skin's pH, and skin's hydration on the selected paws and on two control areas that weren't receiving either the creams (umbilicus and groin). The owners were asked to sign the informed consent, fill the pedal PVAS, and the quality of life (QoL) questionary. At this stage, the two chosen paws of

enrolled dogs were randomized to cream A or cream B, and the owners started the treatment accordingly.

A re-visit was made at Day7 (D7) to score the pedal erythema and measure TEWL, skin's pH, and skin's hydration on the selected paws, umbilicus, and groins. The owners were asked to fill the pedal P-VAS for each paw.

At Day15, the dogs attended the final appointment for the final clinical evaluation. At this last visit, the owners were asked to fill the pedal PVAS for each paw, and the sensory analysis questionnaire. The clinician scored the pedal erythema, and evaluated TEWL, skin's pH, and skin's hydration on the selected paws, umbilicus, and groins.

The study schedule is represented in figure 2.

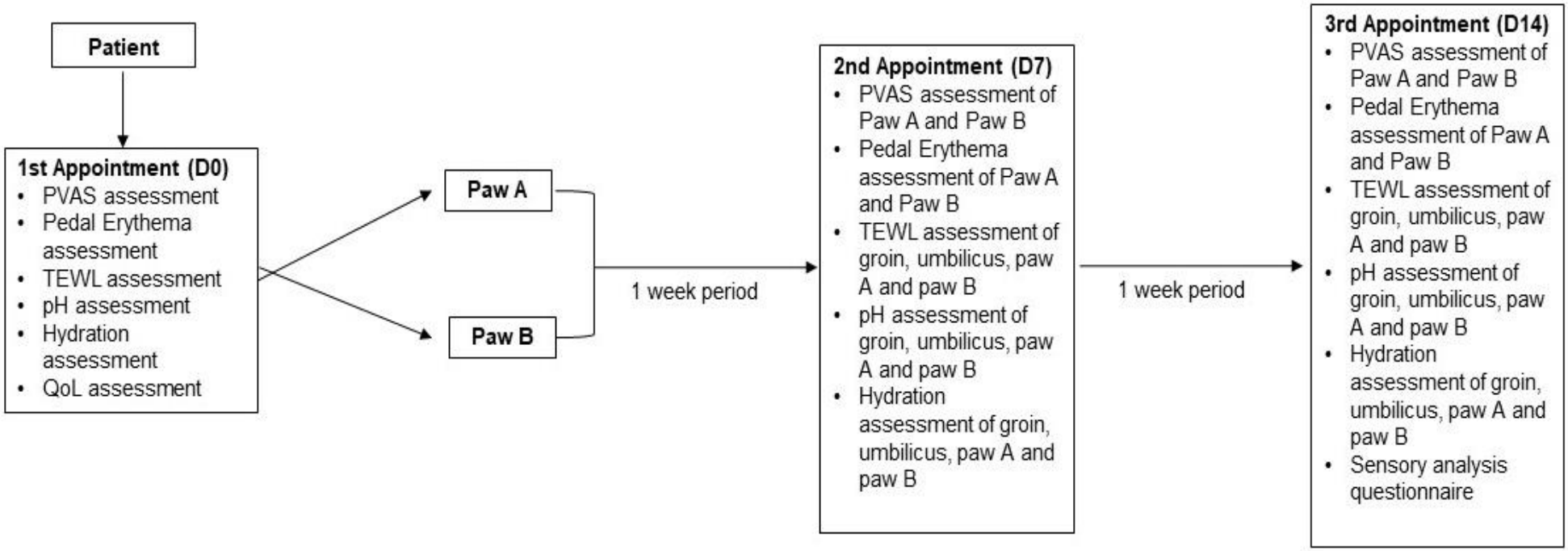


Figure 2 - Study schedule

3.2.5. Outcome measurements

3.2.5.1. Pruritus

The owners assessed each paw's pedal pruritus severity through a validated pruritus visual analogous scale (PVAS) (annex 1). They were instructed to cross a 10cm line, oriented vertically, at the point that corresponds to their dog's pruritus severity, being informed that the scale's start refers to the absence of pruritus and the end corresponds to the most severe pruritus (Rybníček et al. 2009).

The therapeutic success for PVAS was defined as the “percentage of dogs with owner assessed pruritus scores in the range of normal dogs or those with mild cAD at study end", which corresponds to a PVAS <3,6 (Olivry et al. 2018).

3.2.5.2. Pedal erythema

The clinician evaluated the pedal erythema on the three performed appointments. In each appointment, each paw erythema was scored in the following manner: 0 (none), 1 (mild), 2 (moderate), and 3 (severe) (annex 2). Grades 1, 2 and 3 are represented in the figures 3. 4 and 5, respectively. The therapeutic success for pedal erythema was a reduction in one or more levels of erythema grade.

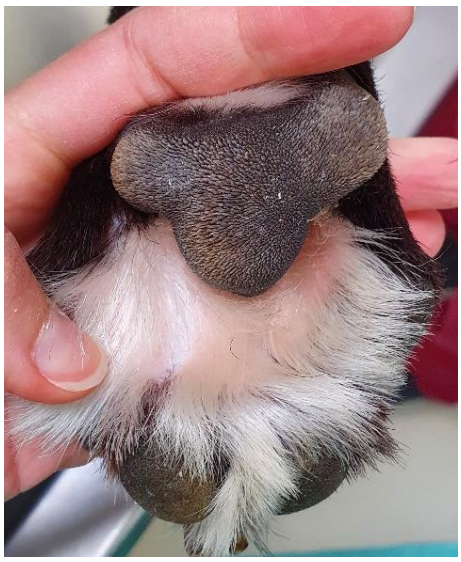


Figure 4 – Mild case of pedal erythema (original picture taken with the owner’s consent)

Figure 3 – Moderate case of pedal erythema (original picture taken with the owner’s consent)

Figure 5 - Severe case of pedal erythema (original picture taken with the owner's consent)

3.2.5.3. Transepidermal water loss (TEWL)

The measurement of the TEWL is a non-invasive method to assess the integrity of the skin barrier. To measure it, the Tewameter TM 210 (Courage & Khazaka Electronic GmbH, Cologne, Germany) was used. This instrument's operating principle is measuring the water evaporation gradient from the skin surface in an open chamber (Barel and Clarys 1995). It is composed of a probe with a cylindrical open chamber system with two sensor units to measure the temperature and relative humidity. It has a small LC screen that shows the TEWL (0-90 grams of water per square metre per hour), the relative humidity (RH) (0-100%), and the temperature (T) (0-50ºC) in the function of time. It is possible to obtain TEWL versus time curves during selected time intervals (Barel and Clarys 1995).

After switching on the Tewameter, it is necessary to wait approximately 15 minutes to heat the probe sensors. Then the probe is placed directly onto the skin, perpendicular to the surface. The patients were acclimated 30 minutes in the observation room before the TEWL measurements, according to the Tewameter operating conditions (RH 30-70% and T 10-30%). The temperature of the room was 22ºC. The patients were restrained in lateral recumbency with no need to be sedated, and the TEWL was measured in 4 areas: the two selected paws, a non-lesional skin site (umbilicus area), and a typical lesional skin site (groin). In order to obtain a stabilized TEWL value, the probe was placed in the measurement location, and five successive measurements were taken every 30 seconds. Then the mean value was calculated. The TEWL measurement procedure is represented in figure 6.

Figure 6 - TEWL measurement (original picture taken with the owner's consent)

3.2.5.4. Skin hydration

The stratum corneum hydration is also a non-invasive parameter that can be assessed using a method based on the skin's capacitance measurement, known as corneometry, which is possible because the skin acts as a dielectric medium (Hester et al. 2004).

In this study the Corneometer® CM 825 (Courage & Khazaka Electronic GmbH, Cologne, Germany) was used. This instrument measures the variation in the dielectric constant due to skin surface water content by differences in the capacitance of a precision capacitor (Hester et al. 2004).

The same conditions (temperature and relative humidity) used for the TEWL measurements were also applied to the skin hydration measurements. After switching on the machine, the Corneometer is ready to operate. The dogs were restrained on lateral recumbency, the probe was placed vertically in the same areas where the TEWL was placed, and the skin hydration was measured. Three measurements were obtained once per second, and the mean value was calculated. The values are expressed in microsiemens (IS) as conductance units.

3.2.5.5. Skin pH

The cutaneous pH is another alternative method to assess the function of the skin as a cutaneous barrier. The Skin-pH-Meter PH 900 (Courage & Khazaka Electronic GmbH, Cologne, Germany) is a useful tool to measure the skin surface's pH. It has a precision of 0.1 pH-unit and shows the pH value in 3 seconds. The measuring principle is based on the measurement of the skin's extractable water-soluble compounds, which are made up of the stratum corneum components and sebaceous and sweat gland secretions, with a connected planar electrode to a voltage meter.

The electrodes are H+ ion-sensitive and are connected to a probe that contains the measurement electronics (Ehlers et al. 2001).

The conditions were the same that were applied to the TEWL and the skin hydration measures. Before each patient's measurements, the probe was washed with distilled water and placed vertically onto the four areas mentioned above for the TEWL and skin hydration measurements. Three measurements were made, and the mean value was calculated and expressed in pH units.

