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Assessing iron parameters in healthy non-atopic dogs and dogs with atopic dermatitis

Master Thesis

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Abstract Deutsch

In der Humanmedizin atopische Dermatitis ist mit einer gestörten Eisenhomöostase verbunden. Es ist bewiesen, dass Allergiker an einem funktionellen Eisenmangel leiden und dass sich ihre Eisenhomöostase von nicht-Atopikern unterscheidet. Ziel dieses Projektes war festzustellen, ob die Eisenparameter bei Hunden mit caniner atopischen Dermatitis (CAD) im Vergleich zu nicht allergischen, gesunden Hunden verzerrt sind. Für diese retrospektive Studie wurden Seren von 36 Hunden mit bestätigter atopischer Dermatitis unter immunsuppressive Therapie mit 102 gesunden, nicht-atopischen Hunden verglichen. Die untersuchten Parameter waren Gesamteisen, Gesamteisenbindungskapazität TIBC, UIBC; Hepcidin, Transferrin, Ceruloplasmin als Marker für die Mobilisierung zellulärer Eisenspeicher, Ferritin und hs-CRP. Allergische Hunde hatten eine signifikant höhere Eisenkonzentration im Serum als nicht allergische Hunde ($p = 0,012$), und dies korrelierte positiv mit Ceruloplasmin und negativ mit dem CRP, was auf einen funktionellen Eisenmangel hinweist. Im Gegensatz dazu waren in der Kohorte gesunder Hunde die Werte für UIBC, Hepcidin und Ceruloplasmin signifikant (alle $p < 0,001$) höher und das Serumeisen war negativ mit Hepcidin korreliert. Die Eisenhomöostase unterschied sich zwischen gesunden und atopischen Hunden. Bei CAD-Patienten, aber nicht gesunden Hunden, ist das Serumeisen von Entzündungsmarkern wie CRP und Ceruloplasmin abhängig, was auf eine Beteiligung von Eisen an der Ätiologie hindeutet.

Abstract English

In human medicine, atopic dermatitis is associated with impaired iron homeostasis. It has been proven that atopic patients suffer from a functional iron deficiency and that their iron homeostasis differs from non-atopic patients. The aim of this project was to determine whether iron parameters differ in dogs with canine atopic dermatitis (CAD) compared to non-allergic, healthy dogs. For this retrospective study, sera from 36 dogs with confirmed atopic dermatitis receiving immunosuppressive therapy were compared to sera from 102 healthy, non-atopic dogs. The parameters examined were total iron, total iron binding capacity TIBC, UIBC; hepcidin, transferrin, ceruloplasmin as a marker for the mobilization of cellular iron stores, ferritin and hs-CRP. Allergic dogs had a significantly higher serum iron concentration than non-allergic dogs ($p=0.012$) and this correlated positively with ceruloplasmin and negatively with CRP, indicating functional iron deficiency. In contrast, in the healthy dog cohort, levels of UIBC, hepcidin, and ceruloplasmin were significantly (all $p<0.001$) higher and serum iron was negatively correlated with hepcidin. Iron homeostasis differed between healthy and atopic dogs. In CAD patients, but not healthy dogs, serum iron is dependent on inflammatory markers such as CRP and ceruloplasmin, suggesting iron involvement in the etiology.

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1. Introduction

1.1 Iron Metabolism and Iron Homeostasis

Iron is an essential nutrient utilized in almost every aspect of normal cell function. All cells require iron to proliferate, iron being essential for DNA biosynthesis, protein function and cell cycle progression. In humans, iron is critical for a wide variety of biological processes as it allows transportation of oxygen, aids in the energy household and is essential for a healthy immune system [1]. Iron metabolism is similar among mammalian species and for this reason, studies of rodent iron metabolism have contributed substantially to the understanding of human iron metabolism.

The iron consumed in the diet is absorbed by duodenal enterocytes lining the absorptive villi close to the gastroduodenal junction, and the absorption is facilitated by the low pH in gastric efflux, which reduces dietary iron and delivers it in a proton-rich milieu. The luminal brush border of the enterocyte contains an enzymatic ferric reductase activity which ensure that iron is available in its ferrous state [2]. The ferrous iron then traverses the two cellular membranes of the enterocyte in order to gain access to the circulation, however not all iron taken up by the absorptive enterocyte is transferred across the basolateral membrane to reach the plasma - a fraction of it is retained within the cell and incorporated into ferritin [3].

Because iron cannot be excreted from the organism in a regulated way, iron absorption represents the critically controlled process. Normally, only 1–2 mg of iron per day are absorbed to compensate for iron losses, for example by sloughing of intestinal epithelial cells, desquamation of skin and urinary cells, blood loss, or sweat. Iron absorption can be enhanced when the needs are higher (for example, because of increased erythropoiesis or pregnancy) and suppressed in iron overload. The lack of an active mechanism for iron excretion accounts for the development of iron overload when the regulation of iron absorption is defective or bypassed (as occurs in blood transfusions) [4]. Therefore, balance is maintained by the tight control of dietary-iron absorption in the duodenum, which is a process that is regulated in at least three ways.

Given that less than 10% of the daily iron needs are met by intestinal absorption, the rest is covered by macrophages that recycle iron internally. The amount of plasma iron is just over

10% of the amount used daily, which means that plasma iron is turned over many times each day. Macrophages phagocytose aged or damaged erythrocytes and catabolize heme using hemoxygenase.

The adult human body normally contains 3 to 5 g of iron (about 55 mg and 44 mg per kilogram of body weight for males and females respectively), with more than two thirds of it (>2 g) incorporated in the haemoglobin of developing erythroid precursors and mature red blood cells [5–7]. Most of the remaining body iron is found in a transit pool in reticuloendothelial macrophages (~600 mg) or stored in hepatocytes (~1000 mg) within ferritin, an iron storage protein. The liver is, therefore, the major depot for iron storage, and the hepatocytes are capable of iron uptake, storage, and export.

A smaller fraction of the body iron is present in muscles within myoglobin (~300 mg), while only a minuscule amount (~8 mg) is constituent of other cellular iron containing proteins and enzymes. Iron bound to plasma transferrin corresponds to less than 0.1% of total body iron (~3 mg) but represents in kinetic terms the most active pool [8].

Most of all circulating iron in plasma is bound to transferrin, a protein that is capable of binding carbonylated iron tightly but reversibly [9, 10]. Iron chelation by transferrin serves three main purposes: i) it maintains Fe3+ in a soluble form under physiologic conditions, ii) it facilitates regulated iron transport and cellular uptake, and iii) it maintains Fe3+ in a redox-inert state, preventing the generation of toxic free radicals. Transferrin has an indirect defensive role against systemic infections by depriving the potential pathogens of extracellular iron, which is essential for their growth [8, 11].

Iron homeostasis is complex, and mammals have evolved homeostatic circuits and specialized molecules to ensure safe and balanced iron acquisition, transfer and storage. There are multiple proteins involved in iron transport and in regulation of iron metabolism, however the most commonly used for routinary evaluation of iron status will be given special attention in the next part of this introduction.

1.2 Iron related parameters

1.2.1 Transferrin

Transferrin is a glycoprotein found in vertebrates which binds to and consequently mediates the transport of iron (Fe) through blood plasma. As mentioned above, transferrin is capable of binding iron in the presence of bicarbonate, and to which most circulating iron is bound to.

Theoretically, under constant rates of transferrin production and catabolism, the saturation of τransferrin with iron is influenced by: i) the amount of iron absorbed from the diet, ii) the amount of iron recycled and released by reticuloendothelial macrophages, and iii) the amount of iron utilized by the bone marrow and other tissues and also iv) by the pH as iron is bound via a bicarbonate-moiety [12–14]. Transferrin saturation reflect the iron supply to tissues, and thus reflects the balance between reticuloendothelial iron release and bone marrow uptake.[8] Under normal conditions, approximately 30% of the transferrin-iron-binding sites are saturated. The relatively low transferrin saturation in conjunction with its high affinity for iron, allow transferrin to efficiently buffer alterations of plasma iron levels and capture unshielded iron, minimizing the risk of toxicity. In humans, values of transferrin saturation under 15% indicate iron deficiency, whereas over 45% are consistent with iron overload [4]. In disorders of severe iron overload (hemochromatosis), transferrin saturation exceeds 60%. Under these conditions, the levels of redox-active non-transferrin bound iron (NTBI) increase dramatically (up to 10–15 μM or higher), and excess iron accumulates in tissue parenchymal cells and leads to organ damage [15, 16]. The liver is the most prominent site of NTBI deposition, although considerable amounts of NTBI accumulate in further tissues, such as heart, adrenal glands and pancreas.

1.2.2 Hepcidin

Tight and accurate regulation of iron absorption in mammals is critical to prevent systemic excess or deficiency. This complex task has hepcidin as its main regulatory molecule.
Hepcidin is a liver-derived peptide hormone of 25 amino acids that responds to multiple regulatory cues that include iron availability, erythropoietic activity, anemia, inflammatory signals and hypoxia [4, 17], [18, 19]. It is generated primarily in hepatocytes, and at much lower

levels in other cell types, upon cleavage of a larger 84 amino acid pro-peptide by the pro-hormone convertase furin [20].

Mature hepcidin is secreted to plasma and circulates bound to α2-macroglobulin [21]. Hepcidin levels increase in response to iron [22] or inflammation [23], the latter considered as a mechanism of innate immunity to deprive iron from rapidly growing invading pathogens. Hepcidin exerts its biological action by binding to ferroportin and promoting its phosphorylation, internalization and lysosomal degradation [17]. Since ferroportin is the one of the major cellular iron exporters in mammals [24], its expression in enterocytes and macrophages determines the degree of intestinal iron absorption and reticuloendothelial iron release. Therefore, hepcidin's physiological role is to act as a negative regulator of iron absorption and release.

Hepcidin levels drop with iron deficiency, hypoxia and increased erythropoietic demand for iron [25]. Hepatocytes integrate diverse signals deriving from these stimuli, which result in transcriptional activation of hepcidin by yet partially characterized mechanisms. However, as an acute phase peptide, it is upregulated during inflammation [26], thereby promoting cellular iron retention, to remove iron from the circulation. Due to its dual role in iron regulation and inflammation, hepcidin levels in the circulation reflect on the one hand ongoing inflammation as well as the need of iron; consequently, in conditions of severe anemia and inflammation, low hepcidin levels will prevail despite the presence of inflammation [27].

