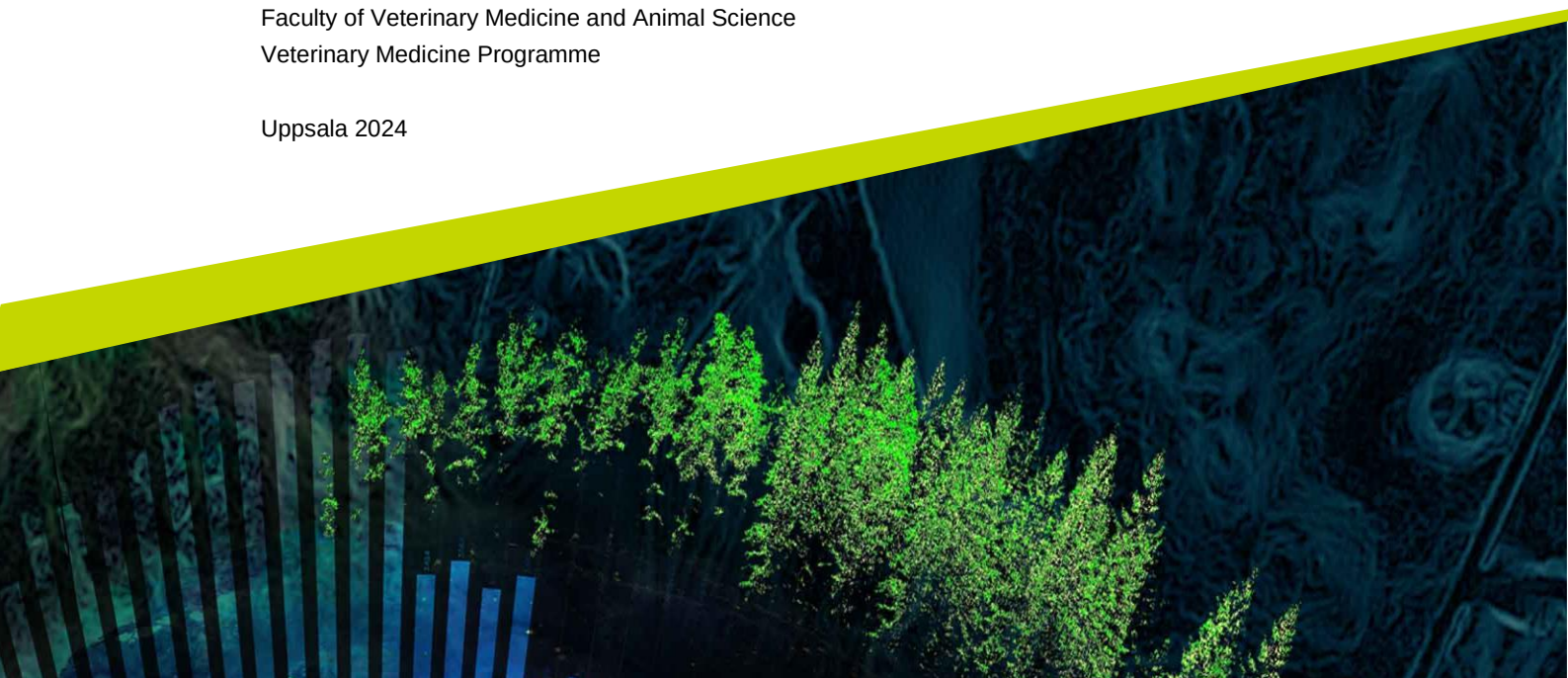
Serum-levels of IL-12 and IL-23 in dogs with lymphoma and solid tumours

Ida Hedman

Independent Project • 30 credits
Swedish University of Agricultural Sciences, SLU
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Uppsala 2024



Serum levels of IL-12 and IL-23 in dogs with lymphoma and solid tumours

Ida Hedman

Supervisor:	**Henrik Rönnberg, Swedish University of Agricultural Sciences (SLU), Department of Clinical Sciences**
Assistant supervisor:	Sara Saellström, Swedish University of Agricultural Sciences (SLU), Department of Clinical Sciences
Examiner:	Jens Häggström, Swedish University of Agricultural Sciences (SLU), Department of Clinical Sciences

Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent Project in Veterinary Medicine
Course code:	EX1003
Programme/education:	Veterinary Medicine Programme
Course coordinating dept.:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2024

Keywords:	cancer, cytokines, immunology, immunotherapy, IL-12, IL-23, IL12-p40, ELISA

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Abstract

Cancer is an increasingly more common disease in both humans and animals and requires several sets of characteristics to grow and metastasize. To obtain these characteristics, signalling via cytokines are crucial. The way that cancer manipulates the environment in which it resides to create a favourable outcome for tumour survival, growth and metastasis is an area of great interest, not only for development of potential cancer biomarkers, but also new and innovative adjuvant cancer therapies. Certain subsets of cytokines, such as IL-17 and IL-23, can favour tumour survival by allowing the tumour to evade immune surveillance, and has been linked to poorer prognosis and response to treatment in several cancers. Likewise, other subsets of cytokines, such as IL-2 and IL-12, are related to better disease outcome and treatment response. The recruitment of immune cells, such as certain populations of T cells, into the tumour tissue alters the tumour microenvironment towards either a pro- or anti-tumour profile depending on which sets of cytokines are being produced. In this study, canine patients with lymphoma, mammary tumours and mast-cell tumours were analysed with regards to the serum levels of IL-12p40, a subset shared between IL-12 and IL-23. Each group were analysed with ELISA and compared to disease-free controls. The levels of IL-12p40 were significantly increased in patients with lymphoma compared to the other tumour groups and controls, but no differences were found amongst the other groups. Since sample size is small, no definite conclusions could be drawn with regards to the usefulness of IL-12p40 as a biomarker in canine cancer, apart from a potential use in canine patients with lymphoma. Further studies are required in all groups, preferably with larger populations and multiple types of cancer, as well as pre and post treatment measurements to evaluate the potential prognostic value of IL-12p40 in canine cancer.