3.2.5.6. Quality of life (QoL) questionnaire

The QoL validated questionnaire (annex 4) was used to assess the QoL of dogs with AD as well as their owners. It is composed of 15 questions and subdivided into three main sections: (i) disease's severity (question 1); (ii) dog's QoL (QoL1, questions 2–8; maximum score: 21); (iii) owner's QoL (QoL2, questions 9–15; maximum score: 21). The owners were asked to answer each question with one of four possible answers: not at all (score 0), a little (score 1), quite a bit (score 2), and very much (score 3) (Noli, Minafò, et al. 2011; Noli, Colombo, et al. 2011).

3.2.5.7. Sensory analysis questionnaire

The sensory analysis questionnaire's objective was to characterize the product in terms of sense, ease of use, product absorption, skin's appearance after the cream's application, among other aspects (annex 3). It is composed of nine questions, and the owners had to rate them, number 1 being the lowest score and the number 4 the highest. The maximum value of the questionnaire was 36 points.

3.2.6. Statistical analysis

All the collected data were recorded and organized using Microsoft Office Excel 2016. The statistical analysis was then performed using the R 4.0.2 program (R Core Team 2020). A p-value<0.05 was considered statistically significant for all the analyzed data.

Descriptive statistical analysis was performed to characterize the studied variables, and absolute frequency, relative frequency, means, medians, variance, standard deviation, range, and percentiles values were calculated. The data were also tested for normality using the Shapiro-Wilk test. As this assumption was not confirmed, the non-parametric Wilcoxon signed ranked test was applied.

Additionally, the correlation between the cAD clinical outcomes (pruritus and erythema) and the skin barrier assessments (TEWL, pH, and hydration) was also determined using the Spearman's rank correlation coefficient rho (ρ). Contingency tables and McNemar's test were also performed to compare associations.

3.3. Results

3.3.1. Sample characterization

Fourteen dogs were enrolled from the Dermatology Service of *Hospital Escolar Veterinário, Faculdade de Medicina Veterinária (HEV-FMV)*. One of the patients (nº13) was excluded from the study due to an adverse reaction (lethargy) that occurred after licking the paws with consequent ingestion of the creams. Consequently, only 13 dogs were included in the statistical analysis.

The group was composed of 3 female spayed dogs (RF =23,08%), 3 intact female dogs (RF=23,08%), and 7 intact male dogs (RF=53,85%). The mean (± standard deviation (SD)) age of the group was 4,59 (±3,21) years old, with a minimum value of 0,7 and a maximum value of 10 years old (Graph 1). The breed characterization is described in table 2.

Graph 1 - Age characterization

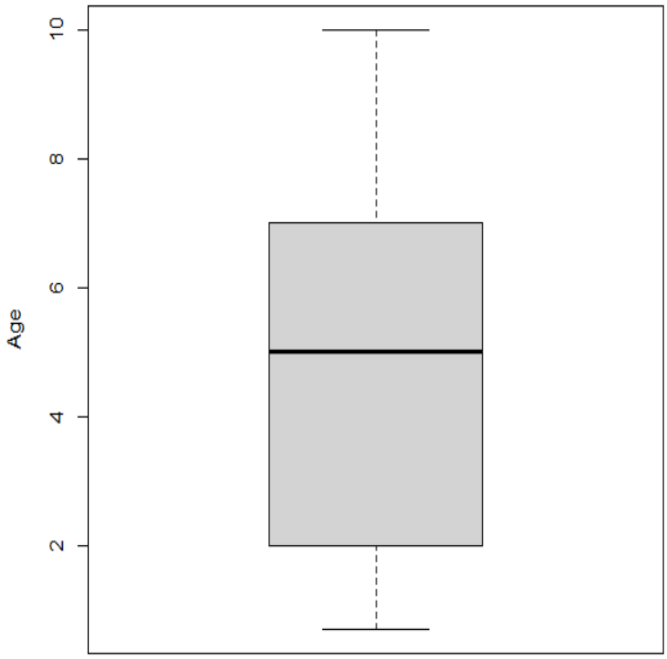


Table 2 - Breed characterization

Breed	n(%)
Miniature Schnauzer	1 (7,69%)
Bull Terrier	1 (7,69%)
French Bulldog	2 (15,38%)
Non-determined	2 (15,38%)
English Bulldog	1 (7,69%)
Boxer	2 (15,38%)
Yorkshire Terrier	2 (15,38%)
West Highland White Terrier	1 (7,69%)
Jack Russel	1 (7,69%)

3.3.2. Study outcomes

The study outcomes during D0 and D14 are represented in table 3.

Table 3 - Outcome measures of the treating paws with cream A (fluvoxamine) and cream B (control) at D0, D7, and D14.

		PVAS		Erythema		Hydration		pH		TEWL	
	Time point	A	B	A	B	A	B	A	B	A	B
Mean	**D0**	5,33	5,33	2,15	2,15	38,53	42,97	6,37	6,63	35,37	30,18
	D7	4,62	4,84	1,38	1,23	44,49	40,69	6,59	6,69	28,99	25,09
	D14	3,65	3,95	1,00	1,15	28,53	32,31	6,69	6,71	26,38	32,07
SD	**D0**	0,86	0,86	0,69	0,69	34,32	36,18	0,74	0,54	22,62	17,50
	D7	1,69	0,99	0,51	0,60	33,25	33,06	0,62	0,44	14,10	13,51
	D14	2,04	1,14	0,58	0,55	26,41	29,52	0,44	0,4	17,69	16,07
Median (50%)	**D0**	5,50	5,50	2,00	2,00	28,33	28,00	6,70	6,67	25,62	27,44
	D7	3,90	4,60	1,00	1,00	44,67	28,33	6,60	6,60	25,05	22,22
	D14	2,80	3,80	1,00	1,00	22,00	20,33	6,70	6,77	20,52	31,10
Interquartile range (IQR)	**D0**	1,20	1,20	1,00	1,00	39,66	47,67	1,13	0,43	32,74	15,80
	D7	2,20	2,10	1,00	1,00	61,00	48,34	0,80	0,53	23,00	8,56
	D14	3,60	0,40	0,00	0,00	16,33	30,33	0,23	0,27	19,77	17,06

3.3.2.1. Pruritus

The pruritus was measured by the owners using the PVAS tool at D0, D7, and D14, and the mean, SD, IQR, and median values are represented in table 3. At the beginning of the study (D0), the values for both paws (A and B) were the same (5,33 ± 0,86). A Wilcoxon signed ranked test was performed, and it was not found a statistically significant difference between pruritus of paw A and paw B between D0 and D7 (p=0,59) and D0 and D14 (p=0,50). The PVAS variation between D0 and D14 for both paws is represented in graph 2.

Additionally, whether or not a difference existed between paw A and paw B was also analyzed regarding the proportion of PVAS values that correspond to success (i.e., according to the definition of “success” described in the section "Materials and methods"). A contingency table with the proportion of successes and failures was drawn for both paw A and paw B, and a McNemar's test

was performed. Neither paw A nor paw B achieved a mean value of PVAS <3,6, although paw A reached a mean value of 3,65 ± 2,04. The association between PVAS <3,6 outcome and group (paw) was also not statistically significant (p=0,48) (table 4).

Graph 2 - PVAS variation between D0 and D14 for paw A and paw B

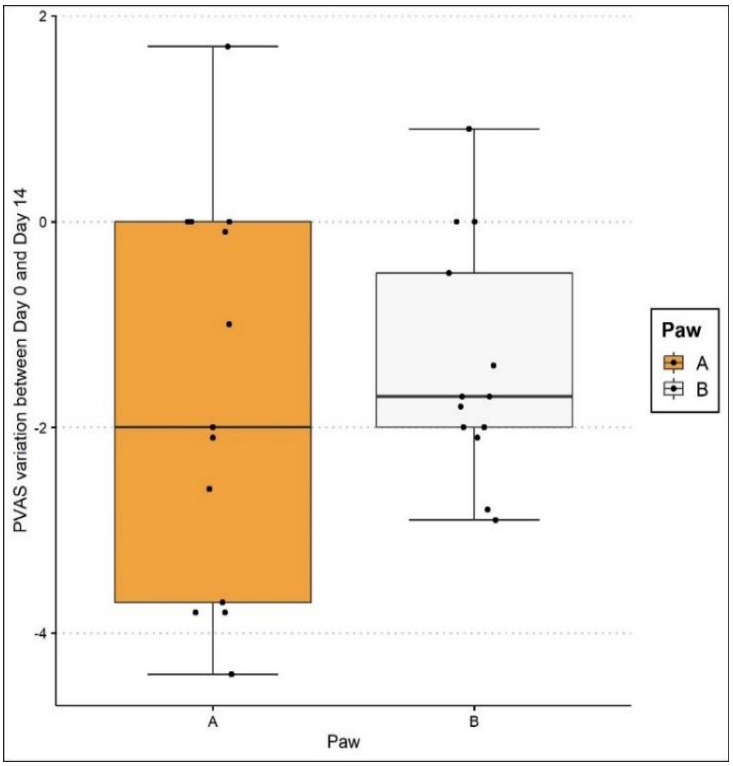


Table 4 - Contingency table and statistical test (McNemar's test) used to verify if there was a statistically significant association between PVAS and group (paw).