1.2.3 Ceruloplasmin

Ceruloplasmin is the major copper-carrying protein in the blood, and plays a significant role in iron metabolism. It is a blue-colored plasma protein that binds up to 95% of circulating copper. The proposed physiological functions of ceruloplasmin include copper transport, oxidation of organic amines, ferroxidase activity, regulation of cellular iron levels, glutathione peroxidase and ascorbate oxidase activities and an antioxidant activity. Furthermore, it has also been reported that ceruloplasmin may scavenge reactive oxygen species such as singlet ($1O_2$), superoxide (O) and hydroxyl radicals (OH). However, it has also been shown to have prooxidant activities, which involve a distinct active site from the antioxidant sites [28].

Individuals who carry a defective Cp gene suffer of aceruloplasminemia, a rare late-onset autosomal disease characterized by partial or total Cp protein deficiency. They have normal copper homeostasis but present a severely impaired iron metabolism [29, 30]. Homozygotes have iron overload mainly in the brain, but also in liver, pancreas and retina and develop retinal degeneration, diabetes mellitus and neurological symptoms, which include ataxia, involuntary movements and dementia. Beside absent serum Cp ferroxidase activity, the signatures of the disease include low transferrin saturation, high serum ferritin and moderate anaemia.

1.2.4 Ferritin

Ferritin is the major iron storage protein in the mammals[31]. Quantitative information about the distribution of ferritin is scarce, although it is found in all tissue. Measurements of non-heme iron concentrations indicate that ferritin can be found in high amounts in the bone marrow, spleen and liver. Each ferritin molecule consists of a spherical protein shell of molecular weight about 450,000 made up of 24 subunits with a variable amount of iron as a crystalline core of ferric-oxide-phosphate[31, 32]. It is composed by two types of subunits: H and L. The letter H originated from original isolation of isoforms of ferritin in human subunit-H rich cardiac tissue, while L refers to ferritin isolated from liver tissue. Genes on the chromosomes 11q and 19q are responsible for encoding the two subunits[33]. The synthesis of ferritin is generally taking place in most tissues, and it is induced by iron, although some mainly functional but also structural differences are reported between ferritin of different tissue origin[34]. Classically, ferritin has been considered as a mainly intracellular-cytosolic protein, that can be identified by a characteristic blue colour after staining with Prussian Blue in bone marrow histological slides. Recently, a mitochondrial form of ferritin was also described[35, 36]. Detection of ferritin in the serum was considered pathological and was associated to extensive liver damage and hepatocellular necrosis. However, ferritin was later proven to be a physiologically common protein in serum with the use of newer radioimmunoassay kits sensitive for serum ferritin[37]. Both in vitro and later in vivo studies have indicated that ferritin can inhibit lymphocyte function[38] and immunity. A study in mice showed that ferritin H can significantly reduce the proliferation rate and the number of granulocyte-macrophage, erythroid and multipotential precursor cells in the bone marrow[39, 40].

Serum ferritin is also perceived as a nonspecific marker for both acute and chronic inflammation. Studies have demonstrated high ferritin concentration in patients with kidney disease, various autoimmune disorders and malignant neoplasia[41–44]. Ferritin is usually good marker for assessing overall body iron stores. However, during inflammation secreted ferritin does not contain iron [45], Consequently, assessment of serum ferritin solely may mask an underlying iron deficiency [46, 47]. Even if this elevation of ferritin levels would mean increased total body iron storage and concentrations in the blood, sequestration of iron is occurring and therefore this reduced iron availability for haematopoiesis, leads to the well described anemia of inflammation[48]. Therefore, ferritin is a valuable diagnostic tool for assessing conditions like iron-deficiency anemia without inflammation, hereditary and acquired iron-overload (hemochromatosis) and other [49].

1.2.5 High sensitivity C-Reactive Protein

C-reactive protein (CRP) is an acute phase protein that is produced in the liver. Its concentration in healthy people is quite low but marked increases occur rapidly in response to acute inflammation. Concentrations also decrease rapidly with resolution of inflammation, thus monitoring serial changes over time can be helpful with documenting resolution or worsening of inflammation. Through binding to the cell wall, CRP activates the classical pathway of complement (C1q), leading to bacterial opsonization, which promotes clearance by phagocytes. It also binds to other ligands, including phosphatidylcholine and membrane lipids and DNA in damaged cells.

Anaemia of inflammation is a highly prevalent syndrome associated with systemic inflammation, and in its common form it is readily diagnosed as a normocytic, normochromic anaemia associated with low transferrin saturation but high ferritin levels. Inflammation is correlated with iron status, and it is not uncommon that patients who present such disease also present iron deficiency anaemia (IDA).

A high sensitivity C-reactive protein (hs-CRP) test is more sensitive than a standard CRP test, being able to detect slight increases within the normal range of standard CRP levels. The hs-CRP test is different from the routinely used CRP test regarding methodology applied, interpretation of the results and purpose of testing, as in humans it is currently being used for

example as a marker of future risk to cardiac disease [50], and even prognostic marker for cancer [51] .

Novel biomarkers of iron metabolism, such as hs-CRP might be useful to distinguish IDA connected with inflammation and absolute iron deficiency [52]. Inflammatory stimuli induce macrophage production of interleukin-6 (IL-6), which stimulates hepatocytes to produce acute-phase proteins including C-reactive protein (CRP) [53, 54] and hepcidin [55]. This inflammation state may lead to extended hypoferremia and cause anemia. [56] A study from Askar et al. found that inflammatory changes occur in elderly women with IDA. Besides pro-inflammatory cytokine levels (CRP, IL-6, TNF-α), antimicrobial protein levels (hepcidin, chemerin, defensin) were found higher in elderly women with IDA because of inflammatory changes [57]. Finally, no significant interaction between iron deficiency and anemia was found in patients who present IDA and increased hs-CRP, suggesting that the results are independent of the anemic status [58], and that hs-CRP can be a good marker for functional iron deficiency.

1.3 Iron Deficiency

Because most of the body's iron is contained in erythrocytes, recovery of iron from senescent erythrocytes is essential. To put it in perspective, it is important that only 1–2 mg of iron normally enters the body each day through the intestine, and this is balanced by iron output from shed cells and lost blood. Even under extreme circumstances, this absorption cannot be increased above 6 mg/day. Meanwhile, the erythron has a daily requirement for 20 mg of iron [59]. Nearly all iron for erythropoiesis is supplied through macrophage recycling, which involves ingestion of senescent erythrocytes and catabolism of their haemoglobin to scavenge iron for reuse. Although macrophages of the liver and spleen perform this function, the pathway iron takes within the phagocytic cell and the mechanism for loading recovered iron onto circulating apo-transferrin are still not fully determined. Animals and people lacking heme oxygenase 1 cannot degrade haemoglobin normally [60, 61]. Patients with anaemia of chronic inflammation (anaemia of chronic disease) also have defective recycling of iron by reticuloendothelial macrophages, with this condition resulting from a change in macrophage iron handling in response to cytokine signals [62, 63]. Iron builds up in macrophages in

anaemia of chronic inflammation and is less available to erythroid precursors, becoming also less available to pathogenic microorganisms, which almost invariably need iron for their proliferation. In this way, iron withholding may be an adaptive and advantageous response; mild anaemia is a relatively low price to pay for avoiding pathogen infestation.

Iron-homeostasis is quite complex, so that to date there is no international consensus that clearly defines iron deficiency even in humans. According to the clinical definition for humans, iron deficiency is an insufficient number of red blood cells, while the WHO, UNICEF and UNU determine absolute iron deficiency on the basis of certain threshold values that depend on age, sex, life-style factors (e.g. smoking) and the altitude a subject is living [1].

However, iron deficiency can also be "functional" and occurs in conditions in which iron is hardly “mobilized" from ferritin stores within the reticulocytes and as a result the functions of cells and tissues is impaired. Only in severe cases does this result in anemia, which is only an extreme example of iron deficiency. In mild to moderate cases of iron deficiency, anemia is not present, although the functions of tissues and cells are already impaired. Iron deficiency can thus be absolute or functional, and mixed forms may also exist. Besides blood loss, a chronic lack of dietary iron and/or a hampered dietary absorption usually as a result of immune activation are the main drivers of iron-deficiency [64], [65]. As the major contributor for systematic iron recycling, shuttling and distribution are immune cells/reticulocytes/macrophages, their iron handling features determine their inflammatory or anti-inflammatory status.

IDA without inflammation is an isolated hypo regenerative microcytic hypochromic anemia, in which reduced serum ferritin concentration and transferrin saturation are indicators for depleted iron stores, respectively [66–69]. With inflammation a functional iron-deficiency is established, in which further dietary iron absorption is hindered by hepcidin, resulting that those subjects with functional-iron deficiency (and inflammation) are in the vicious cycle, in which they need more iron, but must exploit different nutritional approaches to compensate their iron requirements, as otherwise their immune systems remain hyper-active.

Importantly, human patients with functional iron deficiency suffer from underlying chronic or metabolic diseases such as autoimmune[70], atopic diseases [71], [72, 73][74],[75], chronic

kidney diseases [76–78], congestive heart failure [79–81], chronic pulmonary diseases [82–84], and obesity [85, 86], in which the presence of iron deficiency is associated with a worsened prognosis and outcome [87–89].

1.4 Iron assessment in dogs

Similar as in humans, also in dogs there is no single standardized test that can diagnose iron deficiency without anemia, and even the use of multiple tests can only partially overcome the limitations of individual tests, especially because many iron markers are elevated during inflammatory responses or mild immune activation.

IDA is characterized as microcytic and hypochromic, and generally is regarded as hypo proliferative or non-regenerative. Because of an ample storage pool and strict conservation of iron by the body, iron deficiency in adult animals usually is caused by chronic blood loss rather than by inadequate dietary iron alone. Nursing animals and young, rapidly growing animals develop iron deficiency more easily than adults on an iron-replete diet: iron stores in young animals tend to be marginal, milk is low in iron, and rapid growth demands expansion of blood volume.

Diagnosis of uncomplicated and chronic iron deficiency is based on classic hematologic and biochemical abnormalities, ie, microcytic, hypochromic anemia and decreased serum ferritin concentration [90–92]. Also, a study conducted retrospectively with 833 dogs found that low reticulocyte haemoglobin content and low reticulocyte volume are associated with hematologic and serum biochemical abnormalities indicative of iron deficiency, which is suggested to be a noninvasive, cost-effective mean of assessing iron status in the dog [93].