Keywords: cancer, cytokines, immunology, immunotherapy, IL-12, IL-23, IL-12p40, ELISA

Table of contents

Abbreviations

CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CTL	Cytotoxic T-lymphocyte
MMP9	Matrix metallopeptidase 9
MMT	Mammary tumour
NK	Natural Killer Cell
OSA	Osteosarcoma
PD-1	Programmed cell death protein 1
STAT	Signal transducer and activator of transcription
TMT	Tumour microenvironment

1. Introduction

Development of cancer requires the capacity of normal cells to undergo transformation into a neoplastic state. These capabilities have been described as eight "hallmarks of cancer" and can be summarized as the ability of the tumour cells to communicate, spread, and avoid immune detection and cell death (Hanahan & Weinberg 2000). Activation and maintenance of inflammatory pathways, or rather a transformation from an acute to a chronic inflammatory state, is critical for a tumor to maintain these hallmarks. It is of great importance to understand these pathways to discover potential biomarkers aimed specifically at detecting, treating, and monitoring cancer (Demaria et al. 2010). Tumors are known for their ability to downregulate immune surveillance by inducing a favorable cytokine-environment and recruiting immunosuppressive cells such as T-regs, facilitating their growth, progression, and metastasis (Ostrand-Rosenberg & Sinha 2009).

The proteins IL-12 and IL-23 are proinflammatory cytokines that play an important role in the development and dynamics of several autoimmune disorders as well as cancer (Croxford et al. 2014:12). The serum levels of IL-12/IL-23 have not yet been studied in relation to canine cancer as potential biomarkers, although over-expression of IL-23 is seen in many types of human cancer, sometimes in con-currence with low levels of IL-12 (Yan et al. 2018). IL-12 is recognized for its antitumor-immunity promoting effects. It drives a cell-mediated cytotoxic Th1 response through activation of NK cells and CD8+ T lymphocytes in tumour tissue. (Tugues et al. 2015) Conversely, IL-23 is known to contribute to a favourable tumour microenvironment, partly by reducing CD8+ T cell infiltration into tumour tissue (Langowski et al. 2006). Hence, the IL-12 and IL-23 signalling cascades seem to regulate the tumour environment antagonistically, although the dynamics of their interplay seem to vary depending on the type of tumour (Langowski et al. 2006).

The dynamics of the IL-12/IL-23 cytokines have been studied mainly through experimentally induced tumours in mice. Given that tumours arise spontaneously in dogs and in humans, studying the balance between these cytokines in dogs might aid translation of their use for developing treatments and their potential use as biomarkers in both human and canine patients. (Pavlin et al. 2012)

The purpose of this study is to investigate the serum levels of IL-12 and IL-23 in canine patients and discuss whether the serum levels of these cytokines may be useful as potential biomarkers for tumour detection, prognosis, and treatment.

2. Literature review

2.1 Hallmarks of cancer: enabling characteristics

According to the latest review released by Douglas Hanahan and Bob Weineberg, the eight hallmarks of cancer, or rather characteristics of cancer, are the following; sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism and avoiding immune destruction. To acquire these characteristics, genome instability and inflammation must be present. These two requirements have been labelled as "enabling characteristics", that is conditions that allow tumours to take on the above mentioned eight hallmarks. Another recently discussed enabling characteristic is the microbiome, and it is primarily the gut microbiome and its role in cancer that has been recognized. It has been discovered that certain dysbiotic bacteria are present in excess in patients with colon cancer. They supress the adaptive immune response by producing immunomodulatory factors that stimulate production of cytokines that create a favourable environment for acquiring the hallmarks of cancer. (Hanahan 2022)

2.1.1 Stabilization of the genome

Stabilization of gene expression is important to avoid DNA damage. At the same time, the ability of the genome to respond and adapt to outside signals is also crucial for biological processes to function. To avoid responding to signals that are damaging and increase the risk of developing disease such as cancer, DNA must be stabilized. By being coiled onto millions of proteins called nucleosomes, that are in turn organised into chromatin, DNA is protected from genome instability. Simultaneously, chromatin also provides an important medium through which cues from cell-signalling pathways and transcription factors can reach the nucleus and alter gene expression. (Margueron & Reinberg 2010).

2.1.2 Transcription factors and genome instability

Transcription factors, such as STAT3, are proteins that bind to DNA and alter gene expression. They are either synthesized or are already present in the cell. When cytokines bind to their receptors on the cell surface, cell signalling pathways are activated that induce production of certain transcription factors. Transcription factors do not only turn on the transcription of certain genes but can also turn them off. In cancer, certain transcription factors are abnormally activated or synthesized, leading to destabilization of the genome through activation of oncogenes and inhibition of anti-oncogenes. Thus, genes that promote production of growth factors so that normal cells can transform into a neoplastic state, by avoiding cell death and sustain proliferative signalling, are turned on. Simultaneously, genes that inhibit those characteristics in normal non-neoplastic cells are turned off. Alterations in the cytokine-signalling environment, and thereby the activation or suppression of certain transcription factors, are crucial for developing genome instability that is required to induce and maintain cancer progression. (Latchman 1993)

2.1.3 The tumour microenvironment

It is acknowledged that a tumour itself operates as a proclaimed organ by creating its own environment, the tumour microenvironment (TMT). The TMT is characterized by a state of chronic inflammation, consisting of a constant production of inflammatory mediators and chemotaxis of certain immune cells that favour tumour survival. The TMT enables the tumour to acquire and maintain the hallmarks presented earlier. Creation of the TMT is largely attributed to normal non-neoplastic cells. This includes immune cells from the innate immune system, such as NK cells, and from the adaptive immune system, primarily T-lymphocytes, as well as pericytes, fibroblasts and endothelial cells that make up the tumor "stroma". Additionally, as organs of the body possess their own microbiome, the constitution of the microbiome also contributes to the TMT. The balance of the microbiome can either promote or inhibit cancer by triggering immune responses via stimulation of production of certain subsets of cytokines, that can either be tumoricidal or tumour-promoting. (Hanahan 2022)

The interactions between the tumor cells and the stromal cells are necessary for metastasis and cancer progression (Baghban et al. 2020). The tumor cells use cell-signaling via cytokines to stimulate the stromal cells to support tumor invasion and growth, which includes stimulation of endothelial cells to create new tumor vasculature. Not only is this essential for transportation of nutrients to the cancer cells so that they can proliferate but is also vital for metastasis as tumour cells must enter the circulation to migrate to other organs beyond the initial cancer site (Kartikasari et al. 2021). Thus, the cytokine-signaling in the TMT is an important

area of investigation, as more than 90% of human fatalities from cancer is due to metastasis (Grivennikov et al. 2010).