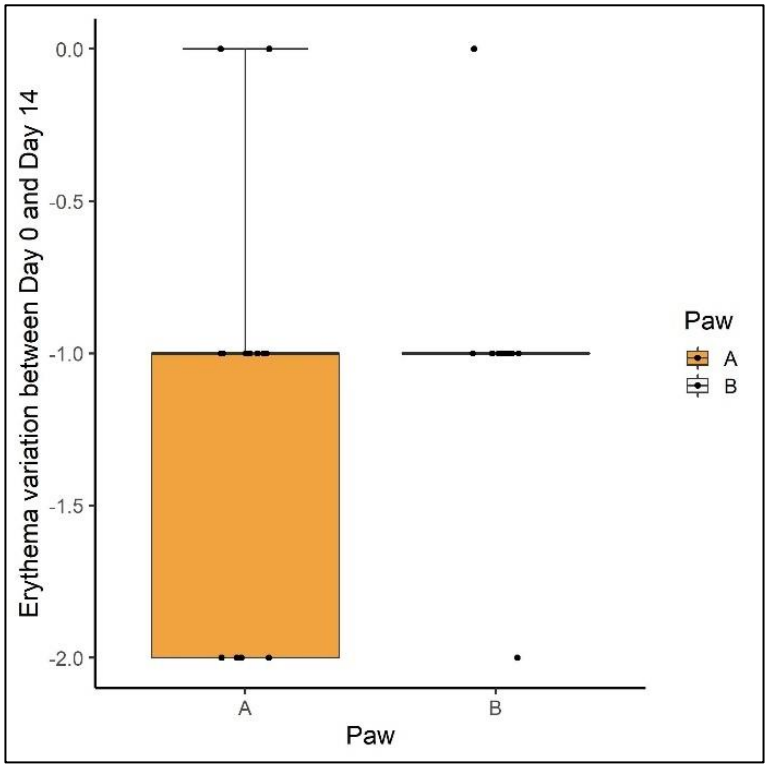
Outcomes	A	B	McNemar's test
Reduction in one grade or more of erythema	84,6	92,3	p=0,0093
Non-reduction in one grade or more of erythema	15,4	7,7	

3.3.2.2. Erythema

The values of erythema were measured by the clinician according to a score that ranged from 0 (none) to 3 (severe) on D0, D7, and D14. The mean, SD, IQR, and median values at D0, D7, and D14 are represented in table 3. The erythema mean values at D0 were the same for paw A and paw B (2,15±0,69). A Wilcoxon signed ranked test was performed, and a statistically significant difference was not found between the erythema of paw A and the erythema of paw B between D0 and D7

(p=0,48) and D0 and D14 (p=0,42). The Erythema variation between D0 and D14 for both paws is represented in graph 3 and in figure 7, figure 8, figure 9, and figure 10.

Graph 3 - Erythema variation between D0 and D14 for paw A and paw B



Plus, whether or not there was a difference between the paws respecting the proportion of erythema values that decreased one degree or more was also analyzed, which corresponds to therapeutic success (i.e., according to the definition of “success” described in the section "Materials and methods"). A contingency table with the proportion of successes and failures was drawn for both paw A and paw B, and a McNemar's test was performed. Both paw A (1,00±0,58) and paw B (1,15±0,55) at D14 decreased one degree of erythema or more, corresponding to therapeutic success. The association between the erythema outcome success and group (paw) was statistically significant (p=0,009) (table 5).

Table 5 - Contingency table and statistical test (McNemar's test) used to verify if there was a statistically significant association between Erythema and group (paw)

Outcomes	A	B	McNemar's test
Reduction in one grade or more of erythema	84,6	92,3	p=0,0093
Non-reduction in one grade or more of erythema	15,4	7,7	

Figure 7 - Pedal erythema before fluvoxamine treatment (D0) (original picture taken with the owner's consent)

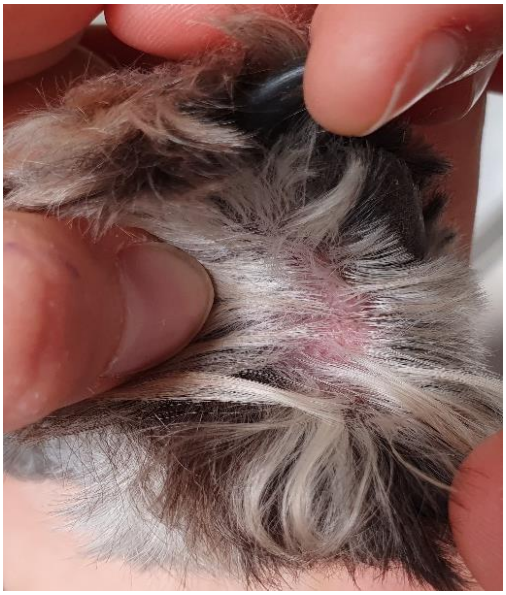


Figure 8 - Pedal erythema after fluvoxamine treatment (D14) (original picture taken with the owner's consent)

Figure 9 – Pedal erythema before fluvoxamine treatment (D0) (original picture taken with the owner's consent)

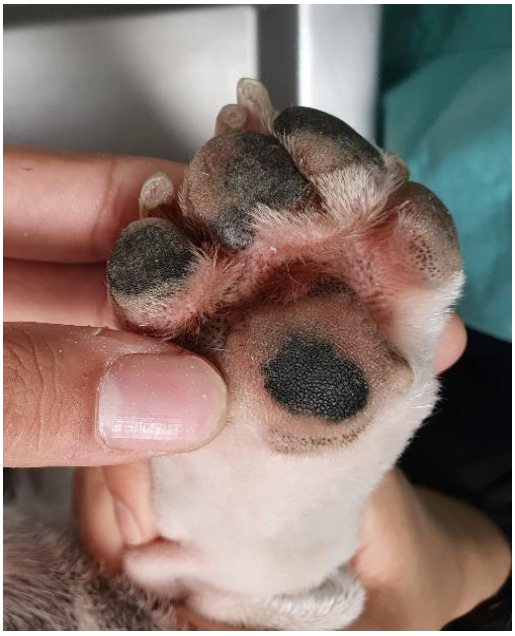
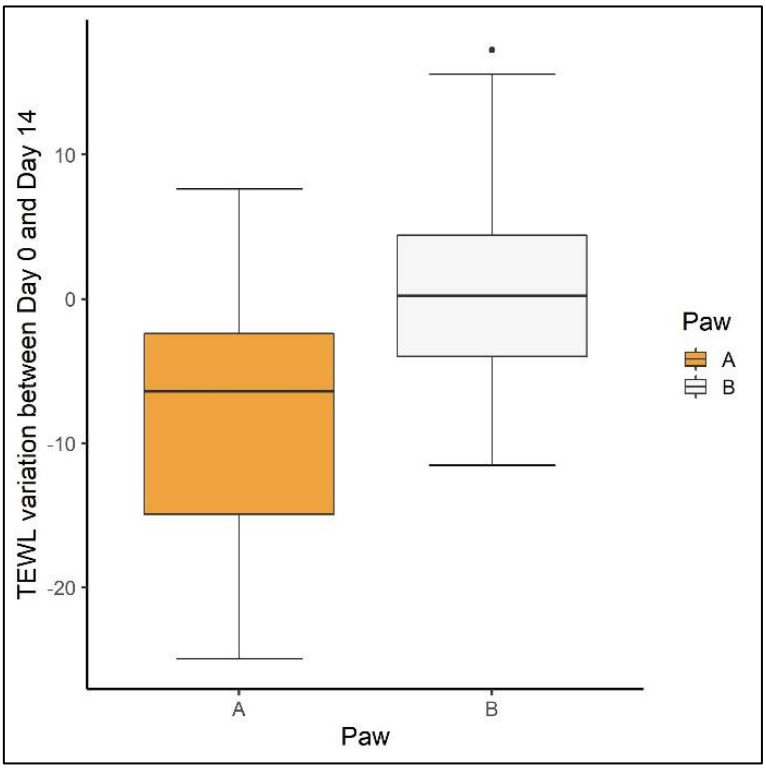


Figure 10 - Pedal erythema after fluvoxamine treatment (D14) (original picture taken with the owner's consent)

3.3.2.3. Transepidermal water loss (TEWL)

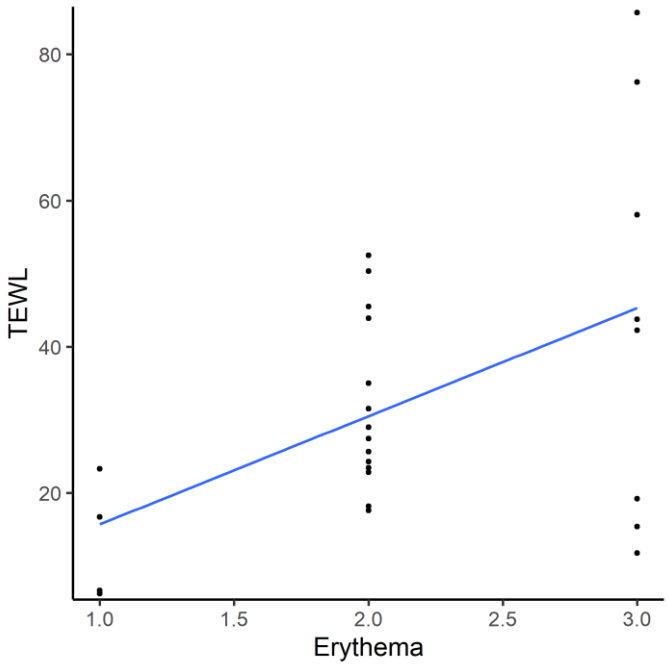
The values of TEWL were measured at D0, D7, and D14 in four areas: paw A, paw B, and in two control areas that didn't receive any of the creams – umbilicus and groin. The TEWL values of the control areas are represented in table 6. It was not found a statistically significant difference between the TEWL of the areas that didn't receive either the cream A or B (umbilicus and groin) between D0 and D7 (p=0,73) and between D0 and D14 (p=0,68). A statistically significant difference between the TEWL of paw A and paw B between D0 and D7 was also not found (p=0,84). However, a statistically significant difference was found between the TEWL of paw A and paw B between D0 and D14 (p=0,008), showing paw A (26,38±17,69) a more significant reduction of TEWL compared to paw B (32,07±16,07). The TEWL variation between D0 and D14 for both paws is represented in graph 4.