However, detection of early iron deficiency, before the appearance of these abnormalities, or iron deficiency in the presence of other diseases (eg, neoplasia and inflammation) is a diagnostic challenge. Because iron is preferentially shunted to hemoglobin formation, typical hematologic changes do not occur until late in iron deficiency[92]. Also, as it was already mentioned

previously, serum ferritin is an acute-phase reactant in mammals and concentrations may be increased with certain neoplastic and inflammatory diseases.

Many researchers consider the evaluation of bone marrow aspirates or biopsies for the presence or absence of stainable iron as a sensitive and reliable test for assessing iron status in people, however scoring systems for marrow iron are not validated in dogs. A pilot study made with 12 dogs was able to partially consider the available staining and scoring methods acceptable [94], but additional studies are still needed to determine if such scoring methods could aid in distinguishing causes of iron restricted erythropoiesis. Also, it is relevant to consider that such procedures are invasive and painful, sometimes requiring general anesthesia.

Regarding evaluation of iron related blood parameters, ferritin is considered a better indicator of total body iron stores in animals than total iron itself. Serum ferritin concentrations are quite stable from day-to-day, in contrast to iron, and the measurement of ferritin can be used to confirm cases of iron deficiency or iron overload. Nevertheless, testing is not frequently performed in dogs and the test is not offered by most veterinary diagnostic laboratories.
The only known cause for a low serum ferritin result is a decrease in the amount of stored iron. However, in dogs the serum ferritin values do not appear to correlate very well with non-heme iron stores in the liver. Indeed, the correlation coefficients between non-heme iron and ferritin are <0.4 [95], indicating that other factors account for more than 60% or more of the variation in ferritin results. Nevertheless, not many studies have investigated ferritin levels in healthy dogs and therefore it the correlation between ferritin and iron deficiency is still not well stablished in dogs.

1.4.1 Transferrin in dogs

In veterinary medicine, percentage saturation of transferrin (Tf) is infrequently measured directly. Instead, it is done through a simple calculation based on the iron and total iron-binding capacity TIBC (*% saturation of Tf = [iron ÷ TIBC] x 100).* In health, roughly 33% of the circulating iron binding sites are occupied, although the actual range (or reference interval) is quite broader than this and varies with species. Since the percent saturation is dependent on iron and TIBC, the results for all three tests should be interpreted together. In dogs, a

transferrin saturation of 15% or below would be compatible with IDA and values between 15 and 20% would be suggestive of the same. Iron values alone would not be helpful for distinguishing IDA from anemia of inflammatory disease in dogs. Total iron binding capacity values are reported to be increased in man [96] and dogs [90, 97] with IDA.

Direct measurement of transferrin is not usually made for dogs, nevertheless there is a study by Nakajima et al. using a commercial ELISA [98], and the reference value considered for the plasma transferrin concentration was 180 mg/dl according to the lower limit of the reference range for healthy dogs that was provided by another study, which unfortunately is only available in Japanese [99]. The dogs used for Nakajima´s study were however malnourished dogs, not constituting a healthy group as we intend to do in our study.

1.4.2 Ferritin in dogs

Ferritin is an acute phase protein and values increase in response to inflammation. This is one of the causes of iron "sequestration" that occurs in animals with chronic or inflammatory disease and will reduce serum iron values.

Sensitive methods are needed to measure ferritin, since serum levels are very low (in nanogram quantities). Immunologic assays requiring species-specific reagents, such as radioimmunoassay and a sandwich ELISA, have been employed for animals, with canine and feline-specific ferritin assays are available through Kansas State University and some commercial laboratories, however ferritin is not regularly tested unless for scientific purposes.

In veterinary medicine, it has been reported that canine serum ferritin levels increase in histiocytic sarcoma and lymphoma [100] [101]. In addition, serum ferritin concentration is reported to be a useful serological marker of histiocytic sarcoma and Immune Mediated Hemolytic Anemia in dogs [101].

A paper from Kazmierzki et al. [100] has a considerably large control group of healthy dogs, consisting in 50 healthy subjects, obtaining a mean ferritin concentration of 805.8µg/L +/- 291.1µg/L, P <.0001. Aside from this, data is particularly limited for healthy dogs.

1.4.3 Ceruloplasmin in dogs

Ceruloplasmin is an alpha2-glycoprotein and one of the positive acute phase proteins in dogs [102]. Many quantitative methods based on different principles have been used for Cp measurement in plasma or serum [103], and assays based on oxidation of different compounds such asp-phenylenediamine (PPD) or its N-dimethyl derivative ando-dianisidine dihydrochloride have been used most often in veterinary medicine [104]. There are no commercially available reference materials to standardize ceruloplasmin concentrations, so different arbitrary units based on the increase of absorbance per unit of time have been used and expressed as oxidase units or UI/L. In dogs, serum ceruloplasmin levels increase during infection, inflammation or trauma [105]. Measurement of this protein provides valuable information on the inflammatory status to clinicians in canine practice, however ceruloplasmin is not commonly tested in healthy dogs. A colorimetric method to determine ceruloplasmin oxidase activity in cats and dogs was validated based on the method of Sunderman et al. [106] using p-phenylenediamine as the substrate [107]. In order to establish normal ranges, plasma samples were obtained from 102 healthy dogs (mixed breed, age and sex) and 54 healthy domestic short-haired cats (mixed age and sex). The mean plasma ceruloplasmin concentrations determined for dogs was 9.28 IU/L (SD 3.03 IU/L), and using these values, normal ranges for dogs were stablished between 3.22 IU/L and 15.35 IU/L. This normal range determined for dogs is consistent with that previously reported, however in this previous study there were only 15 healthy dogs among the entire sample size [108].

1.4.4 Hepcidin in dogs

The canine hepcidin gene was only sequenced in 2004 [109], and the gene expression was studied in dogs with experimentally induced iron deficiency [110] and portosystemic shunts [111]. Nevertheless, data regarding serum hepcidin measurements in dogs is limited, and hepcidin it is not routinely tested in veterinary medicine.

A study from Vizi et al [112] measured hepcidin for 86 healthy dogs of 25 different breeds using liquid chromatography/tandem mass spectrophotometry in order to determine reference values. Synthetic canine hepcidin was used as the standard reagent, and rreference values were

calculated based on the results. The mean hepcidin concentration of the study population was 16.6 ± 7.7 ng/mL, and the reference interval (RI) was defined as 5.3-36.4 ng/mL. There was no significant difference found between male and female dogs, or between different age and body weight groups. As this test was performed using a different method than the one we perform, our data aims to provide confirmation on the reference values and validity of a different method of measurement for dogs.

1.4.5 Hs-CRP in dogs

In order to investigate minor changes in CRP concentrations in the low range in dogs, routine CRP tests are not suitable, as these tests are developed for assessing moderately to markedly increased CRP concentrations in ill dogs, and they are neither sufficiently accurate nor precise at low CRP concentrations. Hs-CRP tests able to measure concentrations in a low range have been performed for dogs in a research setting – for example, it has been investigated if obesity in dogs is associated with increased CRP concentrations [113] and the association between cardiac disease and CRP [114]. However, hs-CRP is nor routinely performed in veterinary medicine, and commercial ELISA kits are not broadly available. Involving healthy animals, one study was performed with 40 patients in order to validate a canine-specific automated hs-CRP protein assay [115], and another tested a commercially available ELISA for determination of CRP in dogs. This last study was made with 46 subjects that were assigned to five groups, i.e. clinically healthy dogs, dogs with neoplastic diseases, dogs with infection, dogs with endocrine/metabolic disorders and dogs with miscellaneous diseases not belonging to any of the former groups, and the values were found to be (1.1–6.3µg/ml). Other previous study had found the range for normal canine sera to be 0.8–22.6µg/ml [116]. To the best of our knowledge, it has not been yet investigated CRP levels in canine patients with Canine Atopic Dermatitis.

1.5 Iron deficiency and allergy in people and dogs

It is still unclear why the defense system of atopic subjects reacts so excessively to environmental triggers that their risk of developing allergies is increased. Female gender, low microbial exposure and the molecular properties of allergens themselves are well-established

risk factors for patients with allergies [1]. In contrast, growing up on a farm [117] with many siblings [118], and pets [119] protects against atopic diseases. Also nutritional deficiencies, particularly of micronutrients such as vitamin D [120], β-carotenoids—which are precursors of vitamin A— [120] as well as deficiencies of iron and folate are associated with atopy [121–125] In humans, a poor iron status at birth is associated with an increased risk of developing allergic diseases [121, 125, 126] with several epidemiological studies showing that allergic subjects are more likely to be anemic than non-allergic individuals [122, 127].

Allergy can only develop when concurrent to allergen exposure (1) a Th2 dominance prevails, which is characterized by the release of Th2-associated mediators such as interleukin-4 and (2) the antibody-producing B cells perform a class switch towards immunoglobulin E (IgE)-antibody production. From cell culture studies it is known that under iron deficient conditions, especially Th2 cells rather than other T-cells survive under these harsh conditions and thus their survival is favored [128–131]. Importantly, the preclinical studies have been replicated in a clinical study that examined blood cells from healthy children with or without iron deficiency. In that study, interleukin-4 was significantly elevated in healthy, iron-deficient compared to iron-repleted children [132]. Consequently, iron deficiency per se favors Th2 dominance and thus the first prerequisite for allergic sensitization. Iron deficiency also affects the cells which are mainly responsible for allergic reactions, the mast cells. Intradermal application of the iron binder, deferoxamine, induces local iron deficiency and results in histamine release and wheal formation [133].

Importantly in the preclinical setting, improving the iron status with oral as well as systemic iron supplementation suppressed airway manifestations in a murine model of allergic asthma in vivo [134] and also in the clinical setting supplementing expecting mothers with iron and folic acid decreases the risk of their offspring for atopic dermatitis by 80% [135], with also a double-blind, placebo-controlled showing that nourishing selective immune cells with micronutrients significant reduced the symptom burden of allergic sufferers in a completely antigen-unspecific manner. [136][141]

1.6 Canine Atopic Dermatitis

Canine Atopic Dermatitis (CAD) is defined as a genetically predisposed inflammatory and pruritic allergic skin syndrome with characteristic clinical features. The typical age of onset is reported to be between 6 months and 3 years, and a typical dog with CAD will exhibit pruritus of the face, ears, paws, extremities, and/or ventrum. The consensus appears to be that some dogs with CAD have no visible primary lesions, even in pruritic areas, and that primary lesions of CAD (when present) would consist primarily of erythema [137]. CAD is associated most with IgE antibodies to environmental allergens, and its diagnosis is based on history, clinical signs, and exclusion of other pruritic diseases [138].