2.1.4 Inflammation in cancer

In the TMT, certain transcription factors are activated that drive the production of inflammatory mediators, cytokines, that recruit more inflammatory immune cells into the TMT. The cytokines activate similar transcription factors in an autocrine fashion in the recruited immune cells and in the stromal cells, to create a chronic inflammatory environment that facilitates growth and spread of the tumor itself. (Mantovani et al. 2008)

The tumor selects the type of inflammatory cells to recruit by altering the cytokine environment. It benefits from immune cells that maintain the chronic inflammatory state without killing off the tumor cells. Immune cells and cytokines that favor tumor survival and progression are therefore often present in the TMT, such as an infiltration of regulatory T cells and higher levels of IL-23. (Grivennikov et al. 2010)

2.2 Immune surveillance in cancer

For tumour cells to be destroyed, they must be recognized by the immune system. As previously discussed, the immune system can both promote cancer and inhibit cancer progression depending on the type of immune cells and inflammatory mediators present (Yan et al. 2018). It is recognized that individuals with cancer harbour T cells with tumour-specific antigens, that recognize and react to the tumour cells themselves (Yamaguchi & Sakaguchi 2006). However, their response is often insufficient in eradicating tumours completely. Moreover, other subsets of T cells in the TMT can induce a form of "self-tolerance" in which the tumour cells are no longer recognized as foreign and is therefore able to hide from the immune system.

2.2.1 Immune checkpoints

Immune checkpoints are regulatory pathways within the immune system itself responsible for self-tolerance, which prevents the immune system from destroying tissue that is not harmful or foreign to the body. Tumours can hijack some of these pathways to induce self-tolerance and thereby hide from the immune system. Certain immune checkpoints are especially important in cancer, such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1). Activation of these checkpoints deactivates cytotoxic T-cell responses that would normally kill tumour cells, and up-regulates regulatory T cells that

favour tumour development by down-regulating anti-tumour immunity. Therefore, many tumour-infiltrating lymphocytes express PD1 and CTLA-4 and their ligands are upregulated on the tumour cells, which down-regulates immune surveillance. Blockade of these checkpoints in cancer have proven useful, as it leads to an activation of the immune cells and thus "reveals" cancer to the host immune system. PD1 inhibitors seem to induce tumour regression in several tumours, and CTLA-4 blockers induces anti-tumour immunity and has been approved for treatment of melanoma. (Pardoll 2012)

2.2.2 CD4+ T helper cells

T helper cells, or CD4+ T cells, are naïve T cells of the adaptive immune system that can differentiate into different subsets of T cells with different effector functions. The effector functions are the specific ways in which the certain subsets of T cells fight off viral, fungal, bacterial, and parasitic infections, as well as cancer. Some subsets, such as regulatory T cells (Treg), down-regulate immune surveillance, while other subsets, such as Th1, stimulates immune surveillance and cytotoxicity. The differentiation of naïve CD4+ T cells is driven by cytokines and activation of certain transcription factors, and the type of cytokine environment determines which subset of T helper cells the naïve T cells will differentiate into. (Read et al. 2019)

The role of T helper cells in the tumour microenvironment is complex, and they may exhibit either pro or anti-tumour immunity depending on the TMT. It is clear however, that certain cytokines and subsets of T-cells, such as IL-12 and Th1 cells, are primarily tumoricidal in their effector functions. On the other hand, regulatory T-cells exhibit pro-tumour immunity properties by allowing tumours to elude the immune system.

2.2.3 Th1 cells

Th1 cells are known for their tumoricidal effects by exposing the tumour to the immune system via secretion of their effector cytokines, IFN-γ and IL-2 (Haabeth et al. 2011). This initiates a cascade of cytotoxic responses via activation of macrophages that kill cancer cells directly. In addition, it stimulates secretion of chemokines by the macrophages that inhibit angiogenesis and thus tumour progression and metastasis. IL-2 secreted by the Th1 cells amplifies CD8+ T cell and NK cell cytotoxicity and thereby activates the immune system to recognize cancer cells as foreign (Jiang et al. 2016). The anti-tumour properties of IL-2 are very well recognized as it is an approved immunotherapy treatment of metastatic renal cell carcinomas and metastatic melanoma in humans.

2.2.4 Th17 and Treg cells

In contrast to Th1 cells, regulatory T cells are known to down-regulate the expansion of effector T cells with tumoricidal properties, and thus induces a form of "self-tolerance" in which the immune-reaction towards tumour cells is inhibited (Sakaguchi 2004).

Another subset of T-cells, Th17 cells, characterized by their production of IL-17, are known to be of great importance in several autoimmune disorders and chronic inflammatory conditions such as psoriasis and inflammatory bowel disease (Korn et al. 2009). Th17 differentiation is driven by the secretion of IL-23 from dendritic cells (Aggarwal et al. 2003). The importance of IL-23 is related to its ability to stabilize and expand Th17 cell lines (Korn et al. 2009).

In cancer, the role of Th17 cells is complex. In human colorectal cancer, the presence of Th1 cells in tumour tissue is correlated with better prognosis (Galon et al. 2006), whereas a high expression of Th17 was consistent with worse clinical outcome (Tosolini et al. 2011). Th17 cells can induce immunosuppressive functions, thereby downregulating tumour immune surveillance as well as stimulating angiogenesis in the TMT which promotes tumour growth and metastasis. In mice, IL-17 promoted a significant increase in tumour growth and vascularization (Numasaki et al. 2003). In patients with B cell acute lymphoblastic leukaemia, increased levels of Th17 cells and IL-17 were observed and contributed to proliferation of cancer cells resistant to immunotherapy (Bi et al. 2016). The higher Th17 cell levels were observed in concurrence with low levels of Th1 cells.