Graph 4 - TEWL variation between D0 and D14 for paw A and paw B

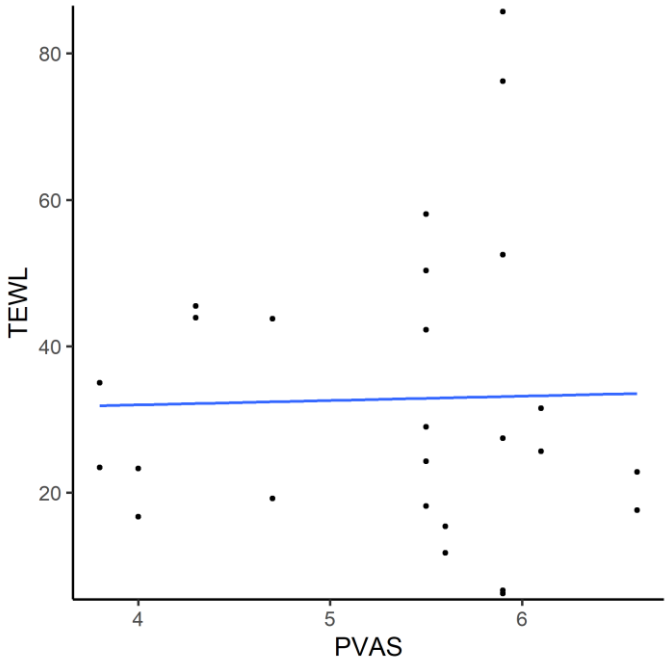


The possibility of a correlation between TEWL and erythema (graph 5) and between TEWL and pruritus was assessed (graph 6). The TEWL was not significantly correlated with PVAS (p=0,63; rho=-0,09), but was significantly correlated with erythema (p=0,04 ; rho=0,404).

Graph 5 - TEWL correlation with erythema



Graph 6 - TEWL correlation with PVAS

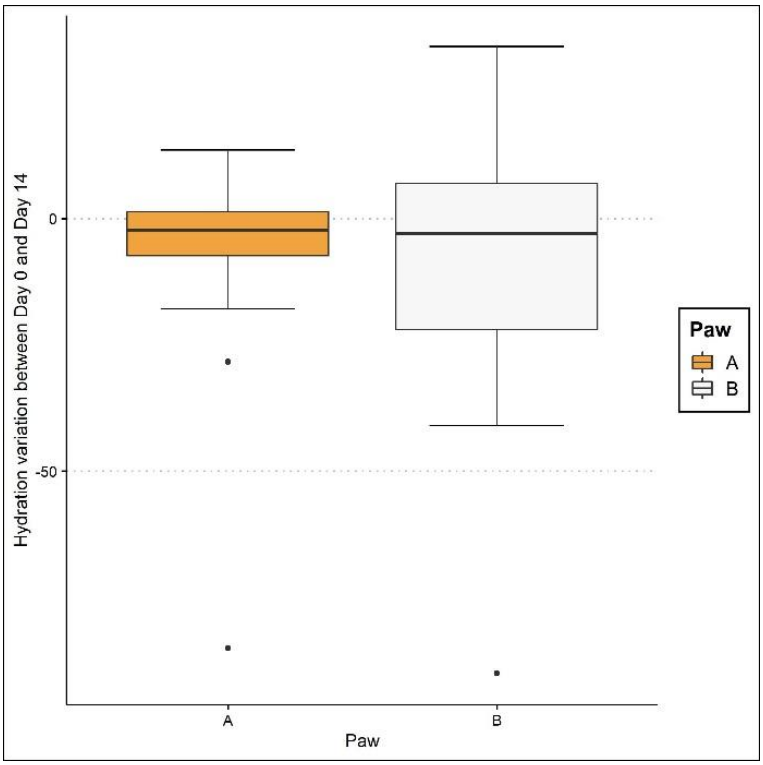


3.3.2.4. Skin Hydration

The hydration values were also measured at D0, D7, and D14 in the four areas referred to in the TEWL section (paw A, paw B, umbilicus, and groin) and are represented in table 3 and table 6. A statistically significant difference between the hydration of the areas that didn't receive either

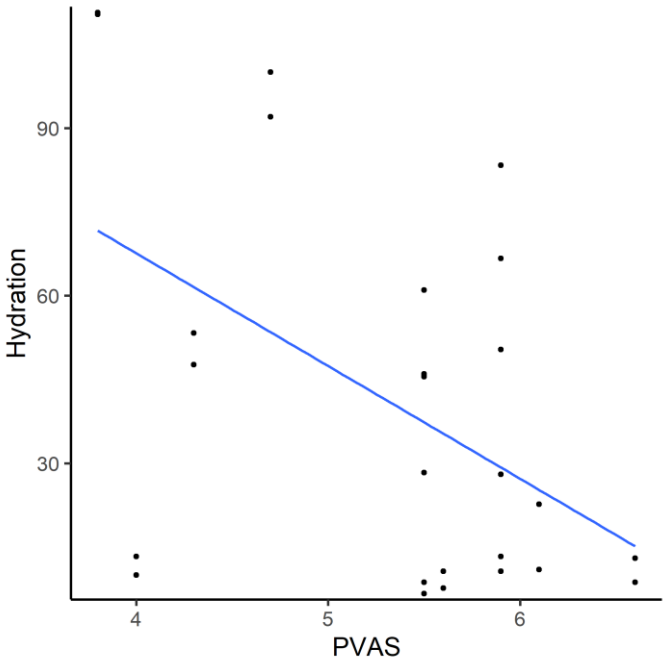
cream A or B (umbilicus and groin) between D0 and D7 (p=0,97) and between D0 and D14 (p=0,094) was not found. The difference found between the hydration of paw A and paw B between D0 and D7 (p=0,057) and between D0 and D14 (p=0,89) was not statistically significant in either of the time intervals. The hydration variation between D0 and D14 is represented in graph 7.

Graph 7 - Hydration variation between D0 and D14 for paw A and paw B

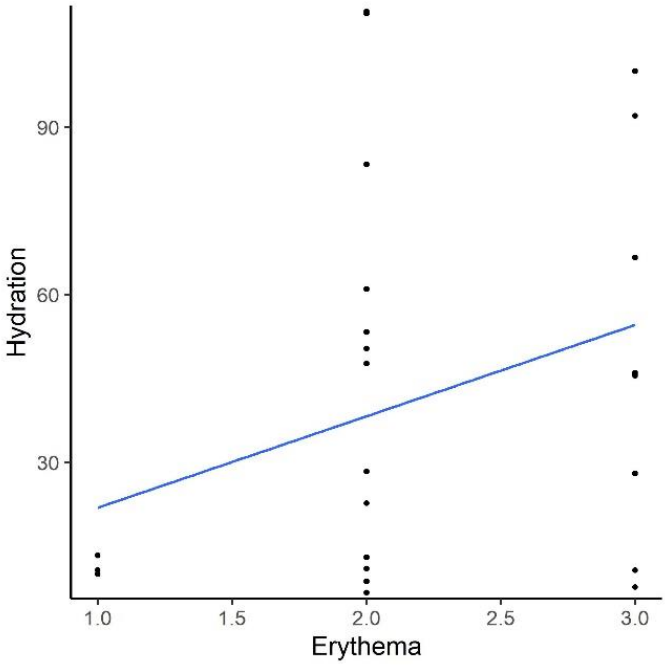


The possibility of a correlation between hydration and pruritus and between hydration and erythema was also assessed, and a statistically significant negative correlation between hydration and pruritus was found (p=0,048 ; rho=-0,390). The erythema was not significantly correlated with hydration (p=0,22 ; rho= 0,25). The hydration-pruritus correlation is represented in graph 8, and the hydration-erythema correlation is represented in graph 9.