Treatment option to CAD depends on the severity of the lesions and on the degree of pruritus, as well as on the level of cooperation of the dog and the owner, bearing in mind that the therapy will need to be reassessed and adapted regularly [139].

Apart from avoidance of the causative allergen [140], in general there are two different treatment approaches: specific allergen immunotherapy or symptomatic with a variety of drugs ranging from immune suppressive to antihistamines. The combination of various drugs can increase the chance of remission [139]. In severely affected dogs, glucocorticoids, ciclosporin, oclacitinib or lokivetmab are used for symptomatic therapy due to their clinical efficacy and high success rates of 70–80% [141].

Glucocorticoids act by binding to cytoplasmic glucocorticoid receptors, then translocate to the nucleus, where they influence gene expression [137]. Systemic administration of glucocorticoids leads to decreased numbers of circulating inflammatory cells as well as reduced production of inflammatory mediators, which allows effective control of cutaneous inflammation and pruritus [137]. Adverse effects of corticosteroid therapy occur most often when daily, high dosage and long-term administration of glucocorticoids is made, therefore their main indication is for control of acute Atopic Dermatitis, for episodic flares of clinical signs, for seasonal CAD and as short-term palliative treatment [139]. It is often, however, that systemic glucocorticoids are used on a long-term basis, when alternative treatments are not effective or when there are financial constraints on treatment options.

As for ciclosporin, it is a calcineurin inhibitor that binds to cyclophilin in the lymphocyte cytoplasm, inhibiting translocation of the nuclear factor of activated T cells (NF-AT) to the nucleus, thereby resulting in down-regulation of synthesis of numerous cytokines, including interleukin (IL)-2 and interferon (IFN)-γ [142–144]. Compared with glucocorticoids, ciclosporin is similarly effective, shows less frequent and less severe adverse effects, but has a slower onset of action (usually 2–3 weeks) [145], which makes it unsuitable for the treatment of acute episodes of clinical signs, but it is one of the drugs recommended for long-term symptomatic treatment of canine AD.

Oclacitinib is a novel Janus kinase inhibitor, widely used for the treatment of CAD. It demonstrates a rapid onset of action and has the advantage of acting against JAK1-dependent cytokines and only minimally against JAK2-dependent cytokines, thus not interfering with hematopoietic process in normal dosages [146]. They are involved in the signaling of the pruritus inducing interleukin (IL)-31, which oclacitinib effectively inhibits, resulting to reduced severity of pruritus [147]. The activity of oclacitinib is not restricted to antipruritic effects. It is a non-selective JAK inhibitor, which through inhibition of the JAK/STAT pathway exhibits anti-inflammatory properties, as determined by its ability to inhibit the function of pro-inflammatory and pro-allergic cytokines, such as IL-2, IL-4, IL-6 and IL-13 [146] and can in higher dosages also inhibit the hematopoietic process (erythropoietin, thrombopoetin).

Finally, Lokivetmab is an injectable anti-canine-IL-31 monoclonal antibody to treat clinical manifestations of CAD. The neutralization of IL-31 has both an antipruritic and anti-inflammatory effect in CAD, as it is a disease associated with IL-31 dysregulation [148].

Treatments are usually lifelong and involve significant side effects ranging from vomitus and diarrhea to immunosuppression. The clinical, immunological, histological, and pathological features in dogs are similar to what is observed in humans, so that canine atopic dermatitis has been suggested as an animal model for human atopic dermatitis [149, 150].

When comparing allergy for people and for animals, both harbor IgE and a similar IgE receptor repertoire and expression pattern. The same cell types are also involved in the triggering or regulation of allergies, such as mast cells, eosinophils, or T-regulatory cells. CAD in particular,

has a remarkable similarity to human atopic dermatitis, to the extent that animals could be used as models to improve our understanding of the pathogenesis of human atopic dermatitis [151].

Given the high degree of similarities, we expect that iron parameters in dogs with atopic dermatitis will significantly differ from iron-parameters of non-atopic dogs. However, as most iron parameter in dogs are poorly defined and usually not assessed, we will also establish reference values for iron parameter, which will help us also to gain greater insights in the mechanistic and etiology of canine atopic dermatitis.

1.7 Hypothesis

As mentioned above iron deficiency can be either absolute, leading eventually to anemia, or functional. In functional iron deficiency, iron reserves are present, however they are unable to be mobilized and used for cellular functions [1].

Studies in human medicine have shown that the concentration, metabolic behavior and bioavailability of iron and the other iron related parameters are considerably different in patients with allergic disease [1, 74]. It has been shown in people that the selective delivery of iron to immune cells can be achieved with whey proteins such as beta-lactoglobulin. Beta-lactoglobulin acts here as a carrier of micronutrients such as iron complexes with the result that providing iron to immune cells ameliorated their clinical symptoms [74].

The status of iron and iron related parameters are not yet evaluated in dogs with atopic dermatitis. Therefore, our goal is to assess the concentration of iron and iron related parameters in healthy dogs and in dogs that present Canine Atopic Dermatitis, and we hypothesize that allergic dogs have different iron status when compared to healthy controls.

2. Materials and Methods

2.1 Animals and Sample Collection

2.1.1 Group 1 – Healthy Animals-Control

A total of 102 plasma samples from adult client-owned healthy canines were used for this study. Data and blood samples originated from a behavioral study from the Interuniversity Messerli Institute of Research, that took place at the same time. In that project, all methods were carried out in according to Austrian guidelines and regulations, and approval was given by the Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry of Science and Research (Ref: BMBWF 20221-0.210.26). Heparin-Plasma samples were centrifuged at 3000rpm for 3 minutes directly after collection and were stored frozen at -20ºC until analysis took place. Any hemolyzed or hyperlipidemic specimens were not used for this study, as hemolysis and hyperlipidemia could influence measurements.

Candidates were defined as healthy after a diagnostic evaluation that included thorough history, physical exam, complete blood count (CBC), and plasma biochemistry analysis. Other inclusion criteria were age above one year old and weight above 5kg. Any dogs that had received treatment with glucocorticoids or other immunosuppressive drugs, antibiotics or medication used for treating atopic dermatitis within the last 4 weeks were not selected for the study. Furthermore, dogs with any chronic disease or acute onset of gastrointestinal or any other clinical symptoms within the last 4 weeks were excluded. Dogs in the control group at the time of blood collection were fed various commercial food, either dry or canned, as diet was not standardized. However, dogs with known food allergy or food responsive enteropathy were also not included in the control group. All blood samples were collected between 7:00 and 12:00, and all animals were fasting for at least 8 hours prior to the blood collection.

2.1.2 Group 2 – Allergic Dogs

For the allergic group 36 blood serum samples were used. All samples were collected in the Dermatology Clinic of the University of Veterinary Medicine Vienna, and all animals had a confirmed diagnosis of Canine Atopic Dermatitis. The diagnose was given in compliance to current guidelines [138], which means that all animals met the clinical criteria and all other

possible causes with similar clinical signs were ruled out. Flea combing, skin scraping, and cytology were performed, where necessary, as part of a thorough work-up. Elimination diet trials were performed for patients with perennial pruritus and/or concurrent gastrointestinal signs. For the animals used in this study, once the clinical diagnosis of canine AD was made, an allergy test to identify circulating allergen-specific IgE was performed for confirmation, to which all animals tested positive.

2.2 Enzyme-linked immunosorbent assay (ELISA) for each parameter

2.2.1 Ferritin (FE)

Plasma ferritin was measured with the commercially available Canine Ferritin Elisa test from BlueGene (E08F0136 Canine Ferritin Heavy Polypeptide ELISA). The above-mentioned kit is a competitive ELISA for quantitative measurement of canine ferritin in samples from blood, plasma, serum, cell culture supernatant and other biological fluids. Competition antigen ELISAs are coated with a captor antibody and a competitive antigen is labelled with the chromogen. Initially a wash solution was diluted with 990ml AquaDest + 10ml 100x Wash Solution Concentrate. The samples and the dilutor were placed in the coated plate wells. All necessary dilutions were made in 0.9% NaCl). (the protocol was tested for different dilutions and the test was finally made with the chosen dilution of 1:4 = 25µl sample + 75µl 0.9%NaCl). Then, 100µl of the standard solutions were inserted in the coated wells and 50µl of conjugate were added to each well except the blank control well. After mixing and incubating for 1h in room temperature the plate wells were washed, and the incubation mixture removed by aspirating contents into a proper waste container. Later, each well was filled with 1x wash solution, and the content was then aspirated. This procedure was repeated for a total of 5 washes. Afterwards, the plate frame was inverted until no moisture was macroscopically visible. Finally, 50µl of Substrate A and 50µl of Substrate B were added to each well, including the blank well, and after covered incubation for 15min, 50µl of stop solution was added to each well and the optical density (OD) was determined at 450nm using the microplate reader immediately.

2.2.2 Canine high sensitivity C Reactive Protein (CRP)

Plasma high sensitivity CRP was measured with the Abcam's C Reactive Protein Dog ELISA kit (ab157698). This is a highly sensitive two-site enzyme linked immunoassay (sandwich ELISA) for the quantitative measurement of CRP in dog serum and plasma samples. In this assay the plasma CRP reacts with the anti-CRP antibodies which have been adsorbed to the surface of polystyrene microtiter wells. The unbound proteins are removed by washing and the anti-CRP antibodies that are conjugated with horseradish peroxidase (HRP) are added to the wells. This leads to the formation of complexes between the already bound CRP and the enzyme-labeled antibodies. After washing, and after the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB), the enzyme bound to the immunosorbent is assayed. The measurement of the plasma CRP concentration is made at absorbance 450 nm. Standards are prepared with a highest concentration of 200ng/ml and as followed: 200ng/ml (3µl stock + 300µl 1x diluent Solution); 50ng/ml (40µl 200ng/ml canine CRP + 120µl diluent); 10ng/ml (20µl of 200ng/ml + 80µl diluent). 90µL of sample or standards was added in 0.1% BSA/PBS in singlets and then incubated for 15 minutes. Plate wells were filled with diluted 1x Wash Buffer and then aspirated, for three times and a total of 4 washes. Then 90µL of the 1x Enzyme-Antibody Conjugate were added in each well in reagent diluent. The plates were left to incubate and were washed again as described above. After adding 90µL of substrate solution in each the reaction was stopped with the addition of 50µL/well of stop solution (1N H_2SO_4). Finally, the absorbance of the contents of each well were determined immediately at 450 nm OD.

