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THE RELATIONSHIPS BETWEEN ENVIRONMENT, DIET, TRANSCRIPTOME AND ATOPIC DERMATITIS IN DOGS

DOCTORAL THESIS

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ACADEMIC DISSERTATION

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*To my beloved Staffie Elvis, who lost his
battle with this disease in June 2014.*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Validating and reliability testing the descriptive data and three different disease diagnoses of the internet-based DOGRISK questionnaire. *BMC Vet Res.* 2016;12:30.
- II Environmental and phenotype-related risk factors for owner-reported allergic/atopic skin symptoms and for canine atopic dermatitis verified by veterinarian in a Finnish dog population. *PLoS One.* 2017;12(6):e0178771.
- III Effect of diet on serum biochemistry and haematology profiles in Staffordshire bull terriers: a randomized, controlled diet intervention study. Submitted.
- IV Diet affects transcriptome differently in the skin of atopic and healthy Staffordshire bull terriers. Manuscript.

The publications are referred to in the text by their roman numerals. The papers are reprinted with the permission of the publishers.

CONTRIBUTIONS

The contributions of all authors in the original articles of this thesis are presented in the following table.

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ABSTRACT

Canine atopic dermatitis (CAD) is a multifactorial disease including genetic predisposition and other predisposing factors like living environment and diet. There is no known cure for this disease. The clinical signs can be managed with medication controlling the pruritus and treating the secondary infections, but many medications can cause unwanted side effects. The right and functional treatment can be hard to find, and more effort should be put into the prevention. The aim of this thesis was to find environmental factors and breeds associated with allergic skin symptoms and atopic dermatitis in pet dogs. In addition, the effect of a raw diet on gene expression, physiology and metabolism was studied with clinical diet intervention trial setup, using client-owned dogs. This was done to increase the understanding of the role of the diet in care and prevention of CAD.

Internet-based data collection is a cost-effective and simple method to obtain epidemiological data. The DOGRISK questionnaire presented in studies I and II was developed to gather information about the associations between diseases, dog characteristics, living environment and diet through a large-scale questionnaire that could be filled out for dogs of all breeds and mixed-breed dogs in Finland. For the reliable utilization of the gathered data, the questionnaire was validated in study I, using three different approaches. The validity of the questionnaire was proven to be good.

In study II, a population of 8643 dogs from the DOGRISK questionnaire was used to analyse the environmental factors and dog characteristics related to CAD. Five breeds with the most owner-reported skin symptoms as a percentage within the breed were found to be i) West Highland white terrier, ii) boxer, iii) English bulldog, iv) Dalmatian, and v) French bulldog. When FCI breed groups were compared to mixed-breed dogs, groups 3 (Terriers) and 6 (Scent hounds and related breeds) had a significantly higher risk for owner-reported skin symptoms. On the other hand, groups 5 (Spitz and primitive types) and 10 (Sighthounds) had a significantly lower risk for owner-reported

skin symptoms. These results indicate that there is a genetic predisposition for allergic skin diseases, and this should be considered when choosing dogs for breeding. The environmental factors found significantly associated with less owner-reported skin symptoms and veterinary-verified CAD in study II were i) being born in the owner family and ii) living with other dogs. In addition, significant association of less owner-reported skin symptoms was found with dogs living in a detached house. Factors that were significantly associated with more owner-reported skin symptoms included extremely clean household and over 50 % of white colour in the coat.

In the clinical diet intervention study, 48 client-owned atopic and healthy Staffordshire bull terriers were fed two different kind of diets (raw and dry food). Haematological and clinical chemistry profiles were analysed from 33 dogs, folate and B12 from 31 dogs, iron from 19 dogs, and transforming growth factor β 1 (TGF- β 1) concentration from 23 dogs (study III). In addition, gene expression profiles were determined from the skin samples of eight dogs (study IV). The high fat-low carbohydrate raw food diet significantly decreased, among others, alkaline phosphatase, glucose, and cholesterol. On the other hand, the low fat-high carbohydrate dry food significantly increased, among others, cholesterol, alkaline phosphatase, and inorganic phosphate. Plasma folate and B12 and whole blood iron were significantly decreased and TGF- β 1 significantly increased by the raw food diet.

In study IV, the gene expression in the skin was also affected by the diet. There were genes related to immune defence, reactive oxygen species, antioxidants, and energy metabolism upregulated in dogs fed raw food. Several genes were found differentially expressed between atopic and healthy dogs, some unrelated to diet fed and some differentially affected by the diet in atopic and healthy dogs. These results are preliminary and should be confirmed using more samples. Nevertheless, they give an interesting and novel information about the effect of the diet on the skin gene expression.

In conclusion, this thesis presents results from different study designs all concentrating on the same disease, canine atopic dermatitis. Features that

come up in many studies in this thesis are related to immune defence and exposure to microbes. Extremely clean living environment as well as minor contact with other dogs might not stimulate the immunity enough and could lead to incorrect development of the immune system in young animals. On the other hand, raw food or diet with high fat and low carbohydrate with only naturally occurring vitamins and trace elements might stimulate the immune system more than highly processed food with added vitamins and trace elements and low fat percentage. The incomplete stimulation could result in atopic dermatitis, hypersensitivities and food allergies later in life, and should be considered when thinking of the lifestyle that might prevent allergic diseases in dogs.

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ABBREVIATIONS

ABCD2	ATP binding cassette subfamily D member 2
AD	Atopic dermatitis
ALOX ₁₂	Platelet-type 12-lipoxygenase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
ASS1	Argininosuccinate synthase 1
CAD	Canine atopic dermatitis
CADESI	Canine Atopic Dermatitis Extent and Severity Index
CBS	Cystathione β -synthase
EDC	Epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FC	Log ₂ fold change
FCI	Fédération Cynologique Internationale
FDR	False discovery rate
FKC	Finnish kennel club
FLG	Filaggrin
LPS	Lipopolysaccharide
FXR	Farnesoid X receptor
LGALS ₁₂	Galectin 12
Hb	Haemoglobin
Hct	Haematocrit
HCAR ₁	Hydroxycarboxylic acid receptor 1
IBD	Intestinal bowel disease
IGHM	Immunoglobulin heavy constant mu
IL-1	Interleukin-1
KRT	Keratin
LOX	Lipoxygenase
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MRRF	Mitochondrial ribosome recycling factor
NFE	Nitrogen-free extract
NRC	National Research Council
NO	Nitric oxide
OR	Odds ratio
PIGR	Polymeric immunoglobulin receptor
PPV	Positive predictive value

P-VAS	Pruritus Visual Analogue Scale
RBC	Red blood cell count
RI	Reference interval
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SIBO	Small intestinal bacterial overgrowth
SLPI	Secretory leukocyte peptidase inhibitor
TEWL	Transepidermal water loss
TGF- β 1	Transforming growth factor –beta 1
VLCFA	Very-long chain fatty acids
WBC	White blood cell count

1 INTRODUCTION

Canine atopic dermatitis (CAD) is a pruritic and inflammatory allergic skin disease with characteristic clinical features and genetic predisposition, and it is associated with IgE antibodies usually directed to environmental allergens (Halliwell, 2006). It has been generally accepted that CAD has a hereditary component along with environmental factors (Bizikova et al., 2015), including the possible influence of the diet (Nødtvedt et al., 2007). CAD affects up to 10 % of dogs (Ka et al., 2014), and pure-bred dogs are reported to have a greater probability of expressing atopy and allergic dermatitis than mixed breed dogs (Bellumori et al, 2013). As there is a strong genetic component behind CAD, there should be no excuse for using atopic dogs for breeding. Environmental and dietary factors might be crucial for preventing this disease especially in dogs with lower genetic predisposition. Nevertheless, there is still only few studies conducted on pet dogs that report predisposing environmental factors for CAD.

To our knowledge, only one validated, internet-based longitudinal canine health questionnaire exists in the UK (Pugh et al., 2015). In addition, cross-sectional studies of separation-related disorder (Konok et al., 2015), risk factors for surgical gastric dilatation-volvulus (Pipan et al., 2012), and risk factors for injury among agility dogs (Cullen et al., 2013) have been studied using internet-based questionnaires. However, in humans, internet-based data collection in epidemiological studies has been widely used (Ekman et al., 2006; Smith et al., 2007). At least three studies have analysed environmental factors related to CAD (Nødtvedt et al., 2006; Nødtvedt et al., 2007; Meury et al., 2011). The presence of other dogs in the household, rural environment, and walking in the forest have been reported to be associated with lower risk of CAD (Meury et al., 2011). On the other hand, living in urban areas and in areas with higher human density, as well as washing the dog regularly, white coat colour, being born in autumn, living in a shed during puppyhood, adoption at age of 8-12 weeks, and a dam eating commercial diet are related to higher

incidence of CAD (Nødtvedt et al., 2006; Nødtvedt et al., 2007; Meury et al., 2011).

Nutritional management of CAD has been widely studied. Clinical symptoms ameliorating effects has been reported with eggs (Jewell and Fritsch, 2015), omega-3 fatty acid supplementation (Saevik et al., 2004; Abba et al., 2005; Müller et al., 2016), vitamin E (Plevnik et al., 2014), and probiotics (Kim et al., 2015; Ohshima-Terada et al., 2015). Also, CAD preventive effect of early usage of probiotics has been reported in one study (Marsella et al., 2012). However, no studies comparing the effect of a raw high fat –low carbohydrate and a highly processed low fat- high carbohydrate dog food on CAD have been conducted.

An abstract by Wynn et al. (2003) compared the haematological and blood biochemical values between pet dogs fed either a dry food or a raw food diet, and found higher haematocrit, urea nitrogen and creatinine levels in dogs fed raw diets. Other studies assessing the effect of the diet on haematology and blood biochemistry have been done using dry kibble diets with differing macronutrient profiles (Kronfeld et al., 1977; Hansen et al., 1992; Davenport et al., 1994; Swanson et al., 2004; Brown et al., 2009; Ober et al., 2016). A comparison of raw and dry diets on plasma B vitamins and whole blood iron has not been conducted in dogs, but different diets have been recently reported to modify the gut microbiota in dogs (Pinna et al., 2016; Sandri et al., 2017), and since intestinal bacteria can produce folate and use B12 (German et al., 2003; Dossin, 2011), plasma folate and B12 could be affected by the diet through gut microbiota.

Altered gene expression in atopic canine skin has been previously studied using mRNA microarrays (Merryman-Simpson et al., 2008; Plager et al., 2012; Schamber et al., 2014) and quantitative PCR (Nuttall et al., 2002; Wood et al., 2009; van Damme et al., 2009; Roque et al., 2011; Schlotter et al., 2011; Theerawatanasirikul et al., 2012; Lancto et al., 2013; Klukowska-Rötzler et al., 2013; Santoro et al., 2013; Mullin et al., 2013). These studies have published several candidate genes for CAD, including genes related to inflammation,

keratinocytes, and skin barrier. To date, no RNA sequencing studies of altered gene expression in CAD has been published nor has there been any studies published about the association of diet with skin gene expression. Nutrigenomics is a field of study which aims to demonstrate the effect of the components in a diet on the expression of genes (Choi and Kwon, 2015). One of the main interests in nutrigenomics is to study the common complex diseases which are multifactorial in origin. Using nutrigenomics, it is possible to characterize pathways regulated by nutrients or to find biomarkers that might allow the detection of the diseases early in pre-disease state (Muller and Kersten, 2003; Choi and Kwon, 2015). The effect of the diet on the gene expression of liver (Kil et al., 2010a), skeletal muscle (Middelbos et al., 2009), adipose tissue (Swanson et al., 2009a), colonic mucosa (Kil et al., 2010b), and brain tissue (Swanson et al., 2009b) has been studied in dogs. Nutrients can change the gene expression directly, acting as ligands for nuclear receptors, or by inducing epigenetic modifications, like DNA methylation and histone tail modifications. Transcription factors, the proteins that bind to a specific DNA sequence and activate or inhibit the transcription of genes, are the main agents through which nutrients influence the expression of genes (Noori-Daloi and Nejatizadeh, 2015). Micronutrients do not necessarily act independently, but have overlapping and complementing actions. For example, vitamin D, E, B2, B6, B12, and zinc, folate and selenium are all involved directly or indirectly in the innate immune response (Larbi et al., 2008). The effects of micronutrients depend on the amount of ingested, the meal matrix, absorption, metabolism and the genetic factors, and all these processes involve a complex interaction with environment, metabolism, and genetic background (Schwartz, 2014).

A significant part of the immune system can have an interaction with what we feed our pets, since the gut is the largest immune organ. Stimulation of the immune cells in the gut likely results in overall activation of the immune system (Satyaraj, 2011). The gut-associated lymphoid tissue (GALT) has receptors for pathogen-associated molecular patterns (PAMPs) which are expressed by microbial pathogens, and these receptors are in most cases the targets of the dietary immune-modulating strategies using applicable ingredients (Satyaraj, 2011). GALT favours the induction of cells that secrete

suppressive cytokines, like interleukin (IL) 4, IL-10 and transforming growth factor beta 1 (TGF- β 1) (Weiner, 1997). Locally TGF- β 1 has an important role in the gut, since it acts as a switch factor for immunoglobulin A production (Kim and Kagnoff, 1990). TGF- β 1 can act as an anti-inflammatory and immunosuppressive cytokine, dampening self-harmful inflammatory responses, but it can also promote proinflammatory responses (Hansen et al., 2000; Travis et al., 2014). TGF- β 1 has an important role in the regulation of immune cells and controlling the T cell responses to environmental antigens (Gorelik and Flavell, 2001; Travis et al., 2014). In the skin of atopic dogs, the expression of TGF- β 1 has been reported to be downregulated (Nuttal et al., 2002; Jee et al., 2013), but after the treatment leading to the rescue of TGF- β 1 expression, TGF- β 1 ameliorated the symptoms of CAD and protected against keratinocyte degeneration (Jee et al., 2013).

Canine atopic dermatitis is an incurable disease and a great burden to both the dog and the owner. The possible prevention of the disease is therefore extremely important and should be studied more extensively. The predisposing elements include the genetic background, the factors related to the living environment, and the diet.

2 OBJECTIVES AND HYPOTHESES OF THE STUDY

The overall aim of this study was to find environmental, phenotype-related, dietary, and genetic factors related to canine atopic dermatitis. The goal was to use different kind of approaches and techniques to study the pathogenesis of CAD, since atopic dermatitis is known to be a multifactorial disease.

The objective of study I was to validate the DOGRISK questionnaire, so it could be reliably used in the future analyses. The validation was focused on diseases, dog characteristics, and environmental questions.