Graph 8 - Hydration correlation with PVAS



Graph 9 - Hydration correlation with Erythema

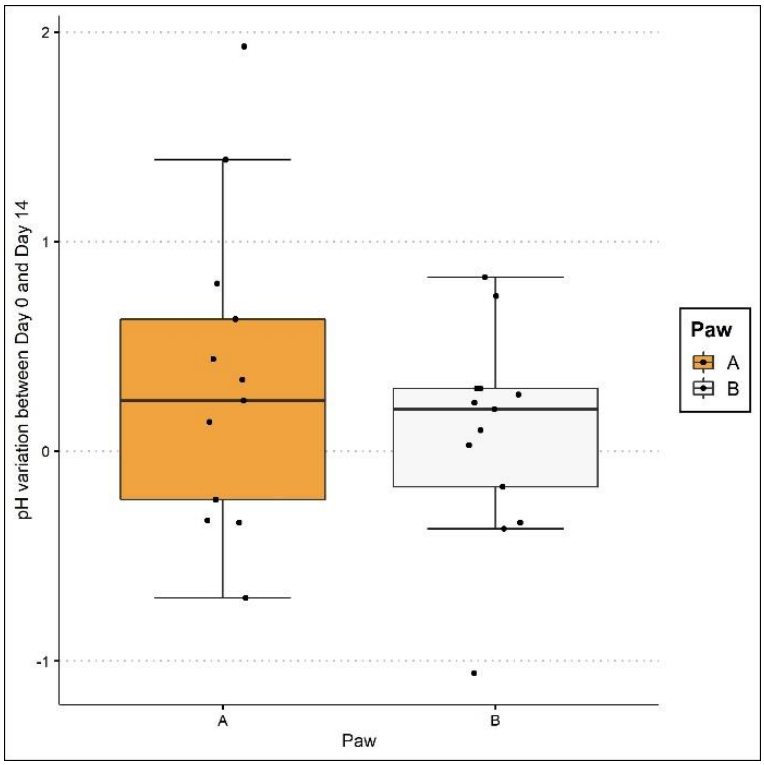


3.3.2.5. Skin pH

Like in TEWL and hydration outcomes, the pH was measured in D0, D7, and D14, and in the same four areas. The pH values for the paws are described in table 3, and the pH values for the

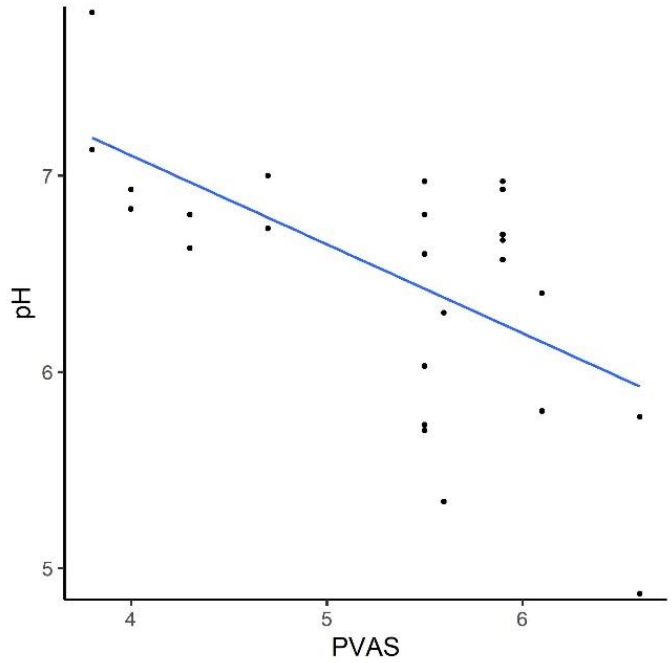
control areas are described in table 6. A statistically significant difference between the pH of the umbilicus and the pH of the groin between D0 and D7 (p=0,38) and between D0 and D14 was not found (p=0,09). Regarding the paws, in both the time intervals D0 and D7 (p=0,50) and D0 and D14 (p=0,22), a statistically significant difference was found between paw and paw B. The pH variation between D0 and D14 is represented in graph 10.

Graph 10 - pH variation between D0 and D14 for paw A and paw B



Regarding the possible correlation between pH and pruritus (graph 11) and pH and erythema (graph 12), a significant correlation between pH and PVAS (p=0,008; rho= -0,51) was found, but a significant correlation between pH and erythema (p=0,67 ; rho= 0,08) was not found.

Graph 11 - pH correlation with PVAS



Graph 12 - pH correlation with Erythema

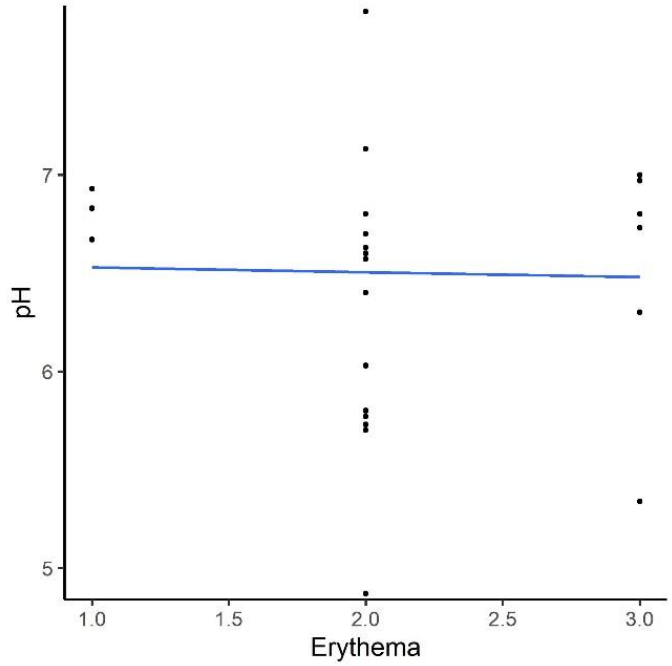


Table 6 - TEWL, hydration and pH values of the control areas at D0, D7, and D14

		TEWL		Hydration		pH	
	Time point	Umbilicus	Groin	Umbilicus	Groin	Umbilicus	Groin
Mean	D0	9,03	8,07	14,81	18,06	6,85	6,76
	D7	11,14	8,79	15,36	25,06	6,76	6,90
	D14	8,96	10,21	17,71	15,08	6,79	6,55
SD	D0	8,44	4,11	10,95	11,51	0,46	0,57
	D7	9,33	7,41	8,98	21,36	0,44	0,45
	D14	5,06	8,99	8,17	7,06	0,35	0,44
Median (50%)	D0	7,40	8,36	21,75	15,00	6,73	6,80
	D7	8,62	8,12	13,33	21,75	6,90	6,93
	D14	8,50	8,30	17,67	15,33	6,80	6,70
IQR	D0	7,02	7,38	16,33	18,00	0,37	0,80
	D7	7,78	3,83	14,00	16,33	0,27	0,50
	D14	4,19	4,46	10,97	12,67	0,30	0,70

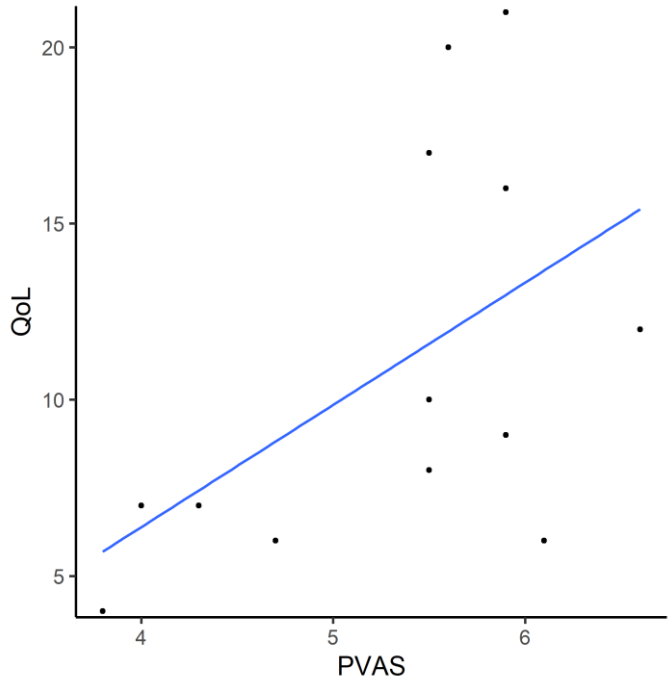
3.3.2.6. Quality of life

The QoL was assessed at D0 in order to find out if there was a correlation between pruritus and QoL. The QoL values are represented in table 7. A significant correlation between QoL and pruritus (p=0,08 ; rho= 0,49) was not found (graph 13).

Table 7 - QoL values at D0

	QoL D0
Mean	11,00
SD	5,68
Median	9,00
IQR	9,00

Graph 13 - QoL correlation with PVAS



3.3.2.7. Sensory analysis questionnaire

The sensory analysis questionnaire was fulfilled by the patient's owners at D14 and the values are represented in table 8.

Table 8 - Sensory analysis questionnaire values

	Sensory analysis questionnaire
Mean	27,23
SD	2,98
Median	27,00
IQR	4,00

3.3.3. Adverse drug reactions

There was one adverse drug reaction reported after the treatment application. The patient nº13 was excluded from the trial at D7 because it appeared lethargic after licking the creams on the paws, which could be due to the action of the serotonergic drug.