Conversely, Th17 cells are also capable of activating cytotoxic T cells and therefore also have an anti-tumour effect, depending on the tumour type (Guéry & Hugues 2015). A study has recently shown that higher levels of infiltratory Th17 cells in human ovarian cancer correlated with improved clinical outcome (Colombe et al. 2022). Hence, it is important to note that Th17 might also play a role in anti-tumour immunity, and it seems to be highly dependent on the TMT. For example, IL-23 levels were found negligible in ascites in ovarian cancer and low levels of IL-17 correlated with worse clinical outcome. Therefore, the anti-tumour immunity effector function of Th17 seem to be related to IL-17 levels, and the pro-tumour effect may be dependent upon co-stimulation from IL-23.

2.2.5 STAT3/STAT4 in cancer

STAT3 is a transcription factor known for its tumor promoting properties and is activated by IL-23 and IL-6 (Rébé & Ghiringhelli 2019). In several types of cancers, signaling via STAT3 is crucial to obtain many of the cancer hallmarks presented earlier, including inflammation in the TMT as well as metastasis and angiogenesis.

STAT3 is important for Th17 cell development which, as discussed earlier, can either have an anti or pro-tumor effect (Yang et al. 2007). Interestingly, STAT3 seems to be particularly essential for driving the immunosuppressive effects of Th17 cells in cancer by upregulating certain enzymes (ectonucleotidases) present in the cell plasma membrane responsible for regulating metabolites important in inflammation (Chalmin et al. 2012). Thus, signaling via STAT3 helps the tumor in avoiding detection by the immune system.

Another transcription factor, STAT4, is activated by IL-12 and drives differentiation of naive CD4+ T-cells into IFN-γ producing Th1 cells which, as discussed earlier, increases NK and CTL cell cytotoxicity, and thereby drives anti-tumor immunity (Ebersbach et al. 2021). This is supported by studies in mice, as a deficiency in STAT4 lead to impaired differentiation of Th1 cells and a lack in IFN-γ mediated cytotoxicity (Thierfelder et al. 1996). In humans, studies have shown that a downregulation of STAT4 in hepatocellular carcinoma predicted worse clinical outcome and increased recurrence after tumor resection, while patients with a higher STAT4 expression in tumor tissue had both less metastasis and extrahepatic recurrence of tumor (Wubetu et al. 2014). Similar results were found in both gastric (Nishi et al. 2017) and ovarian cancer (Wang et al. 2023).

2.3 Tumour biomarkers and targets for immunotherapy: comparative oncology

Biomarkers, specific proteins measured in blood and other tissues, are important tools for identifying and monitoring disease. The ideal biomarker should be easy to measure and be able to distinguish diseased from healthy individuals. In addition, especially when it comes to cancer, monitoring progression, metastasis, and recurrence of disease after treatment are desirable characteristics of potential biomarkers.

Several biomarkers have been studied in canine cancers (Henry & Hayes 2012). Some, such as the enzyme serum thymidine kinase 1, have shown to be potentially useful in identifying different types of cancer, such as lymphoma, leukemia (von Euler et al. 2006), and hemangiosarcoma (Kusaba et al. 2006). However, many biomarkers studied in canine patients does not fulfill all the desired criteria for cancer detection and monitoring described earlier (Colombe et al. 2022). However, biomarkers that have been proven useful to follow in humans also seem to be of use in dogs. Thus, studying biomarkers in canine patients might aid in the discovery of new cancer biomarkers and targets for cancer immunotherapy in humans and vice versa.

2.3.1 Cytokines

Cytokines created in the TMT will eventually reach systemic circulation and can therefore be measured by means of a simple blood sample. As briefly integrated in the discussions with regards to T helper cells and transcription factors, certain distinct sets of cytokines seem to be related to either pro- or anti-tumor immunity. As such, detection of certain subsets of cytokines may help indicate presence of disease and even predict recurrence and treatment response and may therefore serve as potential cancer-biomarkers. (Kartikasari et al. 2021)

In human renal cell carcinoma, high serum levels of IL-6 and IL-17, cytokines associated with pro-tumor immunity as previously discussed in relation to STAT3 signaling, positively correlated with both metastasis and recurrence of tumor after treatment (Gudbrandsdottir et al. 2021). In human breast cancer, higher serum levels of IL-6, IL-8 and IL-10 corresponded to severity of disease as well as metastasis, survival and predicted worse response to treatment (Wang & Yang 2017).

Similarly in dogs, a recent study showed that serum levels of IL-8 and TNF- α were found significantly higher in individuals with malignant mammary tumors (MMT) compared to controls (Baba et al. 2019). Another study in canine patients showed a significant increase in serum levels of IL-10 in dogs with malignant melanoma compared to controls (Hutchison et al. 2019). In addition, significantly higher serum levels of IL-6 and IL-10 were found in dogs with lymphoma compared to healthy individuals, which is consistent with findings in human lymphoma patients (von Euler et al. 2006).

Osteosarcoma (OSA) is the most common primary malignant bone tumor in both dogs and humans (Irac et al. 2019). Although canine osteosarcoma behaves more aggressively than human osteosarcoma, the prognosis is still poor in both species. In particular, the cytokine TGF- β correlated with worse clinical outcome and more aggressive OSA disease in humans. In dogs, blocking TGF- β inhibits proliferation and migration of OSA cells (Portela et al. 2014). Although the TGF- β levels specifically were measured not in serum but in the tumour cells, it still proves the point that altering the cytokine-environment by blocking pro-tumorigenic cytokine receptors may provide potential additional immunotherapy treatments for cancer.