In study II the validated DOGRISK questionnaire was used to find environmental factors and phenotype characteristics associated with canine skin problems reported by the owner and with veterinary diagnosed CAD. The hypothesis was that the risk of allergic/atopic skin symptoms and CAD are associated with environmental factors related to puppyhood, household, and dog care-related conditions, in addition to dog characteristics.

The aim of study III was to compare the effect of raw and dry diet on hematology and clinical chemistry in atopic and healthy dogs using a clinical diet intervention study model. In addition, the aim was to analyse serum folate, vitamin B12 and whole blood iron concentrations before and after the trial. The hypothesis was that the diets have different kind of effect on blood parameters.

In study IV, eight dogs from the same diet intervention study (III) were used in order i) to compare the gene expression profiles in the skin of atopic and healthy dogs, and ii) to analyse the effect of raw and dry diet on the skin gene expression. RNA-seq was performed on non-lesional skin samples from four atopic dogs and on healthy skin samples from four healthy dogs, all before and after the diet intervention. The hypotheses were that the gene expression will differ between healthy and atopic dogs and the diets will differentially change the gene expression in the skin.

3 MATERIALS AND METHODS

3.1 The DOGRISK questionnaire

The DOGRISK questionnaire is an ongoing, large, internet-based, cross-sectional study of dogs' individual phenotypes, canine nutrition, living environment, and health and it contains over 1300 variables. It was launched in December 2009 by the DOGRISK research group of the Department of Equine and Small Animal Medicine, University of Helsinki, Finland (www.ruokintakysely.fi, only in Finnish). Up to March 23, 2013, a total of 8813 questionnaires were completed by Finnish dog owners. This study population was used in studies I and II (Table 1), and it consists of 261 different breeds and 1155 mixed-breed dogs (13.1%) of all age groups, from puppies to senior dogs. The study population includes dogs from all over Finland. Not all questions were mandatory, so the respondents had the opportunity to leave questions unanswered.

3.1.1 Validation of the questionnaire

The validity of the owner-entered descriptive information and disease status was analysed comparing it with the same data from the official Finnish Kennel Club (FKC) register. In addition, the reliability was tested through internal consistency by comparing the answers to two related questions within the questionnaire, and by comparing owner-entered information about their dog having skin symptoms with responses to a short email questionnaire, later sent to the owners. The test-retest repeatability was ascertained by using the questionnaires that were filled in twice for the same dog.

3.1.1.1 *Criterion validity: Inter-rater reliability against an official register*

From the whole DOGRISK questionnaire study population, 487 dogs with an owner-entered official FKC canine registration number were chosen as a convenience sample, starting from the first dog and moving forward until the 487th. Breed, gender, date of birth, and results of official hip radiographs (if

available) were confirmed from the official FKC register and compared with the answers given to the corresponding four questions in the DOGRISK questionnaire. The date of birth taken from the register was recoded into four variables to match the season answers given in the questionnaire: winter (from December to February), spring (from March to May), summer (from June to August), and autumn (from September to November). The hip radiograph results, both the owners' answer and the result from official register, were recoded so that A/B and B/A indicated the same, B/C and C/B indicated the same, etc., as most of the owners probably did not remember if the left or the right is marked first. Cohen's Kappa (κ) was used to evaluate the reliability of the answers for all descriptive variables.

3.1.1.2 Internal consistency within the questionnaire

To assess the internal consistency in the owners' answers, two answers on the questionnaire that would always be expected to coincide were compared. As a first comparison, hypothyroidism was chosen, being one of the 117 diseases asked about in the questionnaire. It is a quite common disease requiring specific diagnostic blood work that is done by veterinarians and it always needs medication. The questionnaire includes a question on whether the dog has the disease or not, and another question on ongoing medication (open question where owners were to fill in all medication used). Cronbach's Alpha (α) was used to evaluate the reliability of the answers to the owner-reported disease status and medication.

As a second comparison, the dog's age was used. The questionnaire included a question on the dog's age (with three response options: puppy/0-6 months, young/7-18 months, or adult/choose the age in years between 1 and 21 years), and another question on the date of birth. The difference between the date when the owner filled in the questionnaire and the birth date of the dog was calculated, and recoded into years: 0.1-0.5 as 0.25 year (puppy), 0.6-1.9 as 1 year (young dog), 2.0-2.9 as 2 years (adult), etc. This value was compared to the owner's answer of the dog's age in years using Cronbach's Alpha (α).

3.1.1.3 Reliability against additional questions

All dog owners who had answered 'yes' to the question 'Does your dog suffer from atopy/allergy (skin symptoms)?' and had provided either their email address or street address were contacted by email/mail in June 2014 (n=1354) and asked whether their original answer concerning the diagnose of atopy/allergy (skin symptoms) was correct and whether the condition had been diagnosed by a veterinarian. The owners also had the opportunity to explain their dog's symptoms more thoroughly. In addition, all dog owners who had not ticked an answer 'yes/no' but had answered one or more of the related questions (has had the symptoms rarely/has had it often; started at the age of; is still having the disease; the disease cured after changing the diet/ I haven't noticed that a diet change would have helped in this disease) were also contacted by email/mail (n=197) and asked whether their dog suffered from atopy/allergy or not. Positive predictive value (PPV) was calculated to evaluate the percentage of true positives of all positive answers of owner-reported disease status. From all owner-reaffirmed positive answers, the percentage of dogs with a diagnosis set by a veterinarian was calculated.

3.1.1.4 Repeatability by a spontaneous, non-prospective test-retest

There were 244 owners in the study sample who had answered the questionnaire twice of their own initiative. For some questions, the owner might not have had all information at hand when they filled in the questionnaire the first time and for this reason they might have filled in the questionnaire twice. Because of adding new information to the questionnaire, the test-retest data could not be used to analyse the repeatability of questions on diseases or of many of the living conditions. Repeatability of ten questions was analysed using Cohen's kappa. These included gender, season of birth, colour of coat, born in owner family, body condition score under two months of age, time spent outside under two months of age, puppy vaccinations, tidiness of the household, adult vaccination, and other dogs in the household. For adult vaccinations, only dogs aged over 18 months were included.

Table 1. Summary of the experiments.

Experiment	Number of animals	Trial period, median days	Mean age, years	Sample type	Objectives	Study
DOGRISK questionnaire						
Validation	8813	-	4.1	Questionnaire	Validation of the questionnaire.	I
Environment and skin symptoms	8643	-	4.1	Questionnaire	Analyse associations between atopy and environment/dog phenotype.	II
Diet intervention						
Haematology and clinical chemistry	33	140	5.2	Blood	Analyse the effect of the diet on haematological and clinical chemistry parameters.	III
Folate and B12	31	140	5.3	Blood	Analyse the effect of the diet on plasma folate and B12.	III
Iron	19	140	5.5	Blood	Analyse the effect of the diet on whole blood iron.	III
RNA-Seq	8	139	4.8	Skin	Analyse the difference in skin gene expression between atopic and healthy dogs, and the effect of the diet on skin gene expression.	IV
TGF- β 1	23	139	4.5	Blood	Analyse the effect of the diet on blood TGF- β 1 concentration.	-
TGF- β 1, transforming growth factor- β 1.						

3.1.2 Environmental and phenotype-related factors associated with skin symptoms

The data used in analyses related to owner-reported allergic/atopic skin symptoms was the same as used in the validation (Study I), retrieved March 23, 2013. A total of 8643 dogs were used in the data analyses. The analyses related to the veterinary diagnosed CAD are based on the follow-up questionnaire sent by email in June 2014.

3.1.2.1 Study population

There were two populations used in this study. The DOGRISK questionnaire population is referred to as the ‘owner-reported allergic/atopic skin symptoms’ population as the owners had answered positively to a question that was given as ‘Does your dog suffer from atopy/allergy (skin symptoms)’ (first case group, n=1585). The second population was based on the follow-up questionnaire sent by email in June 2014 (Study I), and included only dogs whose diagnosis of atopy was verified by a veterinarian (veterinary-verified CAD case group, n=322). The control group for both populations consisted of 7058 dogs with no owner-reported skin symptoms.

3.1.2.2 Statistical analyses

Altogether 20 categorical variables were used in this study. In addition, age was used as a continuous variable. When the answer ‘I do not know/remember’ was available and selected by the owner, it was omitted from statistical analyses. In the dichotomous questions, “Living with other dogs”, “Living with other animals” and “Born in owner family”, which offered “yes” or “no” as options, the missing answer was interpreted as a “no”.

A univariable logistic regression was run individually for each variable to determine its association with both the owner-reported skin-symptom status and the veterinary-verified CAD. All risk factors that had a $P < 0.2$ and less than 1500 missing cases (less than 1500 owners had left the question unanswered) were included into the multivariable logistic regression analysis

using the enter method. In addition, age and Fédération Cynologique Internationale (FCI) breed groups (including mixed-breed dogs as an 11th group) were included in the model as adjusting variables. Statistical significance was declared for values $P < 0.05$. The quality of the fit of the final model was determined by the following criteria: a smaller P-value in the Omnibus test of model coefficients, a larger value in the Hosmer and Lemeshow test, and the closer the value to 100 % in Nagelkerke's R^2 (Dohoo et al., 2012).

The proportion of owner-reported allergic/atopic skin symptoms in this data was calculated breed-specifically for each breed when there were over 40 dogs included in the breed. After that, all breeds were categorized according to FCI breed groups and the risk of owner-reported allergic/atopic skin symptoms was analysed in every group compared to the mixed-breed dogs using a univariable logistic regression model. $P < 0.05$ was considered as statistically significant. All analyses were performed using SPSS software (version 22, IBM SPSS Statistics. Chicago, Ill., USA).

3.2 The diet intervention study

3.2.1 Animals

All animals used in the diet intervention study were client-owned Staffordshire bull terriers. In the DOGRISK questionnaire data, it was one of the breeds with most owner-reported skin symptoms. The dogs were recruited into the trial by the breed club newsletter, by Facebook and by contacting respondents of the DOGRISK questionnaire. Altogether 68 dogs were registered into the study via an electronic form, and all of them were contacted by phone. After the phone interview, the owners of 58 dogs were found suitable and were willing to participate to the study.

3.2.2 Study protocol and sample collection

The diet intervention study was designed for atopic and healthy dogs and most of the study population suffered from canine atopic dermatitis. After the owners had registered with the electronic form, they were contacted by phone

and all except three (n=3) atopic dogs were invited to the inclusion visit (n=36). Those three dogs that were not invited, had recently been or were at the moment on the elimination diet (n=2) or the owner refused to feed the elimination diet to the dog (n=1). At the inclusion visit dogs were examined by a veterinarian, and blood samples were taken for haematology and clinical chemistry. After this visit, four dogs were excluded from the study. Of these dogs, two were not considered suitable for the trial, one dog owner did not want to participate, and one dog moved abroad before the trial started. All atopic dogs remaining in the trial (n=32 + 3) received parasitic treatment with selamectin (Stronghold®, Zoetis, Louvain-la-Neuve, Belgium) every two weeks for three applications. The diagnosis of CAD was done using current accepted standards. Clinical skin lesions were scored using the validated CADESI-04 score (from 0 to 180) (Olivry et al., 2014) and owners evaluated their dogs' pruritus according to the validated pruritus visual analogue scale (P-VAS) (from 0 to 10) (Rybníček et al., 2009). Dogs with atopic dermatitis (AD) went through a six-week elimination diet (Royal Canin Hypoallergenic) and a one-week re-challenge with their own pre-elimination food after the first inclusion visit.

All atopic dogs (after receiving the elimination diet) and all dogs that were registered to the study as healthy dogs, were next invited to the baseline visit (n=54). At the baseline visit dogs underwent a thorough physical examination and blood samples were taken for haematology, clinical chemistry, nutrient analyses, and blood RNA collection. Two dogs reported healthy by their owner where found to be atopic and were re-assigned to the AD dog group. Nine dogs were found to have some form of skin symptoms and where grouped as borderline dogs. For this reason, the number of healthy dogs in the study declined to eight dogs. After their baseline visit, the dogs were randomly divided into two diet groups and stratified for disease severity by the sum of their CADESI-04 and P-VAS score (low ≤ 22 or high > 22), health status (atopic, non-atopic, and 'food as a possible component'), and previous diet (≥ 40 % of raw food, ≥ 80 % of dry food, or neither), using a computerised randomisation list. If there were more than one dog from the same family participating in the study, to avoid accidental 'wrong diet to wrong dog', all the

dogs in the family received the same study diet. There were 30 dogs assigned to the raw diet group and 24 dogs to the dry diet group.

The commercial dry diet used in this study was Hill's Science Plan™ Canine Adult Sensitive Skin with Chicken (detailed composition according to manufacturer shown in Table 2). The two commercial raw diets used in this study were MUSH Vaisto® Pork-Chicken-Lamb and MUSH Vaisto® Beef-Turkey-Salmon (detailed compositions according to manufacturer shown in Table 3). The diets differed by their processing methods, the raw food being only homogenized and frozen, and dry food being processed by high heat and pressure. The diets also differed by their contents, since the raw food included high amounts of protein and fat, and the dry food included high amount of carbohydrates. Nevertheless, they were chosen to this study since they are two very usual ways to feed dogs in Finland, and are indicated as raw and dry diet in this thesis (for clarity). Owners were given coupons which they were able to use in pet food stores to get their study diet free. They were asked to ensure that their dog's diet consisted of at least 99.9 % trial food, and to follow the daily amounts recommended by the manufacturer. All treats and foods given by accident had to be written down on sheets given to all owners. Water was allowed ad libitum. At the end visit, the same protocol was followed as for the baseline visit. All owners signed a written consent form. The study protocol was approved by the Animal Experiment Board in Finland (ELLA) (permit number: ESAVI/3244/04.10.07/2013).

Table 2. *Composition and analytical constituent of food Hill's Science Plan™ Canine adult sensitive skin with chicken.*

Composition: chicken (minimum chicken 23%, chicken and turkey combined 31%), ground rice, ground maize, chicken and turkey meal, maize gluten meal, dried whole egg, vegetable oil, flaxseed, digest, animal fat, potassium chloride, DL-methionine, salt, L-lysine hydrochloride, L-tryptophan, vitamins and trace elements. Naturally preserved with mixed tocopherols, citric acid and rosemary extract.