2.2.3 Hepcidin

Plasma hepcidin was measured with the commercially available Hepcidin-ELISA-Kit, R&D Systems DY8307. The above-mentioned kit utilizes a sandwich ELISA to measure natural and recombinant hepcidin in the plasma or serum. Firstly, the capture antibody was diluted with PBS to the working concentration without carrier protein. A 96-well microplate was coated with 100µL/ well of the diluted capture antibody. The plate was covered and incubated at room temperature overnight. Each well was washed and aspirated with wash buffer for a total of three washes. Any remaining fluid was removed by inverting the plate and blotting it against clean

paper towels. Afterwards, plates were blocked by adding 300μL/well of reagent diluent and then incubated again at room temperature for 1 hour. Then, 100μL/well of sample or standards in reagent diluent were added and the plate was incubated at room temperature for 2 hours. The washing/aspirating step was repeated and 100μL/well of the detection antibody, diluted in Reagent Diluent were inserted. After repetition of incubation and washing, we added 100μL/well of the working dilution of Streptavidin-HRP and 100μL/well of substrate solution. Finally, after adding 50μL/well of stop solution, the optical density of each well was determined immediately to 450 nm.

2.2.4 Transferrin

Plasma transferrin was measured with the Abcam's canine transferrin ELISA kit (ab157704). The assay and technique used are very similar to the ones described for canine high sensitivity CRP. In summary, 100μL/well of each standard, including zero control, as well as 100μL/well of sample were added in the plate. After incubation at room temperature for 30min and after aspiration, each well was filled with appropriately diluted 1X wash buffer. The washing was repeated for 3 times (4 washes in total). After the addition of 100μL/well of appropriately 1X enzyme-antibody conjugate and repetition of the incubation/washing step 100μL/well of TMB substrate solution were added. Finally, after precise incubation in the dark at room temperature for 10 the reaction was interrupted with the addition of 100μL/well of stop solution). The absorbance of the contents of each well were determined immediately at 450 nm OD.

2.2.5 Ceruloplasmin (Cp)

Many quantitative methods based on different principles have been used for Cp measurement in plasma or serum [103]. Assays based on oxidation of different compounds such asp-phenylenediamine (PPD) or its N-dimethyl derivative ando-dianisidine dihydrochloride have been used most often in veterinary medicine. Manual [107] and automated [108] methods based on PPD-oxidase activity have been reported for measuring Ceruloplasmin in canine serum. One of the main problems with Cp assays is the lack of commercially available reference materials to standardize Cp concentrations, such that different arbitrary units based on the increase of absorbance per unit of time have been used and expressed as oxidase units 81or U/L. Plasma ceruloplasmin measurement was performed with the use of N,N-dimethyl-p-

phenylenediamine dihydrochloride, a compound that produces a long-lived radical cation (DMDP, Sigma D4139), as described by Verde at al 2002 [152]. After preparing the plate wells with 100µl acetate buffer/well, 10µl DMPD/well and 2µl sample/standard, Optical Densitometry was measured at 505nm every 5 minutes for 60 minutes. The oxidative activity of Cp was expressed in mg/L according to the following equation: Cp (mg/L) = (A30 − A5) × 752. The rate of formation of the colored product of the enzymatic reaction is proportional to the concentration of plasma Cp; therefore, the enzymatic activity is expressed as milligrams of Cp by liter of plasma instead of commonly used enzymatic units [153].

2.2.6 Total iron binding capacity: Serum iron, serum copper and UIBC

For the measurement of serum iron, serum copper and the "unsaturated iron-binding capacity "UIBC we have used the method described by Yamashita et al [154]. First serum iron and copper is measured, and afterwards UIBC. Ascorbic acid is used to remove iron from transferrin and ceruloplasmin, then nitro-PAPS is added, resulting in serum iron + serum copper being read at absorption maxima at 580 and at pH 5. Subsequently thioglycolic acid is added, which forms a stable uncolored complex with copper without affecting nitro-PAPS-IRON complexes. For the TSAT calculations we used the following equation: [TSAT = (Fe/TF) × 70.9], as described in literature [154]. For the measurement of serum iron, we first added 15µl 0.89% NaCl into each well of transparent 96 well plates, then we pipet 15µl sample/iron-standards/copper-standards/water as blank. We added 100µl Reagent 1 (15ml ascorbic acid (0,3mol/L) and 85ml Nitropaps-color (31.6g SDS and 65.9mg NitroPAPS in 1l 0.32 mol/L sodium acetate buffer, pH5), mixed, and incubated for 10 min at room temperature. Then, absorbance was measured at 580nm (A-1S, A-1Fe and A-1Cu). In the sequence, we added 10µl Reagent 2 (2.16g NaOH + 36.8 thioglycolic acid in 1 liter), mixed, incubated for 5min and measured absorbance at 580nm (A-2S, A-2Fe and A-2Cu). The standards for iron and copper were used in the concentration of 2mg/L, which means 35.8µM/l Iron and 31.5µM/l Copper. The calculation serum concentrations of iron and copper was made as follow:

Serum iron (µmol/L) = 35.8 x (A2S/A2Fe)

Serum copper (µmol/L) = 31.5 x (A1S-A2S)(A1Cu – A2Cu)TIBC

The total iron binding capacity "TIBC" were calculated indirectly through summation of measured serum iron to UIBC, which is defined as the excess amount of iron needed to fully saturate transferrin. UIBC is measured as follows: a predetermined amount of Fe3 + is incubated with serum at near neutral pH, which allows Fe3 + to saturate all the available free iron binding sites on serum transferrin. A chromogenic agent is then added to complex with the free unbound iron (the remaining unbound ferrous iron reacts with the Nitroso-PSAP [2-Nitroso-5-(N-propyl- Nsulfopropylamino)phenol] to form an intense green complex).The difference between the resulting change in the measured absorbance and the absorbance for serum iron measurements from the total amount added to serum is equivalent to the quantity bound to transferrin - this is the Unsaturated Iron Binding Capacity (UIBC). Therefore, TIBC and TSAT are subsequently calculated as follows:

Serum FE + UIBC = TIBC

(FE/(FE+UIBC))*100 iron saturation

The reference ranges for UIBC in adult serum are 112-347ug/dL, for TIBC (calculated) are 250-400 ug/dL and for Iron saturation (calculated) are 14-50%. The measurement range is 17-700 ug/dL. For the measurement of UIBC we added to the wells 15µl sample. + 15µl neutral ferrous-soluti on 62 umol/L (sodium hydrogen carbonate: 75 mmol/L; TRIS buffer: 375 mmol/L, ph 8.4). Then we added 100µl of Reagent 1, mixed, incubated for 5 min at room temperature and measured the absorbance at 580nm (A-1S, A-1Fe and A-1Cu). Afterwards, we added 10µl Reagent 2, mixed, incubate for 5min and measured again absorbance at 580nm (A-2S, A-2Fe and A-2Cu). The calculations of UIBC concentrations of iron and copper were done as follow:

UIBC iron (µmol/L) = 35.8 x (A2S/A2Fe)

UIBC copper (µmol/L) = 31.5 x (A1S-A2S)(A1Cu – A2Cu)

3. Results

3.1 Control Group

A total of 102 plasma samples from adult client-owned healthy canines were used for this study and composed the control group. The median age of dogs in the control group was 6 years old (range 2-11). Most common breed in the control group was mix breed (N= 39), followed by Australian Shepherd (N=8), Collie (N=4) and Golden Retriever (N= 3). All the represented breeds and their number are displayed on Figure 1. 54% (N=55) of the dogs in the control group were female and 46% (N= 47) were male. Most of the dogs were female sprayed (N= 36), followed by male neutered (N= 35), female intact (N= 19) and male intact (N =12) (Figure 2).

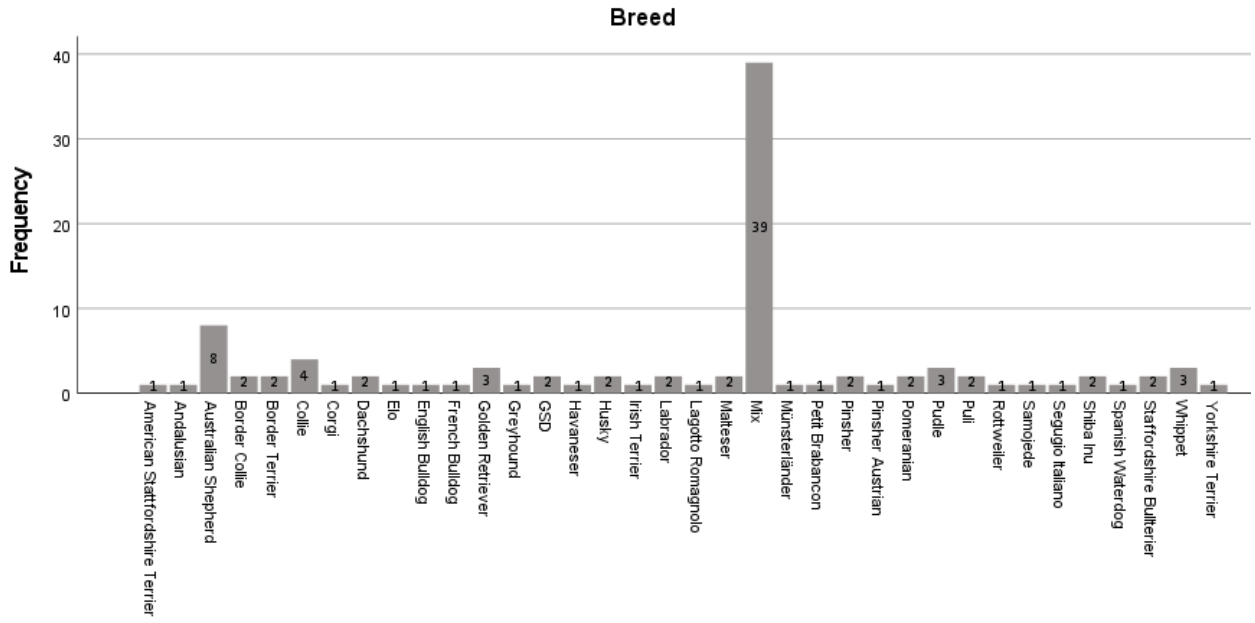


Figure 1. Bar Chart of the breed distribution in the control group.