3.4. Discussion

To our knowledge, this is the first study evaluating the use of a topical SSRI in dermatological conditions in dogs. The present study investigated whether topical fluvoxamine application would benefit dogs with atopic dermatitis, improving pruritus, erythema, and the skin barrier. We found a significant difference in TEWL (p=0,008) values between the fluvoxamine and the control cream between D0 and D14.

The enrolled atopic dogs served as their own control and active control group, allowing a larger sample and less variability. Each of them received the fluvoxamine cream (cream A) and the control cream (cream B) in different paws according to the investigator's indications. During the study, from D0 to D14, several variables were measured: pruritus, erythema, TEWL, pH, and hydration. On D0, the QoL was also assessed, and on D14, a sensory analysis questionnaire was also answered by the owners. Whether or not there was a correlation between the cAD clinical outcomes (pruritus and erythema) and the skin barrier assessment measurements was also assessed (TEWL, hydration, and pH). The possibility of evaluating the OGATE (Owner Global Assessment of Treatment Efficacy) on D14 was considered by the investigators. OGATE is a tool used to assess the owner's perception of the treatment efficacy on their pets (Olivry et al. 2018). However, as the patients received both the fluvoxamine and the control cream, the investigators concluded that it would become difficult for the owners to evaluate both creams' efficacy since both creams were applied on the same animal and, therefore, the investigators decided not to include this outcome measure in the study.

Regarding the results of the present study, a significant difference in pruritus (p=0,50) and erythema (p=0,42) levels was not found between the fluvoxamine and the control cream when applied to the paws for a two-week period. A significant difference in pH (p=0,09) and hydration (p=0,094) was not found, but a significant difference in TEWL (p=0,008) values between the fluvoxamine and the control cream between D0 and D14 was found. Regarding the possible correlations, a significant positive correlation was found between TEWL and erythema (p=0,04; rho=0,404), and a significant negative correlation between hydration and pruritus (p=0,048; rho=-0,390) and between pH and pruritus (p=0,008; rho= -0,51) was also found. No significant correlation was found between QoL and pruritus (p=0,08; rho= 0,49).

The apparent lack of efficacy of topical fluvoxamine in reducing cAD manifestations (pruritus and erythema) showed in this study converges with several studies' results that have already demonstrated that systemic SSRIs are inefficacious in cAD treatment. Fujimura et al. showed that systemic SSRI (fluoxetine) did not affect pruritus in atopic dogs (Fujimura et al. 2014). Saridomochelakis and Olivry also referred to systemic SSRIs as a low-efficacy cAD treatment (Saridomichelakis and Olivry 2016). Indeed, since long ago, the possibility of this treatment is a controversial theme (Marsella and Olivry 2001).

There are several explanations for the lack of efficacy of fluvoxamine in the reduction of pruritus and erythema. As this is the first study evaluating the effect of topical SSRIs in dogs there

is no information available about neither the optimal concentration of fluvoxamine nor the optimal treatment duration that might cause anti-inflammatory and anti-pruritic effects in dogs. Since the optimal fluvoxamine concentration for the reduction of cAD symptoms is unknown, it is not possible to predict if this formulation would have had positive effects in dogs in a different concentration. There is also no information about how much time the cream effect lasts on the skin, being impossible to predict how many applications are necessary to induce the desirable effects. A conservative option was made, that only one application should be performed. However, the question remains: would more frequent applications induce higher benefits? The same doubt remains for the duration of the therapy. Would a more prolonged treatment lead to cumulative effects and higher benefits? Further studies on dogs are needed to assess if a more or less concentrated formulation has different effects and if more frequent applications and more prolonged therapy have higher benefits.

The lack of significance of the results can also be due to the small sample size (only 13 dogs were enrolled with a consequent N=26). As this is a pilot study, a small sample size is acceptable and even expected due to its exploratory nature. There are several rules of thumb for sample size estimation that favor a value of N that range from 12 to 35 individuals per arm in pilot studies (Bell et al. 2018). Nevertheless, caution must be taken in the interpretation of the results and studies with larger sample sizes are needed to raise the statistical power if this line of work is to be pursued.

Another possible explanation for these results is the lack of anti-pruritic effect of fluvoxamine in dogs, which could be explained by the possibility of serotonin being a pruritic mediator with less power in dogs than humans, being a minor cause of pruritus in dogs. Indeed, serotonin appears to be less pruritic in dogs compared to humans, not eliciting pruritus but causing wheel and erythema when injected intradermally in dogs (Carr et al. 2009). There is still a lot of missing information regarding the dog's pruritic pathways. It is, however, known that dogs have serotonin receptors in the dorsal root ganglia, and although they express them, they manifest a minor pruritic reaction to serotonin when compared to the reaction created by IL-31, which is known to cause a significant pruritic reaction (Wheeler et al. 2019).

An alternative hypothesis for the nonsignificant statistical results is the lack of differences between the fluvoxamine and control creams due to the positive effects of the lipid vehicle present in both formulations that might have improved the skin barrier. It has been proved that the topical application of creams with lipid vehicles has shown positive effects on the skin barrier integrity, improving the stratum corneum lipid lamellae, decreasing the TEWL, and sometimes even reducing skin lesions and pruritus (Tamamoto-Mochizuki et al. 2018). The success of the treatment can be related to the topical formulation itself, which is directly related to the capacity of the drug present in the formulation to permeate into the layers of the skin. This permeation capacity of the drug is also related to the physicochemical characteristics of the topical vehicle (Martins et al. 2020b). The topical formulation can be presented in different pharmacological forms (e.g. creams, sprays, lotions, etc.), and in this study, the chosen form was the cream. A sensory analysis questionnaire was fulfilled by

the owners (annex 3) to characterize the cream in terms of sense, ease of use, product absorption, skin's appearance after the cream's application, among other factors. The questionnaire has a maximum value of 36 points, and the obtained median value was 27,23±2,98, which demonstrate that, in a general way, the owners find the cream easy to apply, with a good appearance, and with a rapid absorption, conferring smoothness to their pets' skin. Thus, the improvement in both paws and the consequent lack of statistical significance might be related to the real positive effect of the lipid vehicle. If this is case, it stresses out the importance of emollients in cAD treatment.

The skin barrier assessment in this study was performed through TEWL, pH, and hydration measurements. The values of both paws and two control areas, which did not receive either the creams (umbilicus – a nonlesional cAD area, and groin – typical cAD lesional area), were assessed. A statistically significant difference was not found between D0 and D14 between the control areas for the skin barrier assessment measurements (TEWL p=0,68; pH p=0,09; hydration p=0,094), strengthening the hypothesis that the differences shown in the areas that received the creams were a consequence of the application of the creams.

This study found a nonsignificant reduction of hydration between D0 and D14 (p=0,094). According to what is described in human medicine, a more hydrated skin would be expected at the end of the study (Zajac et al. 2015). However, the results showed a mean hydration values for both creams at D14 (A=28,53; B=32,31) inferior to the hydration values at D0 (A=38,53; B=42,97). These results are in conformation with the results of the study performed by Cobiella et al., where they evaluated possible tools for skin barrier assessment in dogs. Although they considered the Corneometer (the instrument used to measure the stratum corneum hydration) as a useful tool for skin barrier assessment in dogs, they found no significant difference between healthy and atopic dogs and mentioned that the Corneometer was not reliable on the interdigital space assessment, probably due to the reduced skin surface available and consequent instability of the probe (Cobiella et al. 2019). Another possible explanation for the lack of significance in this study's hydration results is the lack of control in the climacteric conditions during the study. Although the animals were acclimated in the observation room with a controlled temperature of 22ºC 30 minutes before measurements, when it rained, the dogs had their paws wet, which led to unreal hydration values and a wide range of hydration values between the enrolled patients. Regarding the correlation between hydration and clinical outcomes, a significant negative correlation was found with pruritus (p=0,048; rho=-0,390), which could mean that, as far as the hydration increases, the pruritus decreases and vice-versa. On the other hand, hydration values were not significantly correlated with the erythema. The later finding converges with the results of Cobiella et al., which found no correlation between hydration and the cAD clinical outcome measured - Canine Atopic Dermatitis Extent and Severity Index, 4th iteration (CADESI-04) (Cobiella et al. 2019).

Regarding the pH values, a nonstatistical difference between fluvoxamine and control was found between D0 and D14 (p=0,09), with the mean values of skin's pH at D14 (A=6,69; B=6,71) being more alkaline than at D0 (A=6,37; B=6,63). According to Cobiella et al., atopic dogs have a

more alkaline pH than healthy dogs (Cobiella et al. 2019). Thus, we expected to find a more acid pH at D14, which didn't happen. Cobiella et al. also support the use of pH measurement as a skin barrier assessment tool in dogs (Cobiella et al. 2019), which also conflicts with our results. The nonsignificant change in pH values could be due to an insufficient treatment dosage and/or duration. Respecting the possible correlations between pH and pruritus, and pH and erythema, a significant negative correlation was found between pH and pruritus (p=0,008 ; rho= -0,51), which is in conflict with the results from the Cobiella et al. study's. Although the results of Cobiella et al. showed a significant correlation between interdigital pH and the CADESI-04, the correlation is positive (Cobiella et al. 2019), contrary to what was found in the present study. A significant correlation between pH and erythema (p=0,67 ; rho= 0,08) was not found.