Analytical Constituent	In food	In dry matter
Protein (%)	25.3	27.5
Fat (%)	16	17.4
Carbohydrate (NFE) (%)	44.5	48.4
Fiber (crude) (%)	1.3	1.4
Ash (%)	4.9	5.3
Moisture (%)	8	-
Calcium (%)	0.66	0.72
Phosphorus (%)	0.58	0.63
Calcium : Phosphorus	1.1	1.1
Sodium (%)	0.35	0.38
Potassium (%)	0.64	0.7
Magnesium (%)	0.07	0.08
Omega-3 fatty acids (%)	1.2	1.3
Omega-6 fatty acids (%)	4.8	5.2
ADDED per kg:		
Vitamin A (IU)	9600	10435
Vitamin D (IU)	480	522
Vitamin E (mg)	600	652
Vitamin C (mg)	70	76
Iron (mg)	53.7	58.4
Iodine (mg)	0.9	1.0
Copper (mg)	5.3	5.8
Manganese (mg)	5.6	6.1
Zinc (mg)	111	121
Selenium (mg)	0.15	0.16
Beta-carotene (mg)	1.5	1.6

The diet is stated as complete diet by the manufacturer.

Table 3. Composition and analytical constituent of MUSH BARF Vaisto® diets.

Composition (pork-chicken-lamb): Finnish pork 46% (meat, lung, cartilage, heart, liver), Finnish chicken 29% (meat, bone, gizzard, skin, heart, cartilage, liver), Finnish lamb 20% (bone, meat, lung, cartilage, liver), vegetables 5% (spinach, broccoli, lettuce, cold-pressed sunflower oil), egg < 1%.

Composition (beef-turkey-salmon): Finnish beef, 47% (rumen, meat, lung, heart, cartilage, liver), Finnish turkey 38% (meat, bone, cartilage), Norwegian salmon 10% (salmon including bones), vegetables 5% (broccoli, lettuce, apple, carrot, cold-pressed sunflower oil, camelina oil).

Analytical Constituent (pork-chicken-lamb)	In food	In dry matter
Protein (%)	15.2	38
Fat (%)	20	50
Ash (crude) (%)	4.20	10.5
Fiber (crude) (%)	0.60	1.5
Moisture (%)	60.0	0.0
Phosphorus (%)	0.65	1.6
Calcium (%)	1.09	2.7
Calcium : Phosphorus	1.7	1.7
Analysed ingredients from different batch per kg*		
Omega-3 fatty acids (%)		0.4
Omega-6 fatty acids (%)		3.8
Vitamin A (IU)		143050
Vitamin D (IU)		698
Vitamin E (mg)		46.6
Iron (mg)		123
Iodine (mg)		1.86
Copper (mg)		24.2
Manganese (mg)		8.8
Zinc (mg)		119
Selenium (mg)		0.62
Analytical Constituent (beef-turkey-salmon)	In food	In dry matter
Protein (%)	15.0	42.5
Fat (%)	15.8	44.8
Ash (crude) (%)	3.70	10.5
Fiber (crude) (%)	0.80	2.3

Moisture (%)	64.7	0.0
Phosphorus (%)	0.34	1.0
Calcium (%)	0.45	1.3
Calcium : Phosphorus	1.3	1.3
Analysed ingredients from different batch per kg*		
Omega-3 fatty acids (%)		1.1
Omega-6 fatty acids (%)		2.7
Vitamin A (IU)		80890
Vitamin D (IU)		2130
Vitamin E (mg)		54.4
Iron (mg)		82.1
Iodine (mg)		1.64
Copper (mg)		31.5
Manganese (mg)		7.4
Zinc (mg)		79.6
Selenium (mg)		0.73

The diets have been stated as complete by the manufacturer. * Ingredients were analysed by the manufacturer from different food batch and provided to researchers by MUSH Ltd.

During the trial, eight dogs dropped out. One was euthanized (raw diet group), one was diagnosed with immune mediated haemolytic anaemia (dry diet groups), one owner could not be contacted at the time of the end visit despite multiple tries (raw diet group), and five owners did not want to continue because the food was not suitable for their dogs/ the pruritus got worse during the trial (two from the raw food group and three from the dry food group). Altogether 46 dogs finished the study.

3.2.3 Blood samples for haematology and clinical chemistry

From the 46 dogs that finished the study, 13 more dogs were excluded for the blood analyses not to interfere with the haematological and biochemical parameters. Of those 13 dogs, two changed their diet during the trial, one had ongoing ciclosporin medication and five stayed on the study diet less than 85 days (they were excluded to make the study population less variable). In addition, one dog was diagnosed with localized demodicosis, one with

hypothyroidism, and one with azotemia at the end visit. Furthermore, two dogs were excluded from the analyses since blood samples for haematology and clinical chemistry were only available from either one of the visits. Of the remaining 33 dogs, 19 were fed the raw diet and 14 the dry diet. Eighteen dogs were suffering from CAD and they all fulfilled at least six of Favrot's eight criteria (Favrot et al., 2010) (9 in the raw diet group and 9 in the dry diet group). Seven dogs were considered as non-atopic (4 in the raw diet group and 3 in the dry diet group), and eight dogs as borderline dogs since they did not fulfil six of Favrot's criteria (6 in the raw diet group and 2 in the dry diet group). The dietary intervention lasted for 102-188 days (median 140 d) in dogs included in these analyses.

Blood was collected from the jugular vein into a Vacuette® 3 mL EDTA and 6 mL plain serum tubes by a closed method (Vacutainer® Safety-Lok™ Blood collection sets, Becton Dickinson, Meylan, France). For haematology, the blood leukocyte count (WBC), erythrocyte count (RBC), haemoglobin (Hb), mean cell volume (MCV), haematocrit (Hct) (RBC x MCV), mean cell haemoglobin (MCH) (Hb / RBC), mean cell haemoglobin concentration (MCHC) (Hb / Hct), and platelet count were determined from EDTA blood samples. Complete blood cell counts were determined by the ADVIA 2120i Haematology System with multispecies software (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) and by using the cyanmethaemoglobin method for haemoglobin measurements.

For the biochemical analyses, the collected blood was allowed to clot and centrifuged (2100 x g, 15 min.), and serum was used for analysis of the following analytes: alkaline phosphatase (ALP), alanine aminotransferase (ALT), albumin, bilirubin, inorganic phosphate, glucose, potassium, sodium, calcium, cholesterol, creatinine, total protein, and urea. Measurements were performed using Konelab 30i (ThermoFisher Scientific, Vantaa, Finland). All samples were fasting samples.

3.2.4 Folate, vitamin B₁₂ and blood iron analyses

In study III, also blood iron concentration was determined from the lithium-heparin whole blood samples using 11 dogs from the raw diet group (4 atopic, 4 borderline and 3 healthy dogs) and 8 dogs from the dry diet group (3 atopic, 2 borderline and 3 healthy dogs) at the MILA Laboratory (Helsinki, Finland) using inductively coupled plasma optical emission spectrometry (Iris Intrepid, Thermo Elemental). Plasma folate and vitamin B₁₂ were analyzed from the lithium-heparin plasma samples at the animal diagnostic laboratory Movet Oy (Kuopio, Finland) using Siemens Immulite 2000xpi from 17 dogs fed raw diet (9 atopic, 5 borderline and 3 healthy dogs) and 14 dogs fed dry diet (9 atopic, 2 borderline and 3 healthy dogs).

3.2.5 Skin samples

Skin samples were taken from eight dogs before and after the diet intervention study in Study IV (Table 1). Four of them were fed the raw diet (2 atopic and 2 healthy dogs) and four the dry diet (2 atopic and 2 healthy dogs). The eight dogs were on their trial diet for a median of 139 days. Nonlesional skin biopsies were taken from the axillary area under anaesthesia using an 8 mm biopsy punch, during a short anaesthesia of dexdomitor (5-10 µg/kg) and intravenous propofol (5 mg/kg). All eight dogs had been off oral glucocorticoids and cyclosporine for at least four weeks, and off oral antihistamines, topical glucocorticoids, and medicated shampoos for at least two weeks prior to the sample collection. The samples were immediately stored at -80°C.

In addition to skin biopsies, transepidermal water loss (TEWL) was measured in axillae, pinna and inguinal areas using a closed-chamber evaporimeter (VapoMeter, Delfin Technologies Ltd, Kuopio, Finland), and analysed from these eight dogs used in the RNA-seq study.

3.2.5.1 RNA extraction

Before the RNA extraction, samples were transferred to RNeasy®-ICE Frozen Tissue Transition Solution (Life Technologies, Carlsbad, CA, USA) and

allowed to thaw overnight at -20°C. Working on ice, the subcutaneous fat was trimmed from the skin biopsies, and the remaining samples were homogenized using a tissue homogenizer (TissueRuptor, Qiagen, Hilden, Germany). Total RNA was extracted using miRNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To determine the total RNA concentrations, a 260-nm ultraviolet spectrophotometry was used (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). The integrity and quality of the RNA was analysed using the Agilent 2100 Bioanalyser (Agilent Biotechnologies Inc., Santa Clara, CA, USA). After extraction, DNase treatment using DNase I, RNase-free (Thermo Fisher Scientific, Waltham, MA, USA) was performed. Samples were stored at -80°C before sending them to RNA sequencing.

3.2.5.2 RNA sequencing

Transcriptome sequencing was performed to the samples originating from the RNA isolated from the skin samples of eight dogs. Transcriptome sequencing was completed at the Institute for Molecular Medicine Finland (FIMM) according to their protocol using Illumina HiSeq 2500 (<https://www.fimm.fi/en/services/technology-centre/sequencing/next-generation-sequencing/ngs-platforms>).

3.2.6 Samples for ELISA assay for canine TGF-β1

In addition to Studies I-IV, serum concentration of transforming growth factor beta-1 (TGF-β1) was measured from 23 dogs (18 atopic and 5 borderline dogs) (Table 1). Of those dogs, 13 were fed the raw diet (8 atopic and 5 borderline dogs) and 10 dogs were fed the dry diet (all atopic). They were fed the study diets for a median of 139 days. Before and after the diet intervention, blood samples were collected from the jugular vein into plastic serum tubes for the ELISA assay. The tubes were centrifuged 30 minutes after the collection, for 15 minutes at 3,000 x g and then the serum was extracted into Eppendorf vials and stored at -80 °C until used. The TGF-β1 concentration in the serum was measured according to the manufacturer's instructions using the Mouse/Rat/Porcine/Canine TGF-β1 Quantikine ELISA Kit (R&D systems,

Minneapolis, MN). The results were calculated by reference to the standard curve and expressed as picograms per mL (pg/mL).

3.2.7 Statistical analyses

The Shapiro-Wilk test was used to assess normality. A dependent t-test was used for all normally distributed data to compare the change in blood analytes during the diet intervention within the diet groups. For non-normal data, and in case of outliers, Wilcoxon signed-rank test was used. Background characteristics were analysed using the independent t-test/Mann-Whitney U test and Fisher's exact test. SPSS software (version 22, IBM SPSS Statistics, Chicago, Ill., USA) was used in all analyses. Statistical significance was set at $P < 0.05$.

The differential expression analysis was performed in Centro de Supercomputaciòn y Bioinnovaciòn (University of Màlaga, Spain). Quality control step was done using SeqTrimNext (v.s.6) where low quality, ambiguous, and low complexity stretches, adaptors, organelle DNA, polyA/polyT tails, and contaminated sequences were removed using custom Blast to mammals against a different database. Bowtie2 (v.2.2.2) was introduced to align the reads to the reference genome (CanFam3.1 (GCA_000002285.2, http://www.ensembl.org/Canis_familiaris/Info/Index) and to the reference transcriptome (Canis_familiaris.CanFam3.1.cds.all.fa, ftp://ftp.ensembl.org/pub/release-83/fasta/canis_familiaris/cds/). Samtools (v.0.1.19) (<http://www.htslib.org/>) was used to the quantification of known transcripts (read counts per transcripts). Transcriptome reference was annotated using Full-Lengther-Next, a tool adapted to NGS technologies, able to work in parallel and in a distributed way to minimize computing time. It can classify and annotate the unigenes full-length, 5'-end, 3'-end and internal, suggesting which unknown genes are coding or not (http://www.rubydoc.info/gems/full_lengther_next/o.o.8/frames).

Two different alignments to transcriptome was carried out using different filters: Mapping Low (less restrictive), where all alignment is searched for and

reported, and Mapping High (more restrictive) where the discordant alignments are rejected. A discordant alignment is an alignment where both mates align uniquely, but that does not satisfy the paired-end constraints.

For the differential expression analysis, raw read counts generated by Bowtie2/Samtools were used as input to DEG-Hunter (v.2.0.11). The differential expression analysis was performed with EdgeR (Robinson and Smyth, 2007) and DeSeq2 (Love et al., 2010) algorithms, with a log₂ fold change (FC) ≥ 2 , and a false discovery rate (FDR) ≤ 0.05 . Functional analyses were performed using Ingenuity Pathway Analysis Software (Qiagen, Redwood City, CA). The RNA-Seq data is available at the Gene Expression Omnibus page (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number SRP110851.

4 RESULTS AND DISCUSSION

4.1 Validation of the DOGRISK questionnaire

The three most prevalent breeds in the entire DOGRISK study population were mixed breed dogs (13.1 %), German shepherd dog (6.1 %), and Labrador retriever (3.4 %). In the FKC sub-cohort they were German shepherd dog (7.6 %), Finnish Lapphund (4.3 %), and Shetland sheepdog (3.7 %), and in the sub-cohort of retest sample mixed breed dogs (8.2 %), German shepherd dog (8.2 %), and Rottweiler (5.3 %).

Reliability, analysed by Cohen's Kappa (κ), of season of birth, gender, breed, and results of hip radiography when DOGRISK questionnaire answers were compared with official FKC register data was following: $\kappa=0.96$ (n=483), $\kappa=0.99$ (n=468), $\kappa=0.99$ (n=478), and $\kappa=0.95$ (n=115), respectively. These results can be considered excellent.

The internal consistency for hypothyroidism was excellent (Cronbach's $\alpha=0.95$, n=8081). This disease is always diagnosed from a blood sample at a veterinary clinic, and it invariably requires medication. Discrepant answers to

the two questions on diagnosis and medication were very few, but could arise from owners having just visited the clinic, but not yet having the medication, or the owner simply did not remember the name of the disease or the medication.

Altogether 515 (38%) of 1354 owners who had responded 'yes, my dog suffers from atopy/allergy (skin symptoms)' also answered to the email/mail. Of these, 457 (89%) reported having given a correct answer and 58 (11%) an incorrect answer. All 58 answers reported as incorrect were controlled one by one, and based on the owners' explanation, 11 were kept as a 'yes' and the rest were considered false positives (n=47). Thirty-seven of the 47 answers were excluded from future analyses as they could not be categorized as atopic nor healthy. Of the 197 owners who had ticked only one of the disease-specifying questions, but not the disease question itself, 63 (32%) answered to the email/mail; 49 (78%) reported that their dog had atopy/allergy, while 14 (22%) reported that their dog did not have atopy/allergy. The 49 were recoded as 'yes' into the questionnaire data. The 14 'no' answers were controlled one by one, and based on the owners' explanation, one was recoded into 'yes', three were recoded into 'no', and ten could not be assigned to either category. After these checks, 1357 positive answers remained in the data, with a total of 518 confirmed answers (38%). The PPV of the owner-reported disease status was 0.91. Therefore, the owner-reported disease status can be considered quite reliable. Atopy/allergy is much more difficult to diagnose than hypothyroidism and hip dysplasia, and therefore its reliability was expected to be much lower than for the other two diseases. Of the confirmed positive answers, only 69% had a diagnosis set by a veterinarian.