2.3.2 IL-12/IL-23 and their role in cancer

The balance between the cytokines IL-12 and IL-23 can contribute to the creation of an either pro- or anti-tumour microenvironment (Yan et al. 2018). Their signalling seem to regulate the TMT antagonistically, where IL-12 is known to promote tumoricidal effects whereas IL-23 can favour tumour development (Langowski et al. 2006).

Signalling via IL-12 leads to induction of the STAT4 signalling cascade, which drives a cell-mediated immune response via differentiation of naive CD4+ T cells into T helper type 1 cells. The Th1 cells release of IFN-γ which drives the cytotoxic response via activation of NK cells and CD8+ T cells in the tumor (Langowski et al. 2006). The activated NK cells and cytotoxic T-lymphocytes themselves secrete IFN-γ, as well as perforin and granzyme, rendering them directly toxic to cancer cells and amplifying the anti-tumour response (Tugues et al. 2015). IFN-γ release induces an upregulation of adhesion factors in tumour vasculature, thereby facilitating recruitment of immune cells into tumour tissue. In addition, it also inhibits tumour angiogenesis, and thereby prevents metastasis.

IL-12 down-regulates PD-1, as discussed earlier on immune checkpoints, on CD8+ T cells and is also required for PD-1 inhibitors to enable CD8+ T-cell killing of tumour cells in immune-checkpoint therapy (Mirlekar & Pylayeva-Gupta 2021). Although the anti-tumour effects related to IL-12 has been recognized primarily through driving the Th1 cell response, it also seems to have a local antitumor-effect through upregulation of leukocyte adhesion molecules in tumour tissue (Eisenring et al. 2010). In human colorectal cancer, pre-operative serum levels of IL-12 served as a promising prognostic marker for survival, where patients with higher levels of IL-12 showed a significant increase in survival (Stanilov et al. 2014). In addition, levels of circulating IL-12 decreased significantly as the disease progressed. Therefore, IL-12 may serve as a potential biomarker for both disease prognosis and progression.

Conversely, IL-23 activates the STAT3 signalling pathway (Floss et al. 2020), a pro-oncogenic transcription factor related to poorer prognosis in several human cancers (Kusaba et al. 2006). In contrast to the Th1 response induced by IL-12, IL-23 has been shown to induce expansion of Th17 cell lines, leading to a shift toward an IL-17 profile (Yan et al. 2018). IL-23 is known to promote tumour growth and metastasis by initiating angiogenesis and activating inflammation by MMP9 (matrix metallopeptidase 9) upregulation (Langowski et al. 2006). It also reduces infiltration of CD8+ T cell into tumour tissue, thus downregulating immune surveillance.

In humans, a significant upregulation of IL-23 is seen in many cancer forms, such as colon, ovarian, lung, breast, head and neck, stomach cancer and melanoma (Langowski et al. 2006). The overexpression of IL-23 in human cancer is sometimes seen in concurrence with low levels of IL-12 (Yan et al. 2018). In advanced colorectal cancer, a significant increase in serum levels of IL-23 was observed compared to controls (Stanilov et al. 2010). Thus, IL-23 may serve as a potential cancer biomarker as it is not only related to presence of disease but may

also provide prognostic information as it is related to poorer prognosis in several cancers as mentioner earlier.

2.3.3 Targeting IL-12 and IL-23 in cancer immunotherapy

IL-12 as a monotherapy has shown great results in preclinical studies. However, in the clinical setting in human medicine, problems like dose toxicity in combination with insufficient anti-tumour responses in humans has led to disappointing results (Nguyen et al. 2020). The lack of efficacy is thought to occur because it is not systemic but rather local administration of IL-12 in the TMT that provides effective tumoricidal effects without systemic dose-toxicity. In veterinary medicine however, IL-12 has shown to be quite effective for treating several types of tumour in both large and small animals (Pavlin et al. 2012).

In mice with experimentally induced melanoma, administering CD8+ T lymphocytes that have been primed *ex vivo* with IL-12 resulted in tumour regression as well as creation of persisting memory T-cells directed toward tumour antigen (Díaz-Montero et al. 2011). Another study in mice with B cell-lymphoma, CAR T cells, T cells engineered with a receptor specific for tumour-antigen intended to target tumour cells specifically, that were modified to express IL-12 lead to eradication of tumour (Kueberuwa et al. 2017). Similar results with increased survival and improved anti-tumour cytotoxicity in preclinical mice-models were seen in both ovarian (Koneru et al. 2015) and hepatocellular carcinoma (Liu et al. 2019).

In a clinical study in humans with glioblastoma where the resection cavity was treated locally with IL-12, a local increase of IFN-γ producing T lymphocytes was observed, switching the TMT from a pro to an anti-tumorigenic profile (Chiocca et al. 2019). Thus, IL-12 has shown potential as an alternative immunotherapy, especially in combination with other immunotherapies. However, further studies are required to improve the safety of IL-12 to avoid adverse effects, mainly by invention of techniques that enable a local delivery directly into the TMT rather than systemically (Mirlekar & Pylayeva-Gupta 2021).

In animal models, mice with transplanted tumours that were depleted of IL-23 showed a restriction in tumour growth. In addition, by depleting IL-23 through administration of antibodies, an increase in tumour-infiltrating cytotoxic T lymphocytes was observed. In mice with melanoma, injection with IL-23 lead to a decrease in infiltration of CD8+ T lymphocytes. Conversely, injection with IL-12 increased cytotoxic activity in tumour, consistent with results from studies discussed earlier. This suggests that IL-23 also plays a role as a target for immunotherapy since blockage of IL-23 signalling seem to alter the TMT to promote anti-tumour immunity. (Langowski et al. 2006)

3. Materials and method

3.1 Patient groups

The recruitment of controls were done using already collected samples from individuals that could be defined as healthy based on normal serum biochemistry and hematology, which is why blood donors were used as controls. Since analysis of these parameters were performed, dogs with normal values and no clinical symptoms that were deemed suitable blood donors were considered appropriate candidates as controls in this study. For patients with lymphoma, mast cell tumours and mammary tumours, the requirement to participate was a definite clinical diagnosis of the disease for each patient, which varied from case to case. In the lymphoma croup, FNA of palpable lymph nodes or FNA with ultrasonographic guidance of affected organs were a common method of diagnoses. In the mast cell tumour group, FNA was a common diagnostic tool including ultrasonographic diagnosis from one patient in this group. In mammary tumours, surgical removal and histopathological examination was the standard mode of diagnosis of disease.