Regarding the TEWL assessment, a statistically significant difference between D0 and D14 (p=0,008) was found. The paws that received the fluvoxamine cream showed a more significant reduction (AD0=35,37; AD14=26,38) than the paws that received the control cream (B0=30,18; BD14=32,07). In this study, TEWL was also significantly correlated with erythema (p=0,04; rho=0,404), but not with pruritus (p=0,63; rho=-0,09). The literature shows that the TEWL is the most non-invasive parameter used to assess the skin barrier function in dogs. Atopic dogs tendentially have more elevated values of TEWL compared to healthy dogs, the TEWL being inversely correlated with skin integrity. However, its use is also controversial due to its high variability and lower repeatability (Cobiella et al. 2019). Cobiella et al. described TEWL as a non-reliable tool for the assessment of the skin barrier in dogs and don't support its use (Cobiella et al. 2019). Nonetheless, a study performed in 2019 evaluating the influence of lokivetmab in the TEWL measurement shows results that are in conformity with our study results since the authors described a statistically significant decrease in TEWL values during the lokivetmab treatment, including in the interdigital space (Szczepanik et al. 2019). They also found a significant positive correlation between TEWL and CADESI-04 (Szczepanik et al. 2019), which is also in conformity with the present study results that found a significant positive correlation with the erythema. The decrease of TEWL in the paws that received the fluvoxamine treatment could be the result of the possible anti-inflammatory power of the cream, which caused an improvement in the skin barrier integrity and a consequent reduction in the TEWL values. A possible explanation for the difference between this study and other studies, that do not show significance, is the variability of the TEWL values according to the anatomical area that was evaluated. The TEWL values are influenced by several factors, the anatomical areas being one of them, with some regions having more significant correlations with TEWL, such as the interdigital space, than others (Zajac et al. 2014). The lower repeatability associated with TEWL values exposed in several studies, like in Cobiella et al. study, might be influenced by the variability of anatomical areas where the TEWL values were measured. Thus, the significant difference present in this study might be correlated with the anatomical region, the interdigital space having a significant correlation with TEWL.

A possible but more far-fetched explanation for the lack of statistical significance in the anti-pruritic and anti-erythematous effect of fluvoxamine is related to cAD pathophysiology. cAD has been recognized as a complex inflammatory syndrome, which means that different pathophysiological mechanisms can be involved in the disease development (Marsella 2021). This could suggest that different mechanisms are involved in the disease development in different dogs. Furthermore, different patients present different clinical manifestations, which strengthened, even more, this idea. Thus, it might be the case that serotonin is involved in the pathological mechanism of some dog's atopic dermatitis, but not in others. Consequently, some dogs will respond to topical SSRIs, while others will not. For this reason, it is so important to individualize the treatment approach for each patient.

Taken together, the findings of this study cannot elucidate the true value of topical SSRIs in atopic dogs with pododermatitis, and, unfortunately, these results alone do not offer a novel perspective for cAD treatment.

It is expectable that pruritus impairs dog's quality of life. In fact, a former study had already demonstrated a significant correlation between QoL scores and pruritus (Noli, Minafò, et al. 2011). In this study, however, a statistically significant correlation was not found between pruritus and QoL at D0 ($p=0,08$; $rho= 0,49$). This result could be explained by the exclusion of severely affected dogs for this study due to ethical concerns. At D0, the mean value of pruritus of both paws was $5,33 \pm 0,86$, which corresponds to moderate pruritus. It would be expected that higher levels of pruritus would result in a drop of the dog's QoL. Additionally, it could also be due to the small sample size.

Besides the limitations already mentioned (small sample size and influence of climacteric conditions in the assessment of skin hydration), this study had some other limitations. Firstly, the study design. During the study, it became clear that the study's design was complicated for some owners, especially the ones that spent a lot of time away from home during the day and consequently spent less time with their pets. In the two reevaluations performed at D7 and D14, the owners were asked about the different pruritus grades in the selected paws, and it was complicated for some owners to make this differentiation. It became evident for the investigators that the owners that worked from home and spent more time with their pets had less difficulty in the differentiation between the paws with different pruritus grade, in comparison to the owners that work away from their home environment and only paid attention to the pruritus manifestations of its pet during short periods of the day.

Secondly, besides the problematic evaluation of two different pruritus grades in the same animal, some owners also reported that because they paid more attention to its pet manifestation of pruritus (licking the paws), they perceived a more elevated pruritus grade at the second reevaluation and questioned the real pruritus grade at the first appointment.

Thirdly, the acclimatization time. Before the TEWL, hydration, and pH measurements, the patients needed to be acclimated in the observation room for 30 minutes. Due to the ordinary course

of the hospital appointments, sometimes there were appointment delays and not all the patients were acclimated for 30 minutes and sometimes, the acclimatization period was slightly shortened.

Fourthly, the place where the appointments occurred. So that the obtained measurements, especially the TEWL, are precise and reproducible, it is recommended that the environmental conditions (temperature and humidity) are constant and specific. Due to the local where appointments were performed (dermatology office of the *Hospital Escolar Veterinário, Faculdade de Medicina Veterinária (HEV-FMV)*, it was possible to control the temperature room (22ºC) but not to measure or control the humidity of the room. Thus, further studies in a more controlled room, where both the temperature and humidity can be measured and controlled, are necessary.

Fifthly, the measures themselves. The TEWL measurement was very complicated to perform because it was stipulated that it was necessary to take five successive measures every 30 seconds. However, whenever the animal moved, the probe also moved, and an unreal value was obtained, making it necessary to start the measurements from the beginning. All these particularities turn the TEWL measurement into a difficult-to-perform step of the process that required a lot of time. Overall, the tools used to assess the skin integrity (Tewameter TM 210 for TEWL measurement, Corneometer® CM 825 for the hydration measurement, and Skin-pH-Meter PH 900 for pH measurement) were quite sensible and challenging to use in animals that, due to the natural condition of their behavior, do not stay still to perform the measurements. Further studies should be performed with more advanced tools that can take the measures in a faster way.

3.5. Conclusion and future perspectives

This was, to the authors knowledge, the first study evaluating the effectiveness of a topical SSRI – fluvoxamine - in reducing pruritus and erythema and improving the skin barrier in atopic dogs. The results of the study suggest that the daily application of fluvoxamine for two weeks did not appear to have a superior effect compared to the control cream, except for the TEWL parameter, which evaluates the integrity of the cutaneous barrier, and showed a statistically significant improvement in the group that received the fluvoxamine cream.

Although a statistically significant difference between the fluvoxamine and the control cream was not found for pruritus and erythema, the enrolled dogs generally improved, showing less pruritus and less erythema in their paws at D14 compared to D0.

Due to the study's limitations, it is the author's belief that no permanent conclusions (either pros or cons) should be drawn out of this study's results. In the future, further studies should be performed to evaluate the effectiveness of topical SSRIs in atopic dogs.

During the study, it became clear that this study design was the most serious limitation. Although, theoretically, using the same subject as control and as active control group has advantages, such as less variability and a bigger sample, it was also very challenging to perform a thorough evaluation of the subjective parameters (pruritus and erythema), for both owners and investigators. Therefore, future research should be conducted in more practical settings, for

example, using two groups of patients. This assumption should be considered and future investigations should evaluate the true value of topical SSRIs.

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5. ANNEXES

Annex 1 - Owner-assessed Pruritus Visual Analog Scale (PVAS)

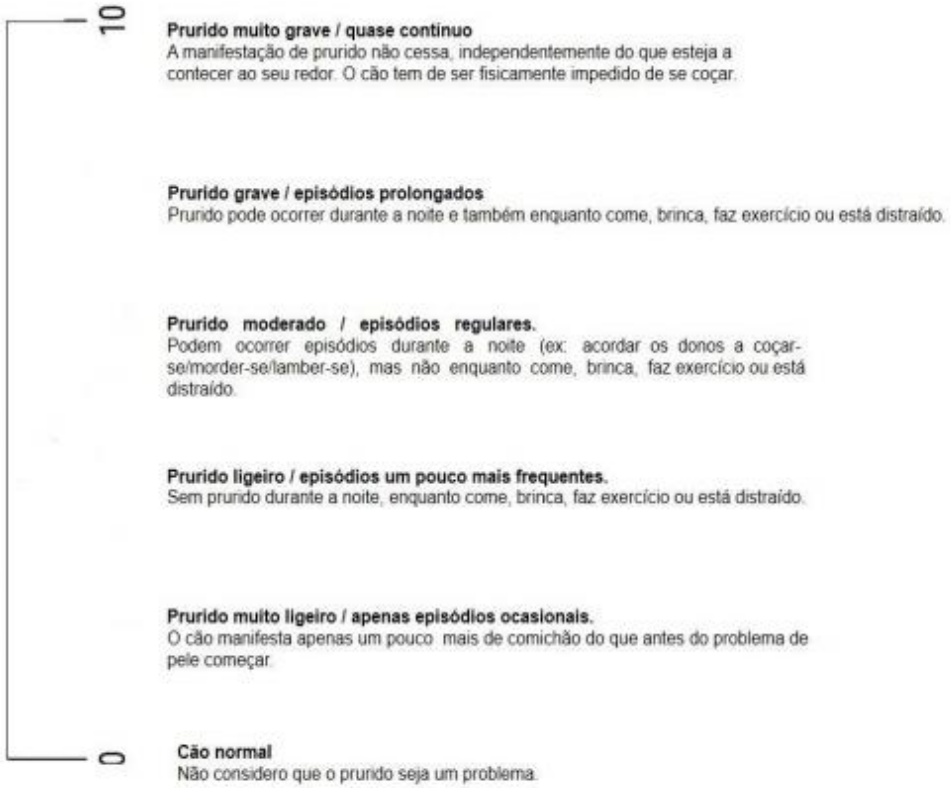
Nº Estudo: _____ **NºGuruvet: _____**

Nome do animal: _____ ___/___/_____

Grau de prurido

Esta escala tem como finalidade medir a gravidade do prurido em cães. O prurido (comichão) pode manifestar-se através de diversos comportamentos, tais como: arranhar-se, coçar-se, morder-se, lamber-se e/ou esfregar-se. Neste caso, vamos avaliar a comichão que o seu cão manifesta nas patas.