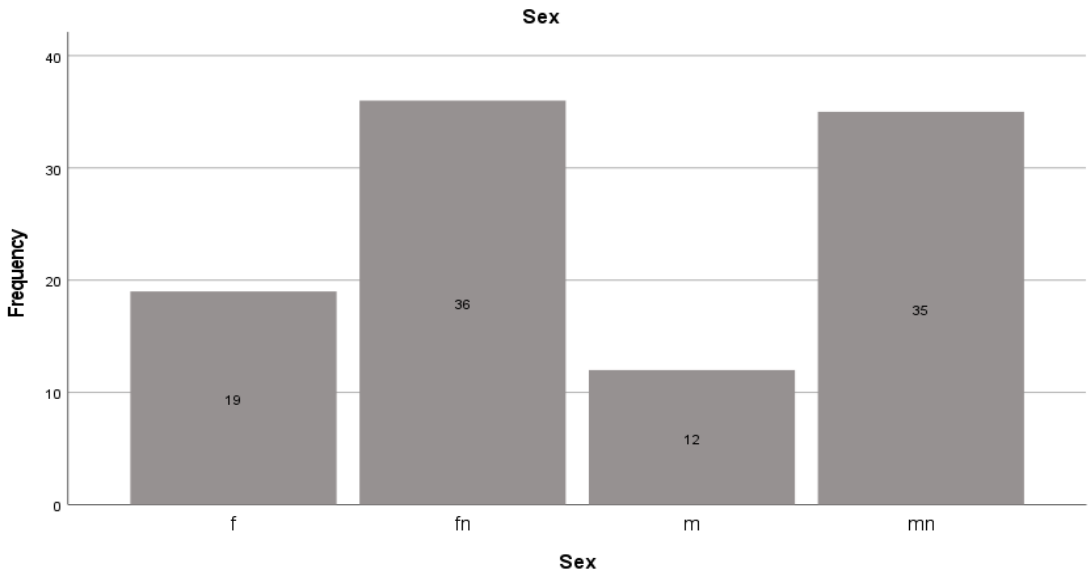


Figure 2. Bar Chart demonstrating the sex distribution in the control group.

3.2 Allergic Group

For the allergic group 36 blood serum samples were used, from dogs diagnosed with atopic dermatitis according to the criteria mentioned in the materials and methods. The median age of the dogs in the allergic group was 4 (range 1-10). Most common breed in the control group was mix breed (N= 7), followed by Labrador Retriever (N=5), French Bulldog (N=4) and West Highland White Terrrier (WHWT) (N= 3). All the represented breeds and their number are displayed on Figure 3. 66% (N=24) of the dogs in the control group were female and 34% (N= 12) were male. Also, in the allergic group most of dogs were female sprayed (N= 12), followed by female intact (N= 10), male intact (N= 9), male neutered (N= 7) (Figure 4).

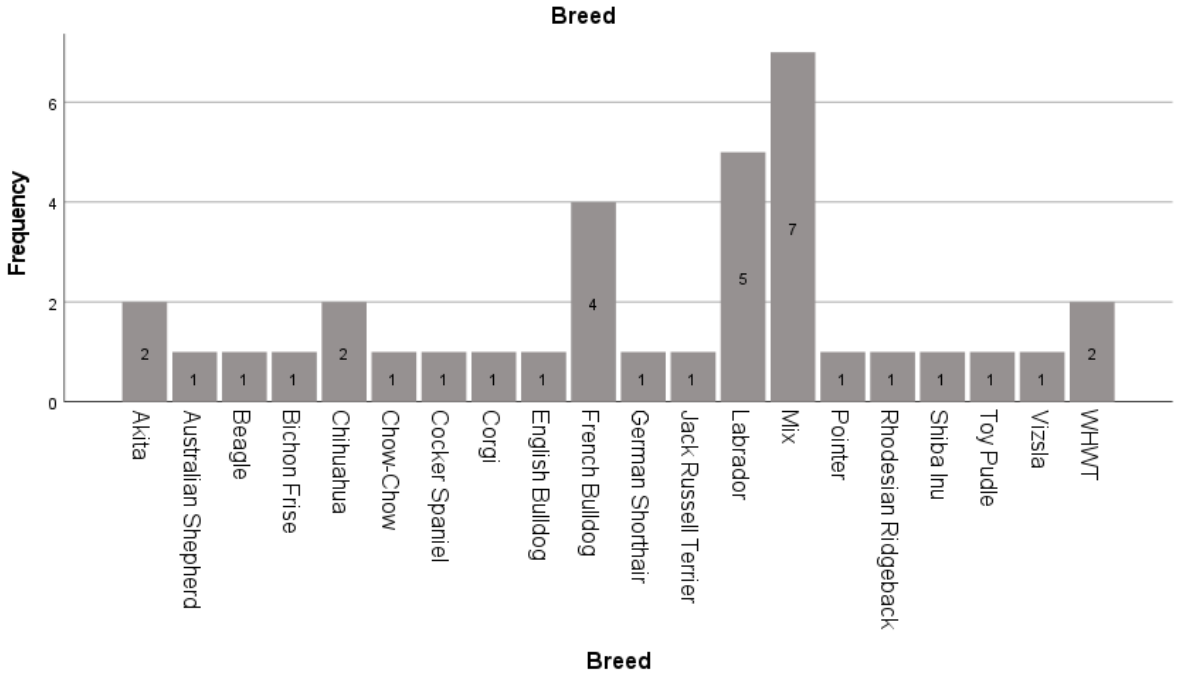


Figure 3. Bar Chart of the breed distribution in the allergic group

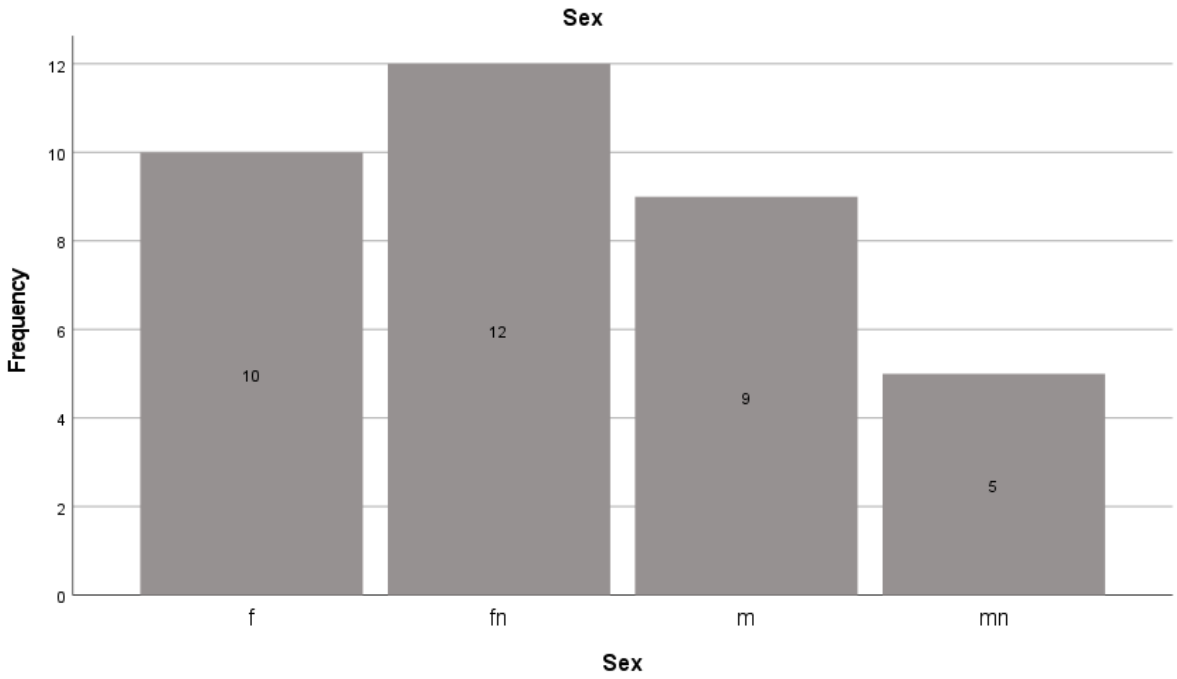
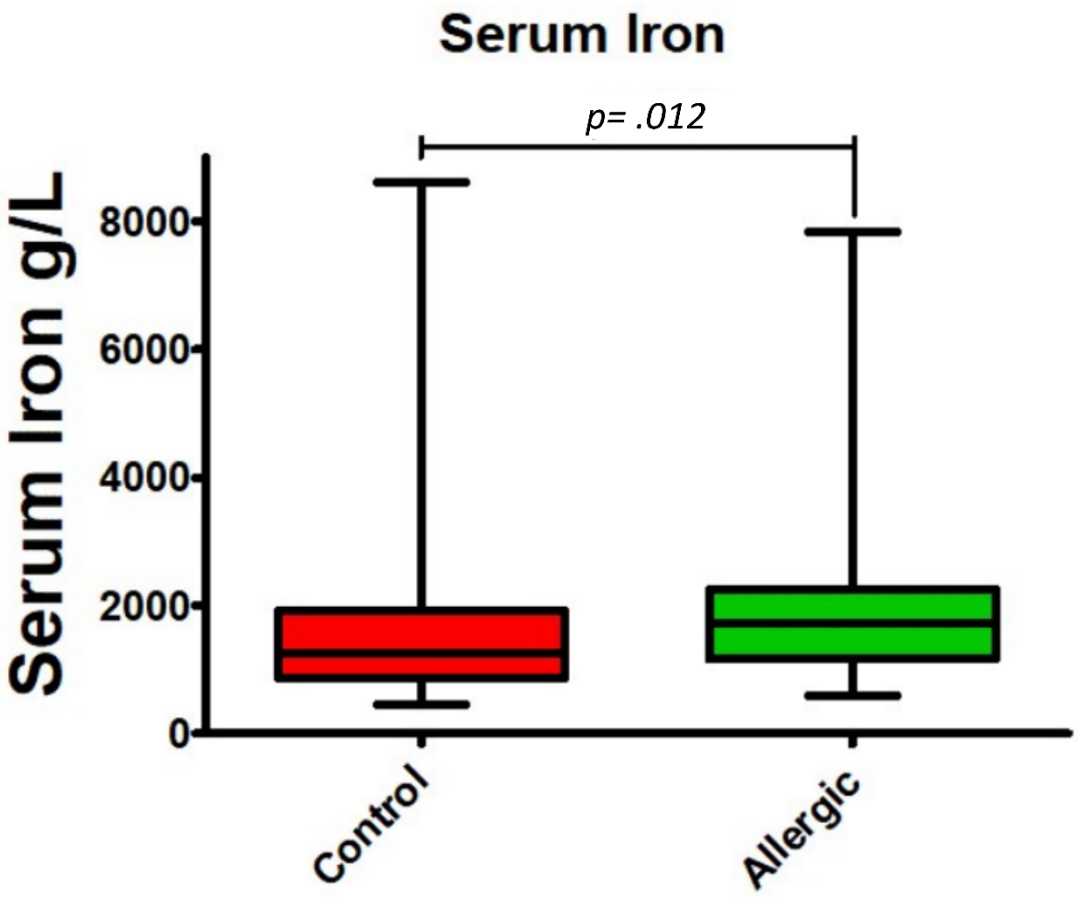