3.2 Sample collection

Appropriate candidates, canine patients of all ages and breeds, were assessed upon presentation of suspected lesions or other symptoms. Before sampling appropriate candidates, owners signed a consent form agreeing on the participation of their dogs in the study. This was aligned with ethical permission according to C103/10, C2/12, C12/15, and 5.8.18-04682/2020. Blood samples were collected in serum and EDTA tubes using a closed vacutainer system. The area was clipped and sterilized before sampling. Serum samples were centrifuged 30 minutes after sampling. The serum was then transferred into separate tubes. Blood samples that required transportation were transported with freeze clamps. All samples were then stored in the canine biobank at SLU Small Animal University Hospital until analysis.

Fine needle aspiration was performed from suspected lesions to confirm malignant neoplasia and sort out patients with benign processes. This was done using a 22G

needle and syringe. The sample was then transferred onto microscopic slides. Intra-abdominal lesions were sampled using ultrasound-guided technique. Cutaneous lesions were sampled directly. The slides were fixed and dyed routinely. In patients that underwent surgical removal of suspected tumours where tissue was sent for pathological-anatomical diagnosis (PAD), only the consent-form and blood sampling was required. The PAD lab-result was then noted to confirm the diagnosis as it arrived in the medical chart from each patient. On some of the patients where lymphoma was suspected, PCR for Antigen Receptor Rearrangements (PARR) was performed as well.

Sampling from appropriate canine patients was undertaken in Östersunds Small Animal Hospital, and at the Oncology Devision at the Small Animal University Hospital (UDS). Healthy individuals used as a control-group were collected from the canine biobank at SLU Small Animal University Hospital, consisting mainly of blood donors. New sampling took place from March 2023 until November 2023. Older samples in which malignant neoplasia was previously confirmed were used to achieve sufficient data for analysis, as some newly sampled patients appeared to have benign lesions upon PAD analysis or cytology. When choosing appropriate samples for analysis, three different tumour groups were analysed: lymphomas, mammary tumours and mast cell tumours.

3.3 Analysis of serum samples

3.3.1 Canine IL-12p40 ELISA

The IL-12 family of cytokines in which IL-12 and IL-23 are included, share several subunits. IL-12 consists of subunits p40 and p35, whereas IL-23 consists of subunits p40 and p19 (Mondal et al. 2020). The analysis used in this study measures the level of IL-12p40 in serum, the subunit shared between both cytokines, using enzyme-linked immunosorbent assay (RayBio® Canine IL-12 p40 ELISA Kit, Atlanta, USA). Each sample from each patient was analysed in duplicates.

Reagents and samples were brought to room temperature before analysis. Blood serum was diluted 1-3 fold with 1X Assay Dilutent from the kit. The 1X Assay Dilutent was diluted 5 fold with distilled water before use. The kit contains 96 wells coated with canine IL-12p40 antibody, to which samples and standards were added and then washed. Any IL-12p40 present in samples bound to their complementary antibodies in the wells. After the first round of washing, biotinylated anti-Canine IL-12p40 antibody was added. The plates were washed again to remove any unbound anti-Canine IL-12 p40 antibody. HRP-conjugated streptavidin was then added to produce a transition in colour from blue to green in those wells that IL-12

p40 was present. The degree of colour-transition corresponded to the concentration of IL-12 p40 in the sample and was assessed at 450 nm. Each sample from each individual was analysed twice.

3.3.2 CRP

C-reactive protein was analyzed in the majority of patients. One patient in each group did not have their serum CRP measured and were therefore not included in the data anlysis of this parameter. CRP in serum was measured at the Clinical Chemistry Laboratory at the SLU Small Animal University Hospital. When calculating average CRP values, patients with values <7 was set equal to 0.

3.4 Staging of tumours

3.4.1 Lymphoma

Staging of lymphoma was performed according to the WHO staging of canine multicentric lymphoma, as seen in table 1. The number of dogs within each stage is also presented. Staging was performed based on clinical examination and diagnosis via FNA and imaging diagnostic tools with appropriate sampling from affected organs.

Table 1: Staging of canine lymphoma patients. A total of 14 patients with lymphoma were analyzed and staged.

Stage	**Number of dogs**
1 - single lymph node involvement	0
2 - enlarged lymph nodes on one side of the diaphragm	4
3 - involvement of lymph nodes both sides of the diaphragm	5
4 - liver and/or spleen involvement	2
5 – involvement of bone marrow, CNS or other distant sites	3

3.4.2 Mast cell tumours

Mast cell tumours were staged on a scale of 1-4 explained in table 2, based on the number of tumours present, if lymph nodes are involved and if there are distant metastases present. The number of dogs within each stage are also represented. Staging was based on clinical examination, histopathological examination of specimen upon surgical removal and imaging diagnostic tools and appropriate sampling when metastases were suspected.

Table 2: Staging of canine patients with mast-cell tumours. A total of 5 dogs were diagnosed and staged.

Stage	Number of dogs
1 - one tumor on the skin with no involvement of lymph nodes	1
2 - skin tumour and lymph node involvement	2
3 - correspond to multiple skin tumors with or without lymph node involvement	1
4 - distant metastasis	1

3.4.3 Mammary tumours

Mammary tumours were staged according to the TNM system of staging, presented in table 3. A total of 4 dogs were analyzed and staged. The number of dogs within each stage are represented. Staging was based on clinical evaluation of tumors and histopathological examination of specimens after surgical removal, including imaging diagnostic tools when appropriate.

Table 3: Staging of canine patients with mammary tumours.