In the internal consistency between the dog's age and date of birth the Cronbach's α was 0.99 (n=3540). Owners seemed to be diligent with basic information, as also the data on gender, season of birth, and breed matched very well with the official register records.

Altogether 244 owners had filled in the questionnaire twice. The time between the answers varied from 1 day to 38 months. Four variables, i) gender, ii) born

in owner family, iii) season of birth, and iv) colour of coat had κ of 0.96 (n=241), 0.96 (n=244), 0.86 (n=234), and 0.80 (n=218), respectively. Answers for these questions can be considered stable since they should not change with repeated response, and repeatability in these was indeed high. Answers to three questions, i) vaccination status as a puppy, ii) time spent outside under two months of age, and iii) body condition score under two months of age might have been unknown when filling in the questionnaire for the first time, and later asked from the breeder. These variables had a κ of 0.67 (n=230), 0.45 (n=105) and 0.37 (n=118), respectively. Three variables, i) vaccination status as an adult, ii) other dogs in household, and iii) tidiness of household could vary over time, and for this reason these questions might be answered differently in two time points. These had κ of 0.76 (n=147), 0.71 (n=198), and 0.42 (n=241), respectively.

This study validated and tested three disease diagnoses and descriptive and environmental factors for reliability. Comparison against an official register showed excellent agreement and internal consistency of the questionnaire was very good. The test-retest repeatability was substantial in some of the questions and good in most of the questions.

4.2 Environmental and phenotype-related factors associated with skin symptoms

The five breeds that suffered most often from owner-reported allergic/atopic skin symptoms as percentage within the breed were: 1. West Highland white terrier, 2. Boxer, 3. English bulldog, 4. Dalmatian, 5. French bulldog. When compared to mixed breed dogs, the risk of owner-reported allergic/atopic skin symptoms among the FCI breed groups was highest in group 6 (Scent hounds and related breeds) and group 3 (Terriers), OR=1.68 (p=0.004) and OR 1.64 (p<0.001), respectively, and lowest in group 10 (Sighthounds) and group 5 (Spitz and primitive types), OR=0.53 (p=0.007) and OR=0.71 (p=0.007), respectively (Fig 1).

The percentage of owner-reported allergic/atopic skin symptoms in the entire dataset was 18.3 %. In the univariable analyses, 14 of the 21 analysed variables showed a statistically significant association with owner-reported allergic/atopic skin symptoms (Table 4). The proportion of owner reported maternal history of atopic/allergic skin symptoms was 13.4 % in the case group and 2.6 % in the control group.

Ten categorical risk factor candidates that presented $P < 0.2$ and less than 1500 missing cases were included in the multivariable logistic regression analysis for owner-reported allergic/atopic skin symptoms. Included factors were season of birth, wood-fired heating system, type of house at the moment, extremely clean household, gender, over 50 % of white colour in the coat, born in owner family, living with other dogs, living with other animals, does the dog have a yard, and age. In the final model, the conditions 'living with other dogs', 'born in the owner family' and 'living in a wooden or non-wooden detached house' were associated with less owner-reported allergic/atopic skin symptoms, indicating that these traits were

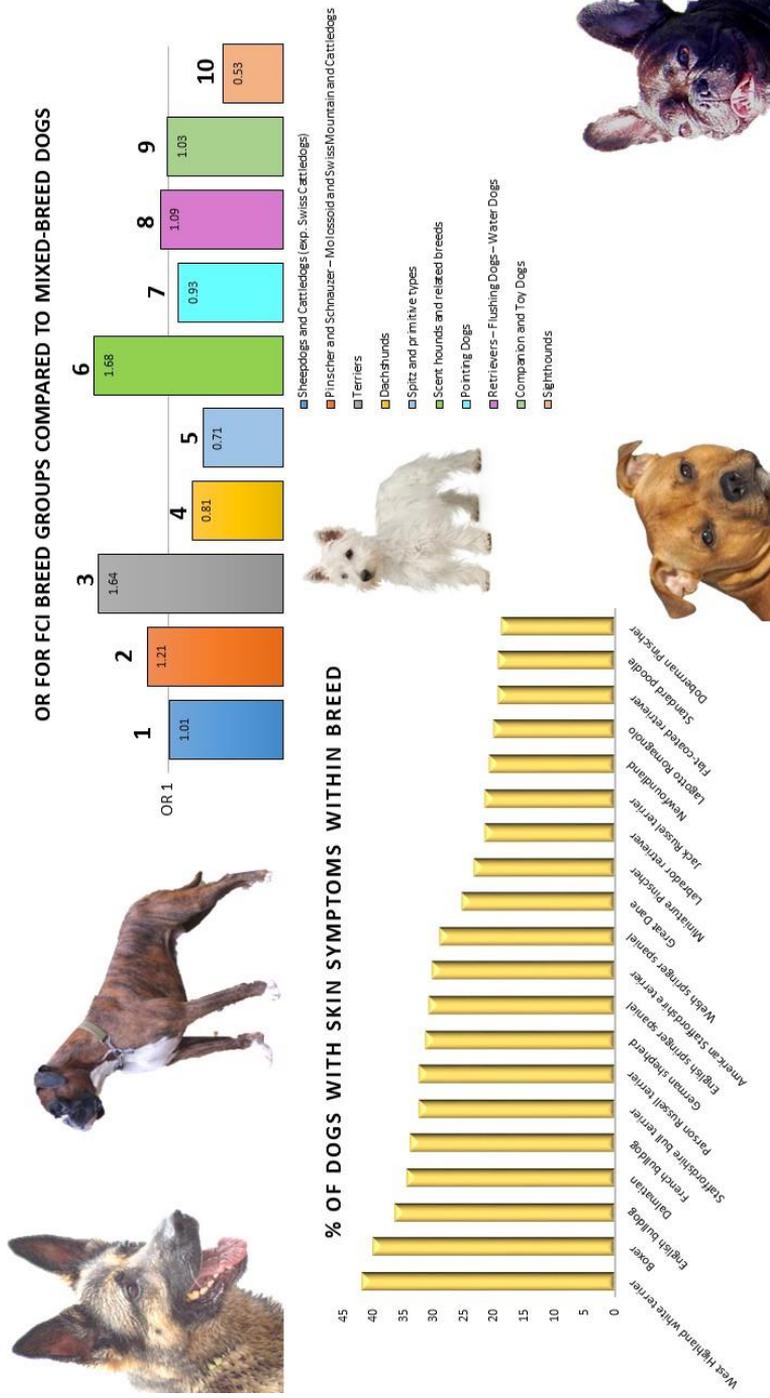


Figure 1. Twenty breeds with the highest percentage of dogs with skin symptoms, and odds ratios (OR) of having skin symptoms for FCI breed groups 1-10 compared to mixed-breed dogs. Statistical significance was reached in groups 3 ($p < 0.001$), 5 ($p = 0.007$), 6 ($p = 0.004$), and 10 ($p = 0.007$).

Table 4. Associations between potential risk factors and owner-reported allergic/atopic skin symptoms in the DOGRISK questionnaire data.

	Potential risk factor	Included dogs (n)	Comparison categories	P-value	OR	95% CI
1.	Season of birth (reference / compared to: autumn)	8372	Winter Spring Summer	0.231 0.145* 0.147*	0.91 0.89 0.88	0.77-1.07 0.76-1.04 0.75-1.04
2.	Wood-fired heating system (reference: no)	7808	Yes	0.016	0.83	0.72-0.97
3.	Type of house the dog has previously lived in (reference: apartment)	6371	Row house Detached house (wood) Detached house (not wood)	0.240 <0.001 0.001	0.90 0.72 0.70	0.76-1.07 0.62-0.84 0.57-0.87
4.	Type of house at the moment (reference: apartment)	8592	Row house Detached house (wood) Detached house (not wood)	0.095* <0.001 0.010	0.88 0.76 0.78	0.76-1.02 0.66-0.87 0.64-0.94
5.	Extremely clean household (reference: all others)	8592	Yes	0.025	1.51	1.05-2.15
6.	Deworming status as a puppy (reference: yes)	7999	No	0.913	0.97	0.55-1.70
7.	Vaccination status as a puppy (reference: yes)	8223	No	0.471	1.22	0.71-2.08
8.	Dam's deworming status pre-birth (reference: yes)	4061	No	0.294	1.24	0.83-1.87
9.	Dam's vaccination status pre-birth (reference: yes)	2401	No	0.901	1.02	0.80-1.28
10.	Gender (reference: female)	8414	Male	0.119*	1.09	0.98-1.22
11.	Over 50 % of white colour in the coat (reference: no)	8188	Yes	<0.001	1.28	1.13-1.47

12.	Born in owner family (reference: no)	8643	Yes	<0.001	0.33	0.24-0.47
13.	Living with other dogs (reference: no)	8643	Yes	<0.001	0.71	0.64-0.79
14.	Living with other animals (reference: no)	8643	Yes	0.028	0.88	0.78-0.99
15.	Where have you been smoking previously (reference: only outside)	3045	Mainly inside Rarely inside	0.120* 0.408	0.62 1.16	0.33-1.14 0.82-1.66
16.	Does the dog have a yard (reference: no)	8149	Yes, a yard where the dog can be loose Yes, an outside kennel where the dog can be loose Yes, a yard where the dog is chained	0.051* <0.001 0.169*	0.88 0.67 0.83	0.78-1.00 0.55-0.81 0.63-1.09
17.	Body condition score under 2 months of age (reference: normal)	5846	Obese Fat Slim Very slim	0.395 0.017 0.963 0.022	1.27 1.26 1.01 1.84	0.73-2.22 1.04-1.52 0.79-1.28 1.09-3.09
18.	Outside under 2 months of age (reference: not at all)	5250	Few days a month Few days a week Once a day Several times a day	0.605 0.018 0.016 <0.001	1.09 0.70 0.71 0.61	0.78-1.54 0.52-0.94 0.53-0.94 0.48-0.78
19.	Walking outside when 5 months old (reference: under 30 min)	5839	30-60 min/day 1-2 hours/day Over 2 hours per day	0.969 0.263 0.012	1.01 0.83 0.63	0.73-1.39 0.60-1.15 0.44-0.90
20.	Dam having a history of skin symptoms (reference: no)	2944	Yes	<0.001	5.86	4.03-8.51
21.	Age	8523		<0.001	1.05	1.03-1.07

OR, odds ratio; *, $P < 0.2$; **bolded**, $P < 0.05$. Modified from Environmental and phenotype-related risk factors for owner-reported allergic/atopic skin symptoms and for canine atopic dermatitis verified by veterinarian in a Finnish dog population. PLoS One. 2017;12(6): e0178771.

possibly protective. On the other hand, having an extremely clean household and over 50 % white colour in the coat appeared to be positively associated with owner-reported allergic/atopic skin symptoms (Fig 2).

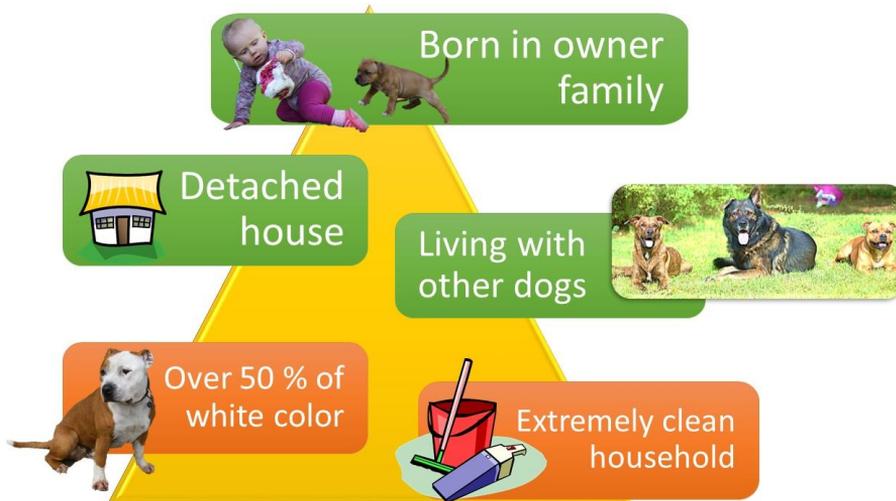


Figure 2. Factors possibly increasing the risk of allergic skin symptoms (in orange) and possibly protecting from allergic skin symptoms (in green).

In the univariable analyses, using veterinary-verified CAD as the dependent variable, 6 of the 22 variables showed a statistically significant association with CAD. Four categorical risk factor candidates that had $P < 0.2$ and less than 1500 missing cases were included in the multivariable logistic regression analysis: i) over 50 % white colour in the coat; ii) if the dog was born in owner family; iii) if the dog was living with other dogs and iv) if the dog had an outside kennel/yard. Altogether, 6407 dogs were included in the final model, including 6131 healthy dogs and 276 dogs with veterinary-verified CAD. In the final model living with other dogs ($P = 0.002$, $OR = 0.665$, $95\% CI = 0.513-0.860$) and being born in the owner family ($P = 0.022$, $OR = 0.408$, $95\% CI = 0.189-0.880$) showed a possibly protective association with the development of CAD.

The incidence of human allergic diseases is much higher in Western countries and urban areas (Yemaneberhan et al., 2004; Flohr et al., 2005; Xu et al., 2012) whereas living on a farm or even having regular contact with a farming

environment seems to have a protective effect (Lampi et al., 2011; Illi et al., 2012; Horak et al., 2014). Also, the attendance to day care in the first year of life or living in a big family have been shown to decrease the risk of allergic diseases in children (Strachan, 1989; Celedon et al., 2003; Rothers et al., 2007). Living with other dogs and being born in the owner family seemed to have a protective role in both owner-reported skin symptoms and veterinary-verified CAD in study II. These things indicate, that in humans the increased contact with other children, and in dogs with other dogs, increases the burden of microbes, and since it has been reported to be inversely associated to atopy in humans (von Hertzen et al., 2006; Janson et al., 2007) there is no reason to doubt that it also would be true in dogs. In addition, living in a detached house was negatively associated and having an extremely clean household was positively associated with owner-reported skin symptoms. People living in a detached house also tend to live in suburban or rural areas. Occurrence of CAD in a Swedish dog population living in urban areas was shown to be 57 % higher than among the rest of the population (Nødtvedt et al., 2006). All these factors can be related to hygiene or microbial exposure. Strachan (1989) proposed the hygiene hypothesis as an important reason behind the atopic and allergic diseases in humans. We may assume that the same is true for dogs. The more they are exposed to microbes in early life, the better. Thereby a more normal immune system also develops. If the immune system only has minimal challenges when it is developing, it does not know how to function normally later at life, predisposing also to atopic dermatitis (Martinez and Holt, 1999).