Depois de ler as descrições abaixo, começando de baixo para cima. Assinale na escala o local que considera descrever o nível de prurido do seu cão.



Annex 2 - Pedal Erythema assessment

Nº Estudo: _____ **NºGuruvet: _____**

Nome do animal: _____ ___/___/_____

Classificação do Eritema Podal

Pata A	***Pata B***
☐ 0 - Nenhum	☐ 0 - Nenhum
☐ 1 - Ligeiro	☐ 1 - Ligeiro
☐ 2 - Moderado	☐ 2 - Moderado
☐ 3 - Grave	☐ 3 - Grave

Annex 3 - Sensory analysis questionnaire

<u>Questionário</u> – Análise Sensorial

Este questionário pretende caracterizar um produto. Para isso pedimos que responda a algumas perguntas de resposta rápida após a aplicação do produto. A sua identidade não será divulgada, agradecemos a sua colaboração.

1	O produto tem bom aspeto?	Mau aspeto	1 2 3 4	Bom aspeto
3	A aplicação foi:	Difícil	1 2 3 4	Fácil
4	Para si, espalhar o produto foi um processo:	Rápido	1 2 3 4	Lento
5	Considerou o produto pegajoso?	Nada pegajoso	1 2 3 4	Muito pegajoso
6	Após a aplicação, a pele do seu cão ficou:	Seca	1 2 3 4	Hidratada
7	Ao tocar na pele do seu cão, após aplicação, sente alguma oleosidade?	Muita oleosidade	1 2 3 4	Nenhuma oleosidade
8	A absorção do produto na pele do seu cão foi:	Rápida	1 2 3 4	Lenta
9	O produto, após aplicado, confere suavidade à pele do seu cão?	Nenhuma	1 2 3 4	Muita

Muito obrigado pela sua colaboração!

Annex 4 - Quality of life questionnaire

Nº Estudo: _____ **NºGuruvet:** _____

Nome do animal: _____ ____/____/_____

Questionário: Qualidade de vida dos cães com doença cutânea e dos seus tutores.

Leia atentamente as perguntas e assinale apenas uma resposta.

1. O quão grave e perturbadora tem sido a doença do seu cão?

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

2. Qual o impacto da doença no comportamento e/ou humor do seu cão? (Mais nervoso, preguiçoso, agressivo, etc.)

☐ Nenhum
☐ Um pouco
☐ Bastante
☐ Muitíssimo

3. O quão afetado pela doença tem sido o sono do seu cão?

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

4. O quão afetadas pela doença têm sido as refeições do seu cão? (Sem apetite para ração/petiscos, coça-se durante a refeição, etc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

5. O quão afetadas pela doença têm sido as atividades de trabalho/brincadeira do seu cão? (Mais preguiçoso, nervoso, com comichão durante as atividades...)

☐ Nada
☐ Um pouco
☐ Bastante

☐ Muitíssimo

6. Qual o impacto da doença na relação do seu cão consigo, com outros membros da família ou outros cães? (Devido a alterações do comportamento/humor, presença de lesões na pele, etc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

7. O quão a doença mudou os hábitos do seu cão? (Mudança de espaço onde dorme/vive/come, mudança nos passeios, etc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

8. O quão afetado tem sido o seu cão pela administração de terapias? (Banhos, sprays, comprimidos, injeções, limpeza ouvidos, etc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

9. Quanto tempo perde com a doença do seu cão? (Em consultas, cozinhar, fazer limpezas, administrar terapias, banhos…)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

10. Que efeito tem a doença do seu cão no seu nível de cansaço? (Em limpezas extra, cozinhar, banhos, etc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

11. O quão afetadas pela doença do seu cão têm sido as suas atividades habituais e/ou as da sua família? (Lazer, férias, passeios, trabalho)

☐ Nada
☐ Um pouco
☐ Bastante

☐ Muitíssimo

12. Qual o impacto da doença do seu cão nas suas despesas? (Custo do tratamento, veterinário, etc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

13. Que nível de sofrimento emocional lhe causou a doença do seu cão? (Sentimento de culpa, impotência, tristeza, arrependimento, ansiedade, incómodo, repulsa, raiva, frustração, atc.)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

14. Que nível de desconforto físico/inquietação tem experienciado devido à doença do seu cão? (Odor desagradável, sensação de casa suja, incómodo estético, etc)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

15. Que impacto a doença do seu cão teve na relação entre os membros da família? (Entre cônjuges, pais/filhos, parentes, amigos, etc)

☐ Nada
☐ Um pouco
☐ Bastante
☐ Muitíssimo

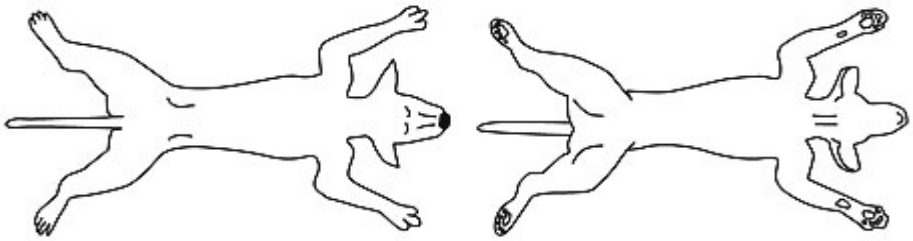
Annex 5 - Owner's guide brochure

Guruvet:_____

ATENÇÃO

- Aplique o produto todos os dias, uma vez por dia, na região anatómica indicada pelo médico veterinário.
- Não altere os hábitos diários de higiene do seu cão (não aplique produtos que não utilize normalmente).
- As bisnagas de produto que recebeu são pessoais. Não aplique este produto noutro animal.
- Se necessitar de administrar qualquer medicação ao seu cão deve avisar o responsável pelo ensaio.
- Anote no verso desta folha qualquer reacção ou sensação observada na pele do seu cão durante o ensaio.

Aplique os produtos nas seguintes zonas:



Caso necessite contacte o investigador responsável:

Raquel Reis

Contactos: 910650222; anaraquelr@campus.ul.pt

No final do ensaio deverá devolver esta folha

Annex 6 - Owner's written informed consent

Consentimento informado para participação em estudo científico/ensaio clínico

Eu,_____
_____, portador(a) do CC nº_____, tutor(a) do canídeo de nome_____, de raça _____, sexo _____ e idade _____, declaro que fui informado e que autorizo a participação no projeto "Creme de Fluxovamina como tratamento tópico adjuvante na Dermatite Atópica Canina", autorizando a colheita, processamento e armazenamento de:

- ___ Dados clínicos e outras informações
- ___ Fotografias

Fui também informado dos procedimentos e condições necessários à realização do estudo:

- Irei aplicar topicamente as formulações medicamentosas (cremes) que me foram entregues, uma vez por dia, durante 2 semanas, a começar no dia da primeira consulta.
- Irei a 2 consultas de reavaliação a cada semana após o início do tratamento e/ou quando o meu animal piorar a sua condição.
- Irei respeitar o protocolo.
- Irei informar os intervenientes acerca de alterações do estado de saúde do meu animal.
- Irei informar os intervenientes caso o meu animal piore a sua condição durante o tratamento.
- Estou ciente de que o tratamento administrado será gratuito.
- Estou ciente que posso desistir do estudo a qualquer momento.

Deste modo dou fé de:

- Ter lido a informação que me foi entregue.
- Ter podido fazer as perguntas que entendi por necessárias sobre o estudo.
- Ter recebido informação suficiente sobre o estudo.

Lisboa, ____ de _____, de 2020 _____

(Assinatura do/a tutor/a)

As informações pessoais recolhidas destinam-se exclusivamente ao projeto e poderão ser utilizadas em âmbito educativo.
As informações estão sujeitas a processamento informático para monitorização do estudo e serão guardadas por um período de 20 anos. De acordo com os regulamentos relativos a dados pessoais, tem o direito de aceder, retificar e limitar o processamento e portabilidade dos seus dados.
Antes do início do estudo poderá anular o consentimento e solicitar a eliminação dos seus dados.
Para exercer os seus direitos poderá contactar dermatologia@fmv.ulisboa.pt