Stage	Number of dogs
1 – one solid tumour <3 cm	1
2 – one tumour 3-5 cm in size	1
3 – tumour >5 cm in size	
4- tumour with lymph node involvement	1
5 – distant metastasis	1

3.5 Data analyses

Results from ELISA were retrieved in excel. The mean absorbance was read and a standard curve was plotted. The y-axis represented absorbance and the corresponding serum concentration of IL-12p40 was presented on the x-axis. Thus, the serum concentration of IL-12p40 could be determined using the known absorbance and the curve. Since each sample was analysed in duplicates, the mean serum concentration of IL-12p40 from each individual was also retrieved from which the inferential statistical analyses were performed. The data presented in the box plots and calculation of mean, median, maximum, and minimum values were performed using the two separate measurements from each individual. Hence, more accurate values could be retrieved as the sample size was generally small for each group, especially in the mammary tumour and mast-cell tumour groups. T-tests were performed in each group and compared to controls, and between tumour groups. A statistically significant difference was defined as a p value <0.05.

Difference in CRP between tumour groups were analyzed, and a p value of <0.05 was considered statistically significant. The correlation between CRP and seum IL-12p40 levels in each group were also analyzed with respect to the same p value.

4. Results

4.1 Physical parameters in tumour groups and controls

4.1.1 Age

Ages at sampling in the lymphoma ranged from 3 to 11 years old, with a mean age of 7.2 years old. In the mammary-tumour group, one patient was 10 years old, two 9 years old and one patient was one year old, with a mean age of 7.25. In patients with mast-cell tumours age at sampling ranged from 3 to 13 years old with an average age of 7-8 years. As the control group was generally made up of blood donors, the individuals were younger with an average age of 2.6 years old, and a range from 1 to 5 years in the group.

Table 4: Mean age in years at sampling in controls and patients with tumours. 13 controls, 14 patients with lymphoma, 4 patients with mammary tumours and 5 patients with mast cell tumours were studied.

	Controls	**Lymphoma**	**MMT**	**Mast cell**
Mean age (years)	2.2	7.2	7.3	7.8

4.1.2 Breed

A total of 21 breeds were included in the study, counting both controls and patients with tumours. The most common breeds were mixed breed and Flatcoated Retriever, followed by Boxer. One patient from the lymphoma and mammary tumour groups were of unknown breed and are not included in the table.

Table 5: Breed distribution in each group of patients including controls. N represents the number of dogs in each group wher breed could be identified.

Breed	**Controls**	**Lymphoma**	**MMT**	**Mast cell**
Mixed	1	3		
Boxer		2		1
Boerboel		1		
Border Collie		1		
Briard			1	
Collie	1			
English Setter		1		
Flatcoated Retriever	3			1
German Shepherd	1			
Golden retriever		1		
Greenland dog	1			
Labrador retriever	1		1	
Malamute		1		
Mastiff	1			
Nova Scotia Duck Tolling Retriever	1			1
Perro de Agua				1
Rhodesian Ridgeback		1		
Rottweiler			1	1
Staffordshire Bullterrier		1		
Saluki	1			
Wachtel	1			
White Swiss Shepherd		1		
	n = 13	*n = 13*	*n = 3*	*n = 5*

4.1.3 Gender

Table 6: Gender distribution in each tumour group and controls

Sex	Controls	Lymphoma	MMT	Mast cell
Male	10	8	0	2
Female	3	6	4	3
	n = 13	*n = 14*	*n = 4*	*n = 5*

A total of 10 males and 13 females were analyzed in the tumour groups. In the control group, only 3 out of 10 dogs were females.

4.1.4 Weight

Table 7: Average weight distribution between groups

	Controls	Lymphoma	MMT	Mast Cell
Average weight (kg)	33.4	34.3	26.7	25.8
	n = 13	*n = 14*	*n = 4*	*n = 5*

Average weight in the control group and lymphoma group appeared very similar. The MMT group and Mast-cell tumour group displayed similar average weight but lower than that of the other two groups.

4.2 Serum concentration of IL-12p40 in canine patients with lymphoma

A total of 13 patients diagnosed with high grade lymphoma and one patient diagnosed with leukemia were analyzed as one tumour-group and compared to a control group consisting of 13 patients. All lymphoma patients were staged according to the WHO staging of canine multicentric lymphoma. Tumour stages in patients in this study ranged from stage 2-5 with an average stage of 3.

The average serum concentration of IL-12p40 in dogs with lymphoma was significantly higher than in controls ($p = 0.0013$), with an average of 1013.67 pg/mL compared to 334.47 pg/mL in the control group. Half of the patients in the lymphoma-group displayed values >1000 pq/mL, whereas only one dog in the control group had a value >1000 pq/mL. Apart from this outlier and two other individuals, the remaining 10 dogs in the control group all displayed values <300 pq/mL compared to the lymphoma group where all patients displayed values >300 pq/mL. The median, minimum and maximum values in dogs with lymphoma dogs

were 1037.99, 311.57 and 1891.02 pg/mL respectively. In controls, the median, minimum and maximum values were 209.4, 109.02 and 1478 pg/ml respectively. Two outliers were identified in the control group, one individual with the maximum value of 1478.4 and another with a value of 636.39. The first individual had leukocytosis of unknown cause, and the other displayed eosinophilia. Both individuals were clinically healthy and, after another sample, still deemed suitable as blood donors.