Having over 50 % white colour in the coat was associated with an increased risk of owner-reported allergic/atopic skin. When CAD was considered as the dependent variable, having over 50 % white colour in the coat seemed to have a protective role in the univariable model but not in the final model. In the study of Nødtvedt et al. (2007) a significantly higher incidence of AD was found in white bull terriers than those of other colours. No clear explanation for this association has been proposed so far. These findings are, in any case, interesting and should be considered at least in dog breeding.

Since this study was observational, the associations found between environmental factors and allergic skin symptoms cannot be confirmed as causal. However, many statistically significant associations were found, most of which were consistent with previous human research and with previous studies in dogs suffering from CAD. Hence, these environmental factors should be of interest in young puppies, or even in pregnant dams, when considering the prevention of atopy. In addition, the diet should also be considered as an important part of prevention, since there already are studies reporting the protective effect of the diet of dam or puppies on the incidence of CAD (Nødtvedt et al., 2007; van Beeck et al., 2015). Like the modern lifestyle in humans, including diets containing more processed and synthetic foods, has been associated with changes in gut microbiome and immune function (Amarasekera et al., 2013), the same is probably true in case of modern dogs. In humans, the diet of the mother can increase (Hopppu et al., 2000; Kieftede et al., 2012; Miyake et al., 2014) or decrease (Furuhjelm et al., 2009; Pelucchi et al., 2012; Jirapinyo et al., 2013; Miyake et al., 2014) the prevalence of atopic dermatitis and allergic diseases in children. Maternal diet is known to influence on the health of offspring through epigenetic modifications (Vanhees et al., 2014). Epigenetic changes in foetal DNA can lead to alterations in immune development (Waterland et al., 2007; Porrás et al., 2008; Odaka et al., 2010), which in turn could result in atopic dermatitis and allergic diseases later in life. In addition, the influence of paternal lifestyle and diet on the epigenetic modulation of the foetus should not be neglected (Bielawski et al., 2002). Regarding what is discussed above, the importance of the diet in modern day's dog health should be more emphasized.

4.3 The effect of the diet on haematology and clinical chemistry

In the raw diet group (n=19) WBC count, ALP, glucose, total calcium, and cholesterol decreased, while RBC, Hb, MCHC, platelet count, albumin, potassium, sodium, creatinine, and total protein increased significantly between baseline and the end of the diet intervention (study III) (Fig 3). In the

dry diet group (n=14), the MCH, MCHC, albumin, inorganic phosphate, sodium, cholesterol, and total protein increased significantly during the same period (Fig 4).

Study III shows that diet induced significant changes in some of the haematological and blood biochemical values in dogs. The findings of increased erythrocyte count, haemoglobin level, and MCHC value in the raw diet group are comparable to the results of Kronfeld et al. (1977), who verified higher RBC counts and Hb levels in dogs fed the highest protein diet compared to lower protein diets (n=18), and to the results of Ober et al. (2016), who reported a significant decrease in RBC counts and Hb levels in dogs fed a low-protein diet compared to two higher protein diets (n=17). Similar results were found in a study by Brown et al. (2009), in dogs fed a meat-based diet compared to dogs fed a meat-free diet, although statistical difference was not detected, possibly due to the small number of dogs (n=12).

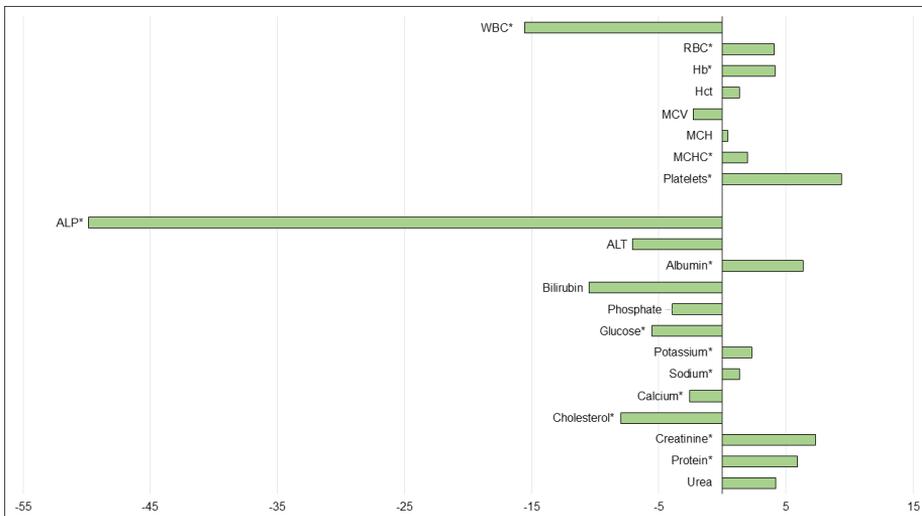


Figure 3. Mean change in percentage from the mean baseline values of the haematology and clinical chemistry analytes in the raw diet group (n=19). * $P < 0.05$.

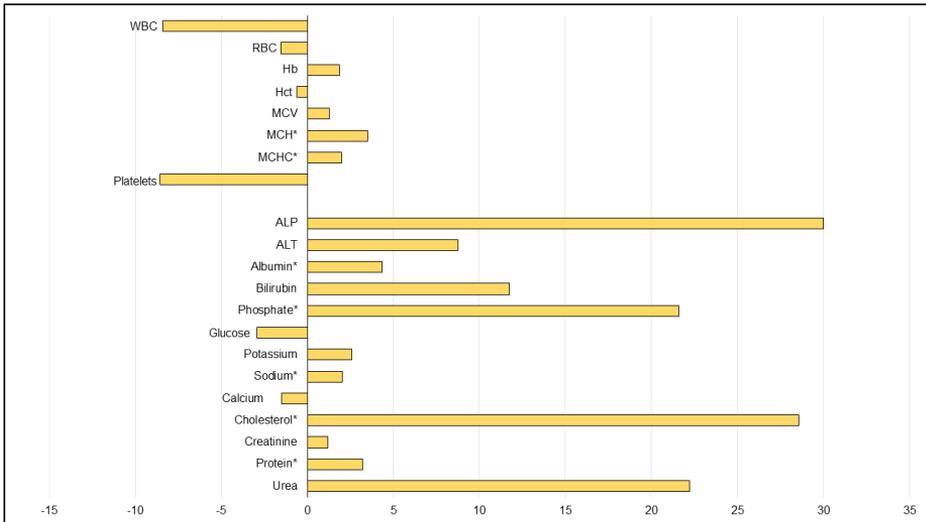


Figure 4. Mean change in percentage from the mean baseline values of the haematology and clinical chemistry analytes in the dry diet group (n=14). * $P < 0.05$.

Erythrocytes are continuously exposed to reactive oxygen species (ROS) that can damage their membranes and impair their function (Mohanty et al., 2014), which makes the elimination of ROS crucial for the survival of erythrocytes (Fujii et al., 2015). ROS are products of normal cellular metabolism, but when they are overproduced the tissue damage leads to an increase in free radical production and to oxidative stress (Valko et al., 2007; Peake and Suzuki, 2004). Oxidative stress has been reported to be associated also with canine atopic dermatitis (Kapun et al., 2012), which makes the possible positive effect of the raw diet on ROS elimination even more important in atopic dogs. However, this is only speculative when it comes to study III, and should be studied further. Cell damage by ROS is counteracted by natural antioxidants produced by the body when in equilibrium, and by antioxidants that are provided in the diet or as supplements (Rahman, 2007). The processing of meat is related to a reduced level of natural antioxidants (Reddy and Love, 1999; Riaz et al., 2009). Processing procedures include the application of heat, and chemical treatments such as the use of oxidizing agents, which might decrease the bioavailability of vitamins (Hamper et al., 2016). On the other hand, synthetically produced vitamins, minerals and trace elements are often

added to processed dry foods to meet the required nutritional recommendations (Case et al., 2011), like vitamin A, E, D, and C in the dry food used in study III. Studies on the bioavailability of natural versus synthetic vitamins are controversial, and especially in humans the difference in their bioavailability might not be relevant (Carr and Vissers, 2013; Borel et al., 2015).

The activity of ALP decreased significantly in the raw diet group. In the dry diet group there was a trend towards significance as the mean ALP activity increased, but it did not reach significance ($P= 0.075$). An increased ALP in adult dogs is usually a sign of functionally disturbed or greatly stimulated tissues and decreased ALP has been reported to be related to nutritional deficiency or genetic conditions (Corathers, 2006). In study III, however, the change in either of the diet groups was not the order of magnitude that could be linked to these conditions, at least not according to reference values. A protein-deficient diet has been reported to increase serum ALP in dogs (Davenport et al., 1994). On the other hand, Swanson et al. (2004) found that ALP increased over time in dogs fed a meat-based diet, but not in dogs fed a plant-based diet. In that study, however, both diets consisted of processed dry foods, unlike in our study. A high-fat diet has been reported to lower ALP more than a low-fat or low-protein diet in dogs (Ober et al., 2016), which is consistent with the results from study III, since the raw diet included more fat and protein than the dry food. Glucocorticoids are known to influence ALP activity, but are reported to return to baseline levels within four weeks (Ginel et al., 2002). In study III one dog in the dry diet group received ongoing glucocorticoid medication during the trial, and it was omitted from the ALP analysis. Any possible short-term glucocorticoid use during the trial should not have had an effect to the results, since none of the study dogs received short-term medication during the four weeks prior to the baseline or the end visit. These results on the effect of the diet on ALP activity are indeed very interesting and further research is needed. Even though the level of ALP activity did not surpass the upper reference value, there might be a possibility that a long-term stress going on in the liver will lead to health issues later in life.

Another interesting finding in study III was the decreased serum cholesterol level in the raw diet group and the increased level in the dry diet group. Three dogs in the dry diet group surpassed the upper reference interval (RI) value for cholesterol. Even though the raw food diets included more meat and animal-based fats, the serum cholesterol concentrations of the raw diet fed dogs decreased during the trial. On the other hand, the cholesterol content has been reported to be higher in cooked meats than in raw meats (Williams, 2007). Nevertheless, recent studies suggest that dietary cholesterol intake does not show appreciable relationship with the serum cholesterol (Fernandez, 2012; Kanter et al., 2012). In a study conducted by Hansen et al. (1992) on dogs suffering from chronic renal failure, dogs that were fed a high-protein dry food had higher blood cholesterol values than dogs fed a low-protein dry food. Swanson et al. (2004) reported elevated blood cholesterol levels in a group of dogs that had eaten an animal product-based diet compared to a plant product-based diet group. Kronfeld et al. (1977) reported increased blood cholesterol in dogs after being on a diet with high protein and fat content. Our results contradict these canine study results. In these three studies, dry dog foods with differing protein or fat content were used, hence their diets are not directly comparable with ours. In humans, both highly processed and protein-deficient diets have been associated with higher blood cholesterol levels (Rauber et al., 2015), and a low-carbohydrate diet has been shown to decrease serum cholesterol levels (Westman et al., 2002). Also, L-carnitine has been reported to decrease both the total and LDL cholesterol levels in human serum (Malaguarnera et al., 2009; Malaguarnera et al., 2010). Especially red meat contains high amounts of L-carnitine (Koeth et al., 2013), which may have had an influence on our results. More research is still needed on the effects of the diet on blood cholesterol of dogs, as the results from different studies are still controversial.

Serum glucose levels decreased significantly in the raw diet group. In the study by Ober et al. (2016), the authors argue that a low-protein-high-fat diet significantly increased serum glucose levels compared to both a low-fat diet and to a high-protein diet. Glucocorticoids have been reported to not have an effect on blood glucose concentration in atopic dogs (Kovalik, et al., 2012a).

These diet related blood glucose results need to be verified and further studied to determine the underlying mechanisms behind them.

Serum inorganic phosphate levels significantly increased in the dry diet group. Nevertheless, it has been described that boiling and other thermal processing methods decrease the phosphorus content of foods (Cupisti et al., 2006; Vrdoljak et al., 2015). In our study, the phosphorus content in the raw foods was higher than in the dry food (1.0/1.6 in dry matter (DM) versus 0.63 in DM, respectively). It should also be noted, that the calcium/phosphorus ratios were higher in the raw food diets than in the dry food diet (1.3/1.7 versus 1.1, respectively), and that calcium and phosphate metabolism are tightly associated with each other (Quarles, 2008). On the other hand, calcium and phosphorus intake seem to reflect serum phosphate levels weakly in humans (Kemi et al., 2010; Braithwaite et al., 2012). In addition, a circadian variation has also been reported to be present for serum phosphate (Calvo et al., 2014), which was not controlled for in the present study. The short-term use of corticosteroids has been shown not to have an impact on serum phosphate or calcium levels in atopic dogs (Kovalik et al., 2012b). The causes underlying the higher phosphate concentration in the serum of dogs on dry food diet is not straightforward. Increased serum phosphate concentration, even when inside the RI, has been associated with increased cardiovascular disease risk in healthy humans (Dhingra et al., 2007; Foley et al., 2009), and hence the results from study III might be clinically important.

In study III, creatinine concentrations increased significantly in the raw diet fed dogs, but remained within the RI. Similar results were seen in the study from Wynn et al. (2003) where significantly higher creatinine levels were observed in dogs eating various raw food diets compared to dogs eating various dry food diets. There are also studies that have reported no effect on blood creatinine concentration due to diet (Polzin et al., 1991; Hansen et al., 1992). Creatinine is derived from both creatine via internal dehydration, as well as creatine phosphate via dephosphorylation (Braun et al., 2003). Creatine is only available in the diet via animal-based foodstuffs, primarily from muscle meats (Brosnan and Brosnan, 2016), and creatine concentrations

has been shown to be significantly higher in raw meat than in commercial dry dog foods (Dobenecker and Braun, 2015). Hence, the increase of plasma creatinine levels in the raw diet group could be due to the endogenous production of creatinine from creatine. Serum creatinine concentration is known to vary with muscle mass (Hokamp and Nabity, 2016), and can be used as an index for muscle mass under normal conditions (Wolfe, 2012). Higher amounts of high quality protein intake allow for the maintenance of muscle mass better than either equal low-quality protein intake, or lesser amounts of total protein intake in dogs (Wakshlag et al., 2003). In the present study, however, the canine muscle mass was not evaluated.