Figure 4. Bar Chart demonstrating the sex distribution in the allergic group.

3.3 Comparison of iron related parameters between allergic and control group

3.3.1 Serum Iron



The median Serum Iron concentration of dogs in the allergic group (N=36) was 1723.33 g/L (range 580.17-7830.83 g/L), while the median Serum Iron concentration of dogs in the control group (N=102) was 1259.50 g/L (range 458-8616.67 g/L). Allergic dogs have significantly higher Serum Iron concentration when compared to the control group ($p = 0.012$, CI 95%) (Figure 5).

Figure 5. Boxplot comparing Serum Iron concentration between allergic and healthy dogs. Allergic dogs have significantly higher serum iron concentration in comparison to healthy dogs ($p = 0.012$).

3.3.2 UIBC

The mean UIBC concentration of dogs in the allergic group (N=36) was 381.48 µg/L (range 154.70-1040 µg/L), while the mean UIBC concentration of dogs in the control group (N=102) was 615.76 µg/L (range 44.30-1399.50 µg/L). Healthy dogs have significantly higher UIBC concentration when compared to the allergic group ($p < .12$, CI 95%) (Figure 6).

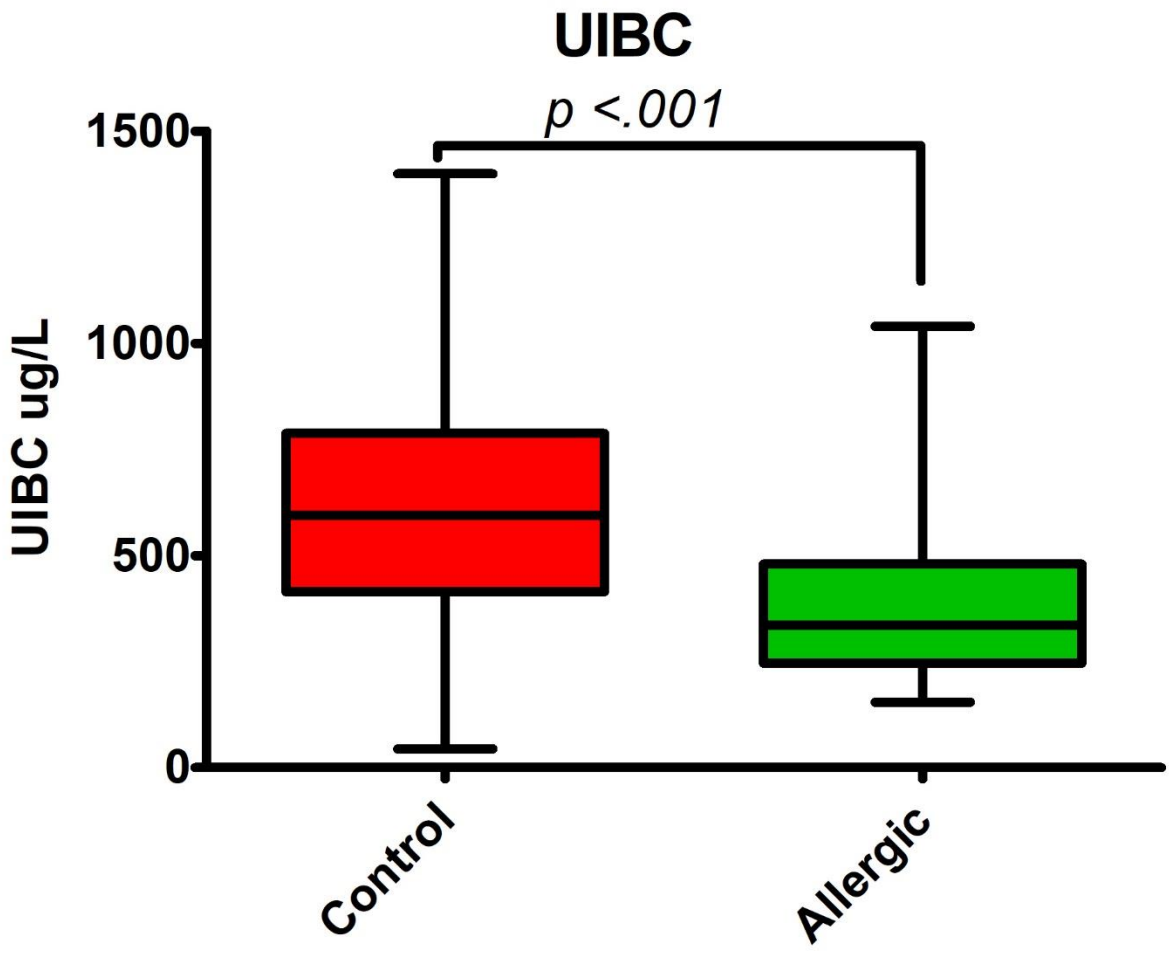


Figure 6. Boxplot comparing UIBC concentration between allergic and healthy dogs. Dogs in the control group have significantly higher UIBC concentration in comparison to allergic dogs ($p < .001$).

3.3.3 TIBC

The median TIBC concentration of dogs in the allergic group (N=36) was 2079.40 g/L (range 968.47-8158.53 g/L), while the median TIBC concentration of dogs in the control group (N=102) was 1887.90 g/L (range 641.42-9186.27 g/L). The difference between the two groups is not statistically significant (*p =.247* CI 95%)

3.3.4 hs-CRP

The median hs-CRP concentration of dogs in the allergic group (N=36) was 1.43 ng/mL (range 0.2-23.32 ng/mL), while the median hs-CRP concentration of dogs in the control group (N=102) was 1.41ng/mL (range 0.5-108.87 ng/mL). The difference between the two groups is not statistically significant (*p =.760* CI 95%).

3.3.5 Transferrin

The mean transferrin concentration of dogs in the allergic group (N=36) was 117.97 ng/mL (range 70.15-173.95 ng/mL), while the mean transferrin concentration of dogs in the control group (N=102) was 121.57ng/mL (range-2.57-249.85 ng/mL). Healthy dogs do not have significantly higher transferrin concentration when compared to the allergic group (*p = .738*, CI 95%).

3.3.6 Ferritin

The median ferritin concentration of dogs in the allergic group (N=15) was 5.8 µg/L (range 3.0-32.30 µg/L). The same median ferritin value is found also in the dogs of the control group (N=73) (range -0.90-67 µg/L). The difference between the two groups is not statistically significant (*p =.837* CI 95%).

3.3.7 Ceruloplasmin

The median ceruloplasmin concentration of dogs in the allergic group (N=36) was 9.05 U/L (range 3.45-28.16 U/L), while the median ceruloplasmin concentration of dogs in the control group (N=102) was 36.44 U/L (range 0.47-1180.70 U/L). Healthy dogs have significantly higher ceruloplasmin level when compared to the allergic group ($p < .001$ CI 95%) (Figure 7).

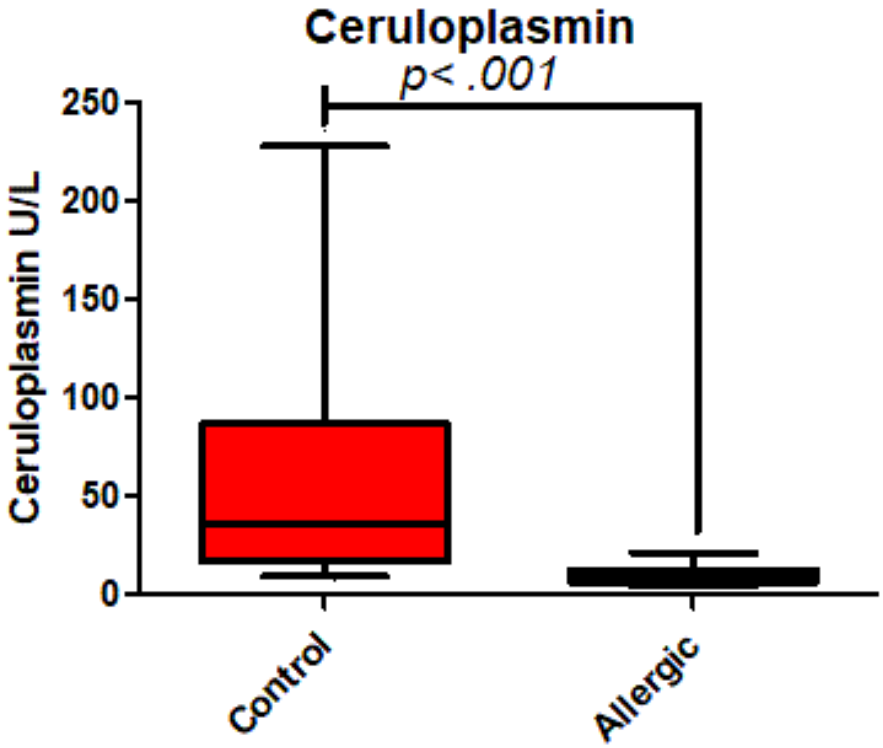


Figure 7. Boxplot comparing ceruloplasmin concentration between allergic and healthy dogs. Dogs in the control group have significantly higher ceruloplasmin concentration in comparison to allergic dogs ($p < .001$).