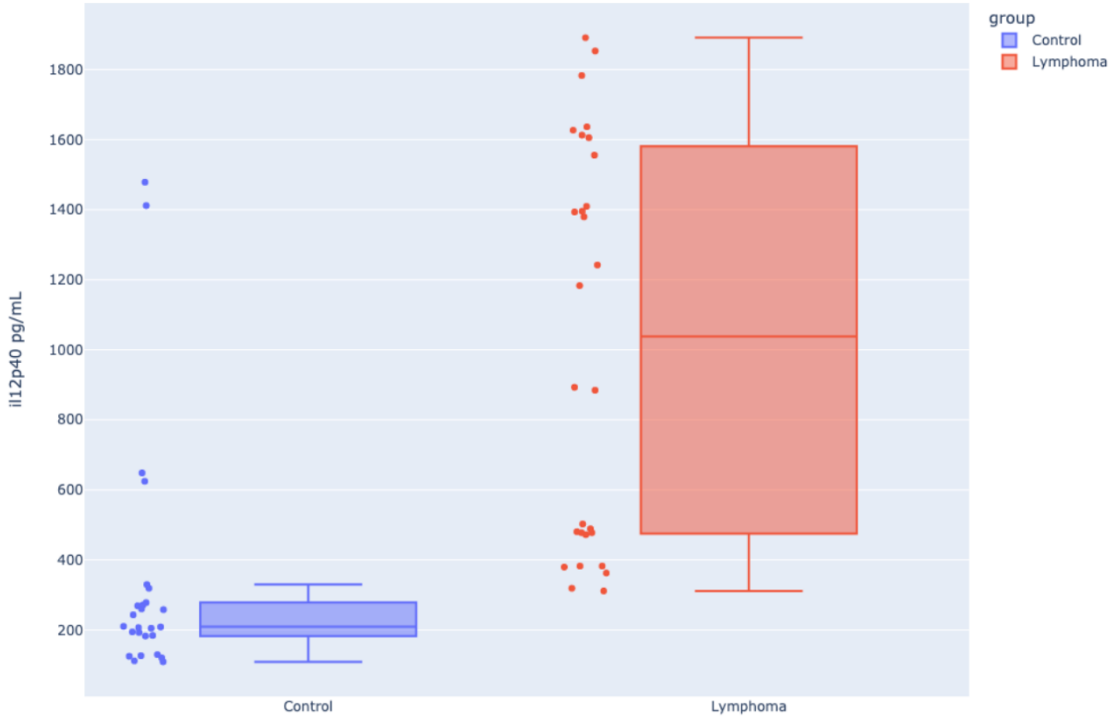


Figure 1: Box plot presentation of serum concentration of IL-12p40 in control group and patients with lymphoma. Data is presented using two separate measurements for each individual as sample size is small. A total of 14 dogs with lymphoma were analyze and compared to 13 controls.

4.3 Serum concentration of IL-12p40 in canine patients with mast cell tumours

Only 5 patients with mast cell tumors were analyzed and compared to the same control group. The tumors were graded on a scale of 1-4 as described in the section on materials and method. Tumours in this group scoped all stages, with a mean stage of 2.4.

The average serum concentration of IL-12p40 in this group was 171.7 pg/mL, and therefore lower than that of controls. No statistically significant difference was found between the groups ($p = 0.145$). All samples in the mast cell tumor group

displayed values <300 pg/mL. Interestingly, the only patient with stage 4 disease had the lowest serum concentration of IL-12p40 of in the group (96.1pg/mL). The median, minimum and maximum values for mast cell tumours were 138.05, 95.2 and 289.5 pg/mL respectively. Thus, it is generally lower than that of controls.

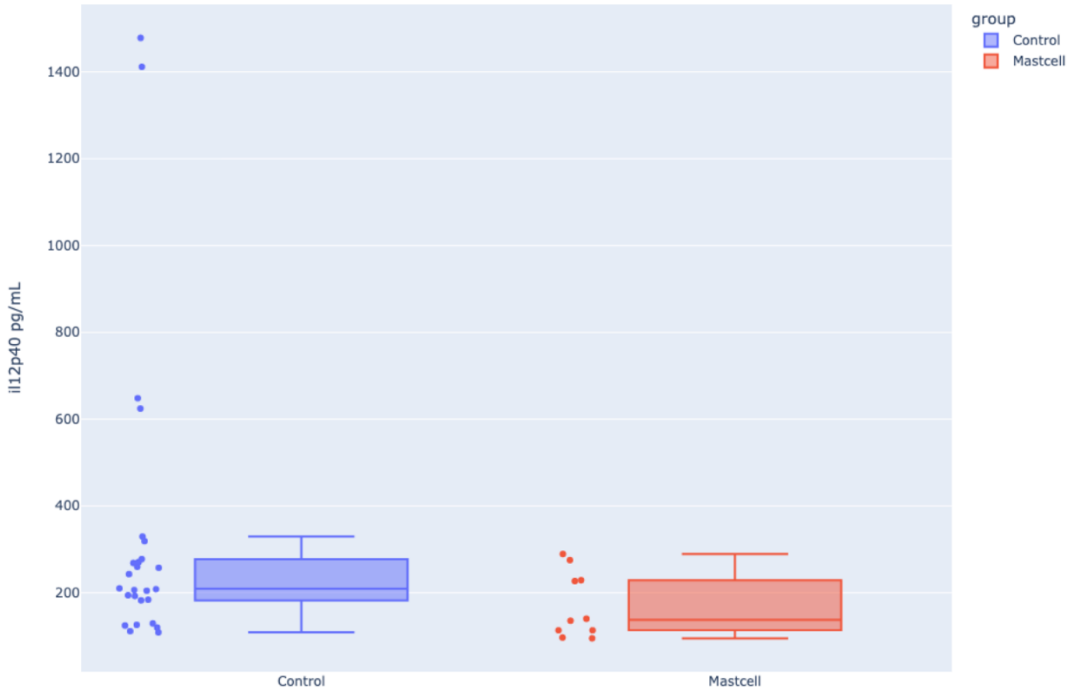


Figure 2: Box plot presentation of serum concentration of IL-12p40 in control group and patients with mast cell tumours. Data is presented using two separate measurements for each individual as sample size is small. A total of 5 dogs with mast cell tumours were analyzed and compared to 13 controls.

4.4 Serum concentration of IL-12p40 in canine patients with mammary tumours

A total of 4 patients with mammary carcinomas were analyzed. The patients were staged according to the TNM system as described in the section on materials and method. The mean stage was 3, as only four patients were analyzed and displayed stage 1, 2, 4 and 5 respectively.

The average serum concentration of IL-12p40 in the mammary tumour group was 227.6, and no statistically significant difference was found when compared to controls ($p = 0.442$). The median, minimum and maximum values in dogs with mammary tumours were 193.0, 72.7 and 455.1 pg/mL respectively.