We can conclude that one of the most significant difference between the diet groups in study III was the activity of ALP. There was a mean 50 % decrease seen in dogs fed raw diet and a mean 30 % increase seen in dogs fed the dry diet, which seems to highlight the different kind of effects that the different diets have on the liver. All activities stayed inside the reference values, but it is important to notice that the reference values of our laboratory have been created using dogs fed a dry diet (Sankari, personal communication). The importance of this finding to dogs' health should be clarified in future studies. Another interesting finding was the increase of cholesterol in dry food fed dogs, since the common belief is that animal derived fats increase blood cholesterol. In fact, in study III, a statistically significant decrease of cholesterol was observed in the raw diet fed dogs, although there were much more animal-based fats in the raw diet. This finding calls for further studies.

An important fact, which makes these results difficult to interpreted, is that the diets differed in more ways than just being raw or dry. The macronutrient profiles were highly different, dry food being rich in carbohydrates and raw food being rich in fats. Also, protein sources differed between diets. The dry food had chicken, turkey and egg as animal-based protein sources and maize gluten and rice as plant-based protein sources. Neither raw diet had plant-based protein sources. The other raw diet included chicken and egg, and the other turkey (all present in the dry food), but in addition they also included

pork and lamb or beef and salmon. In addition, there were added synthetic vitamins and trace elements in the dry food, but not in the raw diets.

The results of the study III are also interesting since the reference values used in the laboratories for blood parameters are calculated based on dog populations fed mainly dry food. If a raw diet (or a diet that has a different macronutrient profile than a typical dry food) leads to different mean values for many parameters, are those current reference values optimal for dogs and their health? If we consider the fact that the highly processed high carbohydrate diet is not optimal for dogs, which is a carnivore, then those reference values should also be reconsidered to make them reflect the values that might maintain a dogs' health and wellbeing in a best possible way. The reference values for dogs fed a typical raw diet should first be created using a larger population of healthy dogs, and if they are found to be significantly different from usual laboratory values, like the study III suggest (Fig 3), more research should be done to reach consensus of optimal reference values for dogs' health and longevity.

4.4 Folate, vitamin B12 and blood iron analyses

There was a significant decrease in the mean plasma folate concentration in the raw diet group (n=17) from the baseline visit (12.0 ng/ml; SD \pm 5.9) to the end visit (5.0 ng/ml; SD \pm 3.8) (p=0.001). In the dry diet group (n=14) no significant change in the plasma folate concentration was observed (baseline and end visit concentrations were 10.8 ng/ml (SD \pm 5.9) and 8.4 ng/ml (SD \pm 1.8), respectively) (p=0.125). The RI for folate was 3-15 ng/ml. Four dogs fell below the RI and one dog surpassed the RI in the raw diet group at the end visit.

Also, the mean plasma B12 concentration in the raw diet group decreased significantly from the baseline visit (620 pg/ml; SD \pm 275) to the end visit (457 pg/ml; SD \pm 178) (p=0.011). There was no significant change observed in the dry diet group from the baseline visit (575 pg/ml; SD \pm 249) to the end visit (585 ng/ml; SD \pm 200) (p=0.891). The RI for B12 was 200-800 pg/ml. None

of the dogs fell below the RI but one dog in the raw diet group and three dogs in the dry diet group surpassed the RI at the end visit.

Unfortunately, the manufacturer of the dry diet was not able to provide the amount of B12 and folate in the diet. The manufacturer of the raw diets analysed B12 and folate from the same food but different batch, and reported the amount of B12 as 11.78 µg/100g DM and folate as 278 µg/100g DM in Pork-chicken-lamb diet, and B12 as 22.9 µg/100g DM and folate as 417 µg/100g DM in Beef-turkey-salmon diet. According to recommended allowance by National Research Council (NRC), the amounts in both raw diets were enough to fulfil the need of an adult dog (NRC).

In addition, the whole blood iron concentration was determined at the baseline and at the end of the study using 11 dogs from the raw diet group and 8 dogs from the dry diet group. The mean concentration in the raw diet group was 10.6 mmol/L (SD ±0.99) at the baseline visit and 9.74 mmol/L (SD ±0.86) after the diet intervention ($p=0.026$), and in the dry diet group 9.73 mmol/L (SD ±0.74) at the baseline visit and 8.96 mmol/L (SD ±0.46) after the diet intervention ($p=0.123$). The added amount of iron in the dry food was 58.4 mg/kg DM and the analysed content of the iron in the raw diets were 123 mg/kg DM in Pork-chicken-lamb diet and 82.1 mg/kg DM in Beef-turkey-salmon. All these amounts should be enough for adult dog (NRC). Changes in folate, B12 and iron are presented in Fig 5.

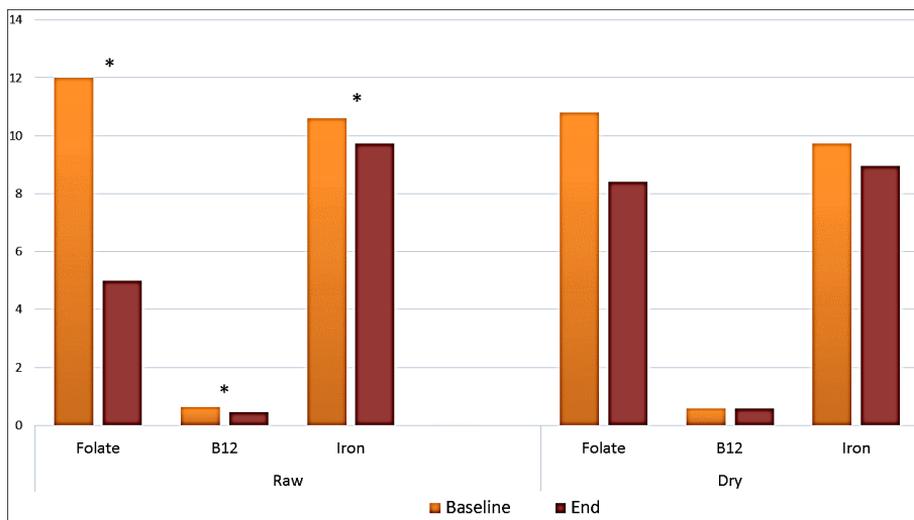


Figure 5. Folate, B12 and iron concentrations in the raw diet and dry diet groups before and after the diet intervention. Folate and B12 are presented in ng/ml and iron in mmol/L. * $P < 0.05$ refers to change from the baseline to the end.

Among important differences between raw and processed meat are alterations in protein structure caused by heat processing, which could consequently alter the digestibility, availability, and biological quality of the protein as well as cofactors, such as B vitamins (Lombardi-Boccia et al., 2002; McNulty and Pentieva, 2004; Lombardi-Boccia et al., 2005; Meade et al., 2005; Case et al., 2011). B-complex vitamins have diverse metabolic functions, including cell division and growth, erythropoiesis and iron metabolism (Bills et al., 1992; Koury and Ponka, 2004). However, there were no significant change seen in the serum folate and vitamin B12 in the dogs of the present study in the dry diet group. These results suggest that there was no folate or B12 deficiency present in the dry diet group, although we do not know the amounts in the food. In fact, three dogs even surpassed the RI of B12. On the other hand, the blood concentration of these vitamins decreased significantly in the raw diet group, and four dogs fell below the RI of folate. This was highly unexpected finding, since majority of vitamin B12 comes from meat and liver, and the amount of B12 and folate in both raw diets was over the recommended level by NRC for adult dogs. Serum folate can increase while B12 can decrease in dogs with small intestinal bacterial overgrowth (SIBO) since intestinal

bacteria can produce folate and use B12 (German et al., 2003; Dossin, 2011). In humans, SIBO has been linked also to skin conditions (Bowe & Logan, 2011). In our study, the decrease in folate in the raw diet fed dogs might indicate the decrease in the number of microbes in small intestine, but the decrease also on serum B12 contradicts this. On the other hand, decreased levels of serum folate have been reported in dogs with inflammatory bowel disease (IBD) (Xu et al., 2016). The mean concentration of serum folate in IBD dogs in that study was 0.011 ng/ml, which still was a lot lower than in raw diet fed dogs in our study (5 ng/ml). Different diets have been recently reported to modify the gut microbiota in dogs (Pinna et al., 2016; Sandri et al., 2017), and the changes in folate and B12 in our study might therefore be due to alterations in the gut microbiota. In one study, the faecal microbes related to folate metabolism were increased in dry diet fed cats compared to canned food fed cats (Young et al., 2016). Based on our results, it is difficult to say which one of the diets might have had an adverse effect to gut microbiota, but it could give the reason to re-evaluate the correct folate and B12 reference values for dogs to reflect the gut health. Considering CAD, this should be studied further, since many dietary effects on gene expression also work through the gut microbiota (Ferreira et al., 2014), which is an important driver of host immunity (Suchodolski & Simpson, 2013). In humans, the potential role of gut microbiota in the pathogenesis of AD has been suggested to work via stimulation and education of immune cell populations (Egawa & Weninger, 2015), again highlighting the role of the diet at young age when the immune system is developing.

A significant increase of the platelet count in the raw diet group was seen in study III, and diet related increased platelet counts have been reported in cases of iron deficiency (Pinna et al., 2016). Iron is needed for the active production of erythrocytes and haemoglobin (Koury and Ponka, 2004), which both significantly increased in the raw diet group (Study III), indicating a sufficient iron supply. Nevertheless, whole blood iron concentration decreased in both diet groups, although significantly only in the raw diet group, even though the absorption of heme-iron, present in meat, is five to ten times greater than that of a non-heme iron, present in grains and vegetables

(Gudjoncik et al., 2014). The concentration of iron in whole blood is not a straight indicator of iron deficiency but could indicate the level of the dietary iron. Considering all the haematological parameters measured in study III, and the reported iron concentration in both raw diets, there is no reason to suspect iron deficiency in the raw diet group, even so the blood concentration decreased significantly. For this reason, the relevance of the findings in blood total iron remains unclear here. In addition, the reason behind the results of decreased plasma folate and B12 concentrations in raw diet fed dogs can only be speculated, since both raw diets fulfilled the recommended allowance of those vitamins. The role of gut microbiota thus might be one of the interesting study subjects in the future.

4.5 The effect of CAD and the diet on transcriptome in the skin

Atopic dogs versus healthy dogs

A total of 149 differentially expressed transcripts were found commonly in EdgeR and DeSeq2 after the diet intervention between atopic and healthy dogs (Fig 6). Top canonical pathways in the functional analyses in atopic dogs are shown in Table 5.

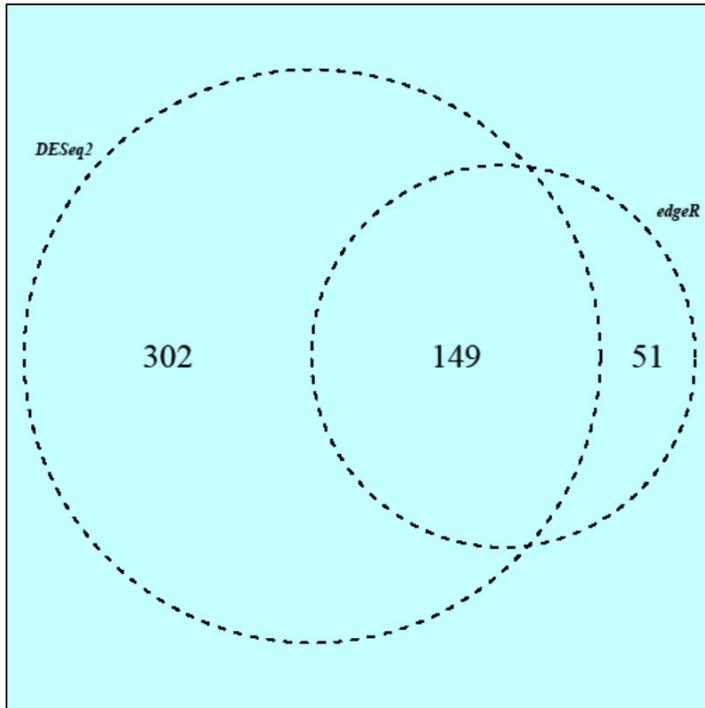


Figure 6. Differentially expressed transcripts between atopic and healthy dogs ($n=8$) found using DeSeq2 and EdgeR algorithms.

Table 5. Top canonical pathways in atopic dogs ($n=8$) in the functional analysis by Ingenuity.

Top Canonical Pathway	p-value	Overlap
Angiopoietin signalling	5.83×10^{-5}	6.3 % (5/79)
EGF signalling	5.48×10^{-4}	5.6 % (4/72)
AMPK signalling	7.44×10^{-4}	2.9 % (6/206)
FXR/RXR activation	7.61×10^{-4}	3.6 % (5/137)
Leptin signalling in obesity	1.21×10^{-3}	4.5 % (4/89)

EGF, epidermal growth factor; AMPK, AMP-activated protein kinase; RXR, retinoid X receptor; FXR, farnesoid X receptor.

A total of 856 differentially expressed transcripts were found in common in EdgeR and DeSeq2 between atopic and healthy dogs fed dry diet (n=4) (Fig 7). Top canonical pathways in atopic dogs fed dry diet are shown in Table 6. Only 60 differentially expressed transcripts were found in common in EdgeR and DeSeq2 between atopic and healthy dogs fed raw diet (Fig 7). Top canonical pathways in raw diet fed atopic dogs in the functional analyses are shown in Table 7.

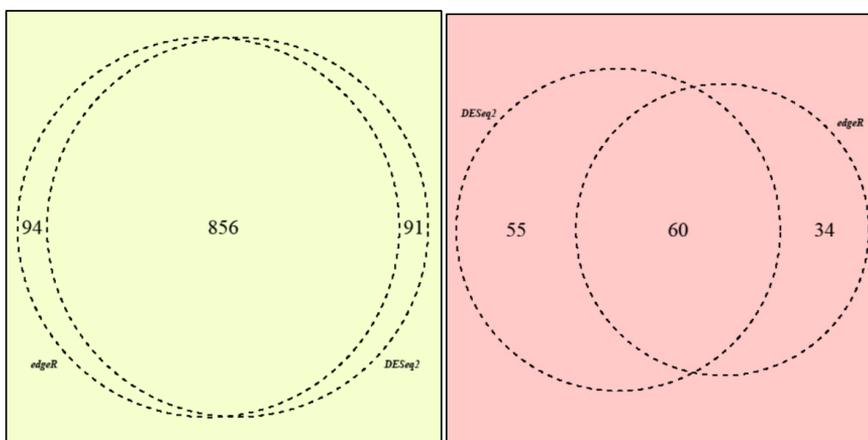


Figure 7. Differentially expressed transcripts between atopic and healthy dogs found using DeSeq2 and EdgeR in the dry diet group (yellow, n=4) and raw diet group (red, n=4).