3.3.8 Hepcidin

The median hepcidin concentration of dogs in the allergic group (N=36) was 1.32 ng/mL (range -2.16-10.16 ng/mL), while the median hepcidin concentration of dogs in the control group (N=102) was 7.76 ng/mL (range -4.31-65.78ng/mL). Healthy dogs have significantly higher hepcidin concentration when compared to the allergic group ($p < .001$ CI 95%) (Figure 8).

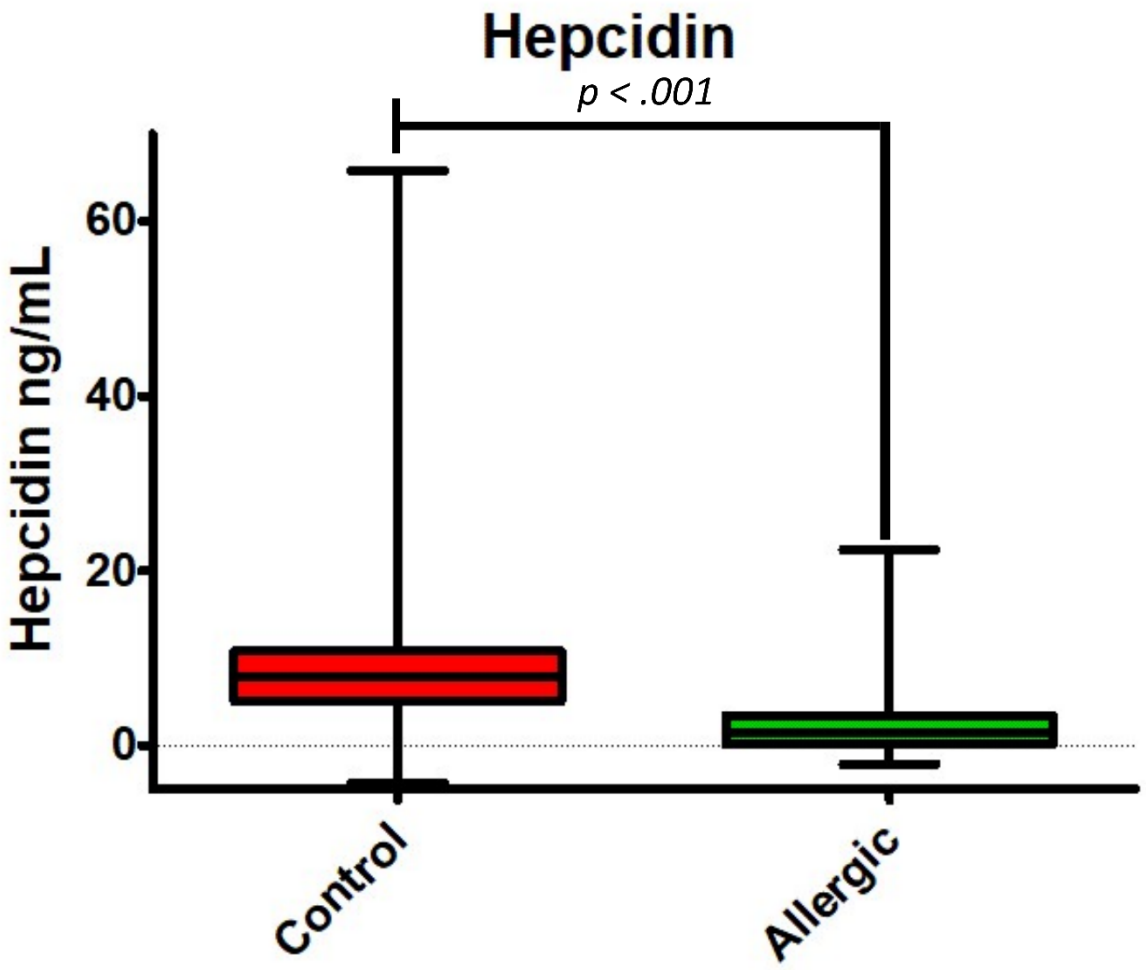


Figure 8. Boxplot comparing hepcidin concentration between allergic and healthy dogs. Dogs in the control group have significantly higher hepcidin concentration in comparison to allergic dogs ($p < .001$).

3.4 Correlation of Iron Parameters in Allergic Dogs

Correlations Heatmap Allergic

		UIBC	SERUM IRON	TIBC	CRP	TRF	FRT	CRLP	HEPCIDIN
Spearman's rho	UIBC	1,000	-,291	-,111	,191	,119	,018	-,219	-,188
	SERUM IRON	-,291	1,000	,960**	-,342*	-,045	-,013	,383*	,052
	TIBC IRON	-,111	,960**	1,000	-,269	-,038	,044	,368*	,022
	CRP	,191	-,342*	-,269	1,000	,073	,046	-,056	,040
	TRF	,119	-,045	-,038	,073	1,000	,427	,210	,183
	FRT	,018	-,013	,044	,046	,427	1,000	-,233	,372
	CRLP	-,219	,383*	,368*	-,056	,210	-,233	1,000	,196
	HEPCIDIN	-,188	,052	,022	,040	,183	,372	,196	1,000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

3.5 Correlation of Iron Parameters in Healthy Dogs

Correlations Heatmap Control

		UIBC	SERUM IRON	TIBC	CRP	TRF	FRT	CRLP	HEPCIDIN
Spearman's rho	UIBC	1,000	,197*	,499**	,004	-,040	,035	,016	,090
	SERUM IRON	,197*	1,000	,917**	-,070	,176	-,052	-,019	-,403**
	TIBC	,499**	,917**	1,000	-,045	,165	,007	-,058	-,349**
	CRP	,004	-,070	-,045	1,000	,075	-,068	,045	,068
	TRF	-,040	,176	,165	,075	1,000	-,142	-,031	,045
	FRT	,035	-,052	,007	-,068	-,142	1,000	,022	,043
	CRLP	,016	-,019	-,058	,045	-,031	,022	1,000	,057
	HEPCIDIN	,090	-,403**	-,349**	,068	,045	,043	,057	1,000

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Abbreviations	
UIBC	Unsaturated iron binding capacity
TIBC	Total iron binding capacity
CRP	Hs-CRP
TRF	Transferrin
CRLP	Ceruloplasmin

These results reveal that for allergic dogs there is a positive correlation between serum iron and TIBC (Spearman Rho .960, $p < .001$), as well as a negative correlation between serum iron and hs-CRP (Spearman Rho -0.342, $p = .048$). Furthermore, serum iron has a significant positive correlation to ceruloplasmin (Spearman Rho .383, $p = .025$) Also, contrary to what is observed in normal physiology of iron replete status, there is no correlation between serum iron and hepcidin. These results reflect physiology of functional iron deficiency.

In healthy dogs a significant negative correlation between serum iron and hepcidin is observed (Spearman Rho -.403, $p < .001$). The same negative correlation is seen between hepcidin and TIBC (Spearman Rho -.349, $p < .001$). These results reflect normal physiology o iron replete status

4. Discussion

Iron is a trace element essential for nearly every organism and needed for oxygen transport, cellular respiration, and that also contributes to immune regulation. Iron related parameters are infrequently measured in veterinary medicine, and this work aimed to measure these parameters in dogs, and to observe whether the findings on iron metabolism would be similar to what is seen in human medicineHepcidin is the main molecule for iron regulation, having as physiological role to act as a negative regulator of iron absorption and release. Hepcidin levels increase in response to iron or inflammation, therefore the significant negative correlation observed between hepcidin and serum iron ($p < 0.01$ CI: 95%) for the healthy dogs is expected and corresponds to the normal physiology of iron regulation in an iron replete status.

The higher levels of serum iron seen in the allergic dogs are not associated with higher ferritin reflecting a better iron-status. Another important finding is that the iron-levels are not associated with hepcidin at all, emphasizing that in dogs with atopic dermatitis a normal iron metabolism for iron replete status is not present.

For the allergic dogs it is possible to observe positive correlation between serum iron and ceruloplasmin. As ceruloplasmin is fundamental in the transport of iron from the cell stores into the plasma, consequently promoting increase of serum iron in the circulation, it would be expected that its levels would increase when plasma iron is low, so to transfer iron from the reservoir into the circulation.Another correlation observed in the allergic group was between hs-CRP and serum iron. Iron deficiency is associated with increases in hs-CRP, regardless of anemia being present or not, and the fact that can observed an increase in hs-CRP can be observed as the iron levels go lower indicate the presence of possible reaction to inflammation in the allergic dogs.

When we compared the healthy dog group to the allergic, significantly higher serum iron ($p = 0.012$) is observed in the allergic group. The result obtained are contrary to what was expected, as from human medicine it is known that atopic individuals lack iron, which profoundly affects their immune system and render them hyper-sensitive. However, several confounding factors existed, which were not present in the healthy cohort: First, most allergic dogs included in this

study were under therapy for CAD with Cytopoint, a monoclonal antibody that represses the inflammatory cytokine 31 and thus impedes the itching cascade. They responded well to the therapy and did not present acute allergy symptoms during the period when the collection of blood samples was made, which does not reflect the usual patient suffering from CAD. Furthermore, the allergic patients had their blood collected throughout the entire day and sometimes post-prandial as opposed to the healthy animals, which had their venipuncture done always fastened and in the morning period. Iron levels in humans considerably fluctuate according to the circadian rhythm and time after a meal [157]. All these factors must be considered when evaluating iron parameters but were not possible due to the nature of this retrospective study. So, the measured serum iron-levels in the allergic cohort reflect rather the repressed immune system and circadian and post-prandial variations, and thus not allow a direct head-to-head comparison with the serum iron -levels obtained from the healthy cohort.

5. Conclusion

In conclusion, our study reveals that iron parameters differ considerably between healthy and allergic dogs. In healthy dogs, the results observed reflect a normal iron homeostasis in an iron replete status. In contrast, the results obtained for atopic dogs are associated with inflammation markers and are not dependent on parameters reflecting an iron replete status.

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