Table 6. Top canonical pathways in dry diet fed atopic dogs (n=4) in the functional analysis by Ingenuity.

Top Canonical Pathway	p-value	Overlap
Atherosclerosis signalling	8.60 x 10 ⁻⁷	12.5 % (16/128)
Role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis	1.65 x 10 ⁻⁶	9.2 % (22/238)
LPS/IL-1 mediated inhibition of RXR function	2.60 x 10 ⁻⁶	9.3 % (21/226)
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	5.25 x 10 ⁻⁶	7.9 % (25/315)
FXR/RXR activation	4.21 x 10 ⁻⁵	10.2 % (14/137)

LPS, lipopolysaccharide; IL-1, interleukin-1; RXR, retinoid X receptor; FXR, farnesoid X receptor.

Table 7. Top canonical pathways in raw diet fed atopic dogs in the functional analysis by Ingenuity (n=4).

Top Canonical Pathway	p-value	Overlap
γ -linolenate biosynthesis II (animals)	1.05×10^{-3}	8.3 % (2/24)
Hepatic fibrosis / hepatic stellate cell activation	6.23×10^{-3}	1.6 % (3/187)
Protein citrullination	1.20×10^{-2}	16.7 % (1/6)
Calcium transport I	2.77×10^{-2}	7.1 % (1/14)
Type II diabetes mellitus signalling	3.55×10^{-2}	1.4 % (2/148)

These results show the importance of the diet when comparing the gene expression between two groups. The difference in gene expression between healthy and diseased was much more detectable in dogs fed the dry diet than in dogs fed the raw diet. This might also be due the individual differences, since the sample size is small, but should be studied further and considered in future studies.

In the functional analysis of the study IV the lipid metabolism was the top molecular and cellular function in AD dogs, when looking at them independent of diet group (data not shown). The cornified envelope, an envelope consisting of proteins and lipids that replaces the plasma membrane of keratinocytes, is embedded in the lipid envelope and lipids in the skin are important for the barrier function when avoiding transepidermal water loss (Candi et al., 2005). Expression of the galectin 12 (LGALS12), responsible for lipogenesis in keratinocytes (Harrison et al. 2007; Xue et al., 2016), and of hydroxycarboxylic acid receptor 1 (HCAR1), suppressor of lipolysis (Cai et al., 2008), were upregulated in AD dogs compared to healthy dogs. ATP binding cassette subfamily D member 2 (ABCD2) is suggested to have a role in transporting very-long chain fatty acids (VLCFA) to peroxisomes to be degraded by β -oxidation in various tissues in mice (Fourcade et al., 2008). ABCD2 was upregulated in the skin of the AD dogs in the study IV. VLCFAs are an important part of the skin ceramides forming the cornified envelope

(Fürstenberger et al., 2007; Mizutani et al., 2009). There were also three downregulated lipoxygenases (LOX) in AD dogs fed the dry diet in study IV. Two of them, ALOX12B and ALOXE3, play a key role in epidermal barrier function and epidermal differentiation (Epp et al., 2007; Krieg and Fürstenberger, 2014; Muñoz-Garcia et al., 2015). Their oxidized products are involved in the transportation of VLCFAs to the cornified lipid envelope in the skin (Krieg and Fürstenberger, 2014). The third downregulated lipoxygenase in the dry food fed AD dogs, platelet-type 12-lipoxygenase (ALOX12), has been shown to affect the permeability barrier function in knockout mice (Johnson et al., 1999).

The top canonical pathway in the skin of the AD dogs fed raw diet was the upregulated γ -linolenate biosynthesis II (Table 7). The same pathway was found among top canonical pathways in a meta-analysis associated with human AD (Ewald et al., 2015). It has been shown that this omega-6 fatty acid pathway is dysfunctional in humans suffering from atopic eczema and AD (Horrobin, 1992; Yen et al., 2008; Chisaguano et al., 2013). The omega-6 / omega-3 ratio in the dry food was 4:1, in the Pork-chicken-lamb diet 9.5:1, and in the Beef-turkey-salmon diet 2.5:1 (Tables 2 and 3). From the two raw diet fed AD dogs, one was fed with both diets and another only with Pork-chicken-lamb diet. This finding of a possible positive effect of the raw diet on fatty acid metabolism in AD dogs could be due to overall larger amount of fat in the raw diets compared to the dry diet, or have something to do with the omega-6 / omega-3 ratio.

In study IV altogether 23 keratins (KRTs) were found differentially expressed between atopic and healthy dogs, most of them seen only in the dry diet group. In a study using quantitative reverse transcription PCR, KRT5 was found upregulated in non-lesional skin of AD dogs compared to normal skin (Theerawatanasirikul et al., 2012). In our study IV, an increased expression of KRT5 was found only in the dry diet fed AD dogs. On the other hand, KRT1 and KRT10, which affect the mechanical integrity of the whole epidermis (Paramio et al., 1999; Moll et al., 2008), were downregulated in the skin of the dry diet fed AD dogs. An interesting finding was KRT4, which had one

transcript highly downregulated in the skin of the dry diet fed AD dogs and three other transcripts upregulated in those same dogs. In addition, KRT84 was overexpressed in AD dogs compared to healthy dogs at the end visit (Fig 8), and the expression of KRT84 increased in atopic dogs in the dry diet group (n=2) during the diet intervention (mean transcript count at the baseline visit was 35 and at the end visit 363, $p < 0.001$). It was also one of the top-downregulated genes in the functional analysis (data not shown). An association between KRT84 and CAD has not been reported previously.

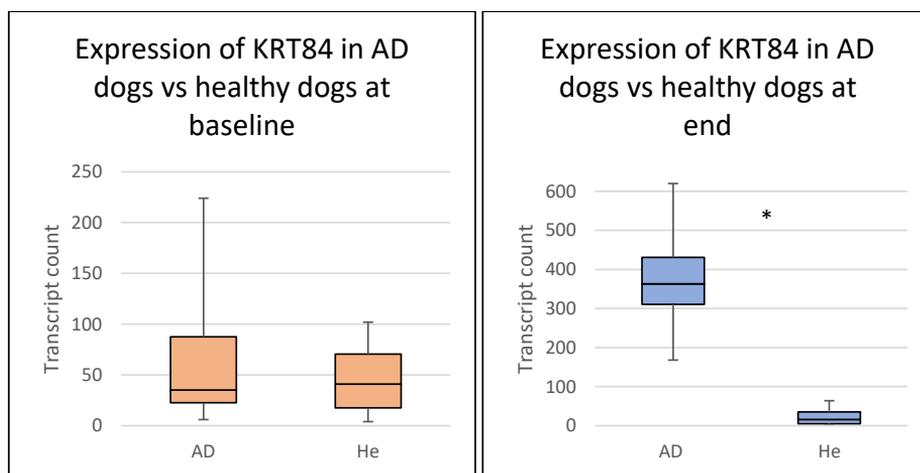


Figure 8. The expression of keratin 84 (KRT84) in atopic dogs (n=4) compared to healthy dogs (n=4) at the baseline visit (orange) and at the end visit (blue). Statistical significance was reached at the end visit (* $p < 0.001$, $\log FC = 3.5$). AD, atopic dermatitis; He, healthy; FC, fold change.

The epidermal differentiation complex (EDC), located in chromosome 1q21 in humans, includes gene clusters responsible for coding proteins involved in skin cell differentiation (de Guzman Strong et al., 2010). These involve for example filaggrin (FLG). Studies have reported conflicting results of FLG in AD dogs, like decreased expression of FLG in atopic dogs (Rogue et al. 2011), increased expression of FLG in atopic dogs (Theerawatanasirikul et al., 2012), association between FLG and CAD in Labrador retrievers in UK (Wood et al., 2010), and no association between FLG and CAD (Barros Roque et al., 2009; Wood et al., 2010; Salzman et al., 2011). In our study IV, FLG2 was

downregulated in AD dogs fed the dry diet compared to healthy dogs. FLG2 might play an overlapping and synergistic role with FLG in the formation of the epidermal barrier, protecting the skin from environment and the escape of moisture (Wu et al., 2009; Makino et al. 2014). Another gene family located in the human EDC is the S100 gene family, of which the S100 calcium binding protein A8 (S100A8) has been associated with lesional skin of CAD dogs in previous studies (Merryman-Simpson et al., 2008; Wood et al. 2009; van Damme et al. 2009; Plager et al., 2012). In study IV, a higher expression of S100A6 (also known as calyculin) was found in the skin of AD dogs than in healthy dogs. The special AT-rich sequence-binding protein 1 (SATB1) was the top-downregulated gene in the functional analysis (data not shown), and it was downregulated in the skin of AD dogs. It is one of the epigenetic regulators of epidermal differentiation (Fessing et al., 2011). None of the three genes above have been previously reported to be associated with CAD, although recent studies have reported decreased expression of FLG2 in the skin of human AD patients (Pellerin et al., 2013; Makino et al. 2014).

Angiopoietin signaling was the top upregulated canonical pathway in AD dogs in study IV (Table 5). AKT serine/threonine kinase 3 (AKT3), angiopoietin 1 (ANGPT1), tyrosine receptor kinase (TEK), and secreted phosphoprotein 1 (SPP1, also known as osteopontin) were upregulated in the skin of AD dogs, all known to regulate angiogenesis (Thomas and Augustin, 2009; Puxeddu et al., 2010; Buroker et al., 2012). Angiogenesis, the growth of new blood vessels from pre-existing ones, has previously also been reported to play a role in atopic dermatitis (Zhang et al., 2006; Chen et al., 2008; Varricchi et al., 2015).

In study IV the key findings in AD dogs seemed to be the alterations in the lipid metabolism, a more proliferative than differentiated state of keratinocytes, as well as activation of angiogenesis and increased wound healing processes. Lipids play an important role in the skin barrier function, which is known to be dysfunctional in atopic dermatitis. In normal epidermis the proliferative and differentiated states of keratinocytes are precisely balanced and a functional skin barrier can be generated (Candi et al., 2005). The dysregulation of keratinocytes is characteristic to atopic diseases and it

has a profound effect on the skin barrier function, also disturbed in atopic dermatitis. The results from study IV partly support the previous findings in canine and human research, and novel genes are reported.

Raw diet versus dry diet

In total, EdgeR found 16 differentially expressed genes between the raw diet fed and the dry diet fed dogs. Only polymeric immunoglobulin receptor (PIGR), secretory leukocyte peptidase inhibitor (SLPI), argininosuccinate synthase 1 (ASS1), cystathione β -synthase (CBS), and immunoglobulin heavy constant mu (IGHM) have so far been identified in the dog (Table 8). DeSeq2 found 6 differentially expressed genes. Of these, ASS1, CBS, IGHM and mitochondrial ribosome recycling factor (MRRF) are identified in the dog (Table 8). All genes were upregulated in the raw food group compared to the dry food group.

Table 8. *Differentially expressed known genes between raw diet fed and dry diet fed dogs (n=8).*

Gene name		Log2 FC	P-value	Algor.
Polymeric immunoglobulin receptor	PIGR	6.4	< 0.001	EdgeR
Secretory leukocyte peptidase inhibitor	SLPI	5.4	< 0.001	EdgeR
Immunoglobulin heavy constant mu (5 transcripts)	IGHM	5.3 / 1.3	< 0.001	ER/ DS
		5.1 / 1.2	< 0.001	ER / DS
		4.9	< 0.001	EdgeR
		2.7	< 0.001	EdgeR
		2.5 / 1.3	< 0.001	ER / DS
Arginosuccinate synthase 1	ASS1	2.1	< 0.001	ER / DS
Cystathione β -synthase	CBS	1.8	< 0.001	ER / DS
Mitochondrial ribosome recycling factor	MRRF	0.7	< 0.001	DeSeq2

FC, fold change; ER, EdgeR; DS, DeSeq2.

Upregulation of IGHM in the raw diet group indicates an activation of humoral mechanisms. Immunoglobulin Ms are natural antibodies which are the first to be produced in an ongoing immune response to infection or immunization (Petrušić et al., 2011). Also, PIGR has an important role in the mucosal immune system and it acts as a transporter for polymeric immunoglobulins (Asano and Komiyama, 2011). The secretory IgA (sIgA), generated at the cleavage site of PIGR, is an inhibitory factor to bacteria on the skin surface, and abnormalities in sIgA have been reported in atopic dermatitis in humans (Imayama et al., 1994).

ASS1 is a rate-limiting enzyme for the biosynthesis of arginine in the urea cycle in the liver (Long et al., 2016), and for the citrulline-nitric oxide (NO) cycle in many other cells, leading to NO synthesis (Husson et al., 2003). NO is a messenger molecule in the immune defence (Mori and Gotoh, 2004), it functions as an antimicrobial molecule (Nathan, 1992), and it can initiate the differentiation of human keratinocytes (Liebmann et al., 2010). It has been suggested that high levels of NO are of functional importance for the resolution of chronic inflammatory processes (Bruch-Gerharz et al., 2003; Bodgan, 2001), and in primary cultures of human keratinocytes, low NO level has been shown to promote keratinocyte proliferation, often seen in psoriatic skin (Krischel et al., 1998).

The expression of SLPI, an antimicrobial peptide neutralizing bacterial and viral pathogens, has been found to be downregulated in the lesional and non-lesional skin of atopic dogs compared to healthy dogs (Lancto et al., 2013). SLPI expression was upregulated in the raw diet fed dogs in study IV. This gene might have an important role in the immune system of the skin, which is an important part of the innate immune system, including a properly working skin barrier system. In fact, in the feeding trial in study IV, an increased TEWL (10.7 g/m² hr) was observed in axillae area in dogs fed dry diet (n=4) compared to the raw diet fed dogs (6.7 g/m² hr) (n=4) (P=0.043), which could be a sign of a dysfunctional skin barrier in these study dogs.

CBS catalyses a reaction in which cysteine is produced from serine and L-homocysteine (Miles and Kraus, 2004; Prudova et al., 2006). Cysteine is the limiting reagent in the production of glutathione, an important antioxidant (Prudova et al., 2006), which scavenges free radicals and other reactive oxygen species (ROS) (Fang et al., 2002). Alterations in this pathway increase inflammatory processes (Rosado et al., 2007), and reduced glutathione is associated with increased vulnerability to oxidative stress (Prudova et al., 2006). In recent studies it was reported that in human keratinocytes cysteine inhibits the upregulation of pro-inflammatory mediators (Lee et al., 2016).

Also, the depletion of MRRF results in elevated ROS production and cellular dysfunction (Rorbach et al., 2008). These results of CBS and MRRF could suggest that the raw food diet inhibited ROS production in the skin cells. Oxidative stress plays an important role in the pathogenesis of AD in mammals (Tsuboi et al., 1998; Niwa et al., 2003), and the induction of oxidative stress is related to excessive ROS and to deficiencies in the antioxidant system (Valko et al., 2007). Since ROS production is increased in CAD during the inflammatory process (Kapun et al., 2012), these possible effects of CBS and MRRF to decrease oxidative stress and ROS production might be of value to atopic dogs.

The raw diet upregulated several genes in the skin compared to the dry diet, showing that the raw food might have enhanced the passive immunity, and protected cells from inflammatory effects and from oxidative stress in the skin of dogs, more so than the highly processed and more sterile dry food did. These effects might be beneficial in dogs suffering from CAD. Antibiotics are often used when treating secondary skin infections common in AD dogs, and antibiotic resistant bacteria are an increasing problem in veterinary medicine (Hillier et al., 2014, Hensel et al., 2016). If the immunity in the skin could be enhanced in other ways, for example through diet, this might decrease the need for frequent use of antibiotics. At the same time, the immunological effect of raw food might also work through the intestinal system. The lack of contact with microbes and parasites that have been a part of the 'human ecosystem' throughout evolution, has been proposed to have an impact on the occurrence

of allergic and autoimmune diseases in industrialized countries, and is described as 'biome depletion' (Parker and Ollerton, 2013). The fact that raw food includes more bacteria than processed dog foods (Strohmeier et al., 2006), might be just what the dogs' body and gut need to stay healthy. This calls for more research in the future. Nevertheless, the findings in study IV are novel and their association with the immune system makes them interesting and important.

4.6 Serum concentration of TGF- β 1

There was a significant increase ($P=0.001$) in the serum concentration of TGF- β 1 during the diet intervention study in the raw diet fed atopic/borderline dogs (Fig. 9). The serum concentration of TGF- β 1 in the raw food group at the baseline was 34,797 pg/mL ($SD\pm 9,846$) and at the end 45,996 pg/mL ($SD\pm 9,624$). In the dry food fed atopic dogs, a statistically significant difference between the start and the end of the diet intervention was not observed ($P=0.153$).

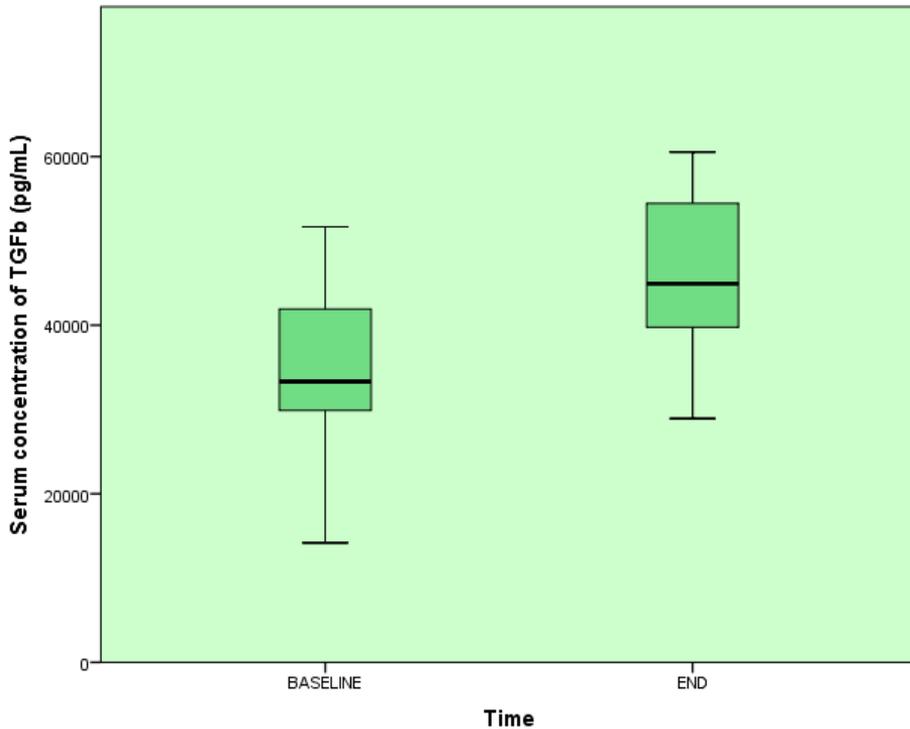


Figure 9. Serum transforming growth factor β_1 (TGF- β_1) concentration measured by ELISA at the baseline and after the diet intervention in raw diet fed atopic/borderline dogs ($n=13$). Difference was found statistically significant ($p=0.001$).

Before the diet intervention there was no statistically significant difference in serum TGF- β_1 concentration between the dogs that were assigned to the raw food group and the dogs that were assigned to the dry food group ($P=0.187$). After the diet intervention the serum concentration of TGF- β_1 was significantly ($P=0.015$) higher in the raw food group than in the dry food group (Fig. 10).

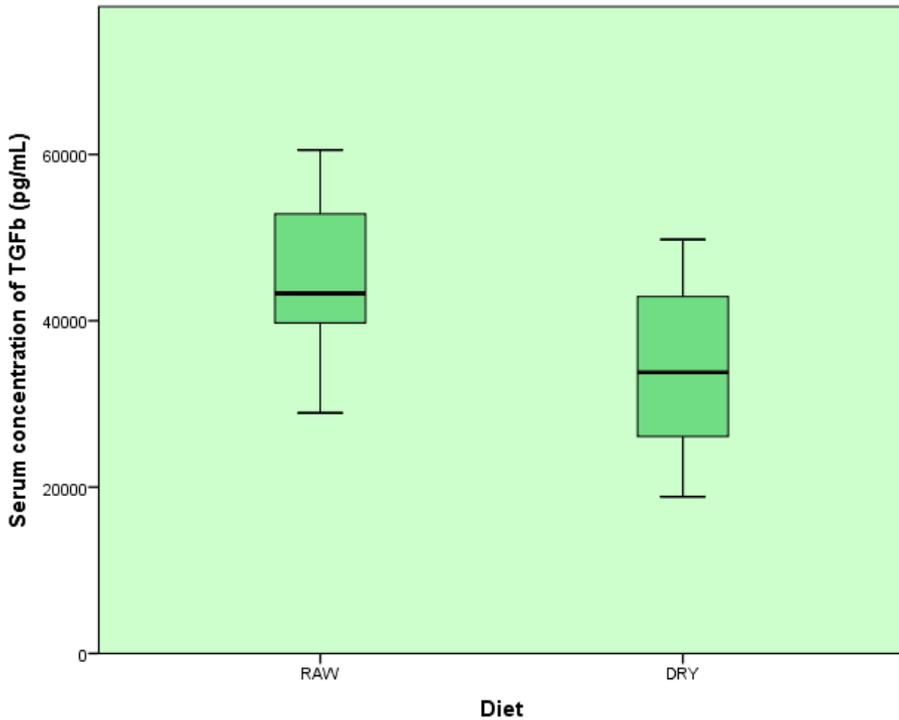


Figure 10. Serum transforming growth factor β_1 (TGF- β_1) concentration measured by ELISA after the diet intervention in dogs fed raw food ($n=13$) and in dogs fed dry food ($n=10$). Difference was found statistically significant ($p=0.015$).

Raw food can be expected to include bacteria, as they are not destroyed with heating, although the bacterial load was not measured in this study. TGF- β_1 controls Th1 cells with immunopathogenic properties and their response to environmental antigens, including non-pathogenic microbial flora (Gorelik and Flavell, 2001). This could be one explanation for the increased TGF- β_1 concentration in the raw diet group, as a reaction to the increased bacterial load, acquired through the diet. In previous studies probiotics were reported to have different effects on the serum TGF- β_1 levels in humans depending on the strain (Isolauri et al. 2000), and to increase the expression of TGF- β_1 in the gut of dogs with inflammatory bowel disease (Rossi et al., 2014). TGF- β_1 can also be obtained straight from the diet, for example colostrum and whey protein are known to be rich in TGF- β_1 (Satyaraj, 2011).

The alleviation of AD symptoms can be seen in the skin of atopic dogs when the expression of TGF- β 1 increases (Jee et al., 2013). Also, helminth infection stimulates local regulatory mechanisms, such as TGF- β production in the intestine (Wilson et al., 2005) and is known to protect from allergic diseases (Yazdanbakhsh et al., 2002; Maizels, 2005). Our findings on elevated TGF- β 1 concentration in the serum of raw diet fed dogs could therefore be considered beneficial for atopic dogs. This association still needs to be verified in further studies.

Considering the results from the skin gene expression and serum TGF- β 1 concentration, it could be argued that raw diet might have modulated the immune response and antioxidant activity positively. There have been several studies reporting positive effects of immune-modulating and antioxidant diets on canine Leishmaniosis (Cortese et al., 2015), canine keratoconjunctivitis sicca (Destefanis et al., 2016), cataract formation in dogs with diabetes mellitus (Williams et al., 2015), cognitive performance in old dogs (Fahnestock et al., 2012), and chronic otitis externa in dogs (Di Cerbo et al., 2016). As discussed at the end of chapter 4.5, the possible role of raw food in the enhancement of the immune system is interesting and more research in this area should be conducted.

There were some limitations in this thesis. The diets differed by their macronutrient contents, by their ingredients, and by their processing methods. Also, when we are talking about atopic dermatitis, which is known to be related to fatty acid metabolism, the different omega-6 / omega-3 ratios of the diets should be considered. This makes the interpretation of the results difficult regarding the factors influencing the changes seen in the gene expression and blood parameters. Some changes might have been due to the high carbohydrate content in the dry food or to the high fat content in the raw food, and some changes might have been due to the different food processing methods that were used in the making of the foods, or maybe due to the different bacterial load. The purpose of this experiment was to study the real-life situation, which is the reason why the commercial foods used by the Finnish dog owners were chosen. This is the reason why they differed also by

their content and macronutrient profile. We wanted to see, if in real life the dogs fed typical but different kind of diets, differ from their metabolism, blood values, and gene expression. This was found to be true, and the next step would be to find the reasons behind these findings. This would mean diets designed particularly for the study, and to differ only in certain aspects from each other. In addition, client-owned dogs living in their home environment were used instead of laboratory animals and laboratory environment. Also with this aspect, we wanted the results to mimic real life as well as possible and to give new ideas for future research. The same study done in laboratory animals using more similar diets would not have given us results that could have been applied to real life situation with pet dogs. In addition, the sample size was small in some studies in this thesis, like in the RNA-seq study (IV). It would be important to repeat the study design with more samples. Since the diet intervention study was conducted using client-owned dogs, it also opens the possibility for uncontrolled environmental factors affecting the results. Nevertheless, the diets were seen to affect to the body metabolism in different ways, and in different levels. This shows, that it is not insignificant what we feed to our pets, and the healthy and unhealthy impacts of different diets should be investigated. Study II highlighted some interesting factors associated with CAD, but it does not prove the causality and the results should be interpreted carefully. However, this thesis introduces interesting hypotheses for the future studies.

5 CONCLUSIONS

In this thesis, the multifactorial aetiology of canine atopic dermatitis was considered, as well as the factors associated with the disease. The environmental factors related to living environment, household conditions, and dog characteristics were found to be associated with atopic skin symptoms and CAD in the large epidemiological study. The role of hygiene and genetics together are likely to have an impact on dogs' health.

Two typical diets used in Finland, a raw and a dry kibble diet, had different effects on blood parameters. These findings are relevant, since the reference intervals in laboratories are based on mainly dry food fed dogs. They might not be optimal for the dog to thrive, since the dry food is highly processed and includes large amounts of carbohydrates, and dogs are still considered carnivores. Raw and dry diets also differently affected the B12, folate and iron concentrations in the blood as well as the concentration of TGF- β 1. All these results show, that the different kinds of diets change the metabolism and the function of the body in a different way, which might change the health outcome of the animal.

Several genes were found differentially expressed between atopic and healthy dogs. We presented findings supporting the previous studies in dogs and humans, but also novel candidate genes were found, possibly since this was the first study using RNA-seq. Also, the effect of the raw diet was seen in many immune-related genes. The study was novel and showed that the raw diet can influence the gene expression of the skin. This finding is relevant in the case of atopic dermatitis, where the function of the skin is disturbed in many ways.

The role of immunity stood out in many studies of this thesis, reflecting the already acknowledged role of a too sterile hygiene in allergic and autoimmune diseases. People and their pets live in the same clean environment and eat more processed and therefore sterile foods, lacking the important contact with microbes, particularly in their childhood and puppyhood.

6 FUTURE RESEARCH

Immune-modulating diets have been studied related to several diseases with promising results. It is of importance for future studies to find out if a raw diet per se could function as an immune-modulating diet or if there are certain ingredients in these kinds of diets changing the immune defence towards a healthier direction. The role of the relation between different macronutrients, and the effect of natural and synthetic vitamins and trace elements should also be investigated. Certain type of diet might be especially important in puppyhood, when the immune system is developing. If the diet of puppies should consist in its entirety of raw ingredients or if having raw food as a part of the puppy's diet might be enough to enhance the immune system, should be studied further. This might be one way to prevent atopic and allergic diseases. If a healthy diet can enhance the immunity, it should be clarified if there is only a certain 'time window' when it can be used for prevention of allergies and atopy, or if it works at any age, also later in life in the management of atopy. More research is needed to study if it might be beneficial to feed raw food to atopic dog to boost the immune system of the skin and gut to decrease the secondary infections and hypersensitivity.

Altogether, this thesis raises a novel and interesting aspect of the effect of dietary and environmental choices that the breeder and the dog owner of a dog suffering from canine atopic dermatitis could make. These factors, along with breeding selection, should be considered when working with prevention of this disease. At the same time, it would be crucial to identify if diet and environment can be modified for prevention in the same way in all dogs, and in all breeds. For those individuals that have a hereditary predisposition to allergic and atopic diseases the prevention methods might not work as well as for other individuals. In addition, since many drugs used in the treatment of atopic dermatitis are affecting to the gene expression, it should be studied more intensively if we could use the diet to generate the same changes in those genes without the side-effects of the drugs. Diet clearly has an effect to the gene

expression, which should also be considered in future studies evaluating the candidate genes for atopic dermatitis and other diseases. This probably has been underestimated in many previous studies.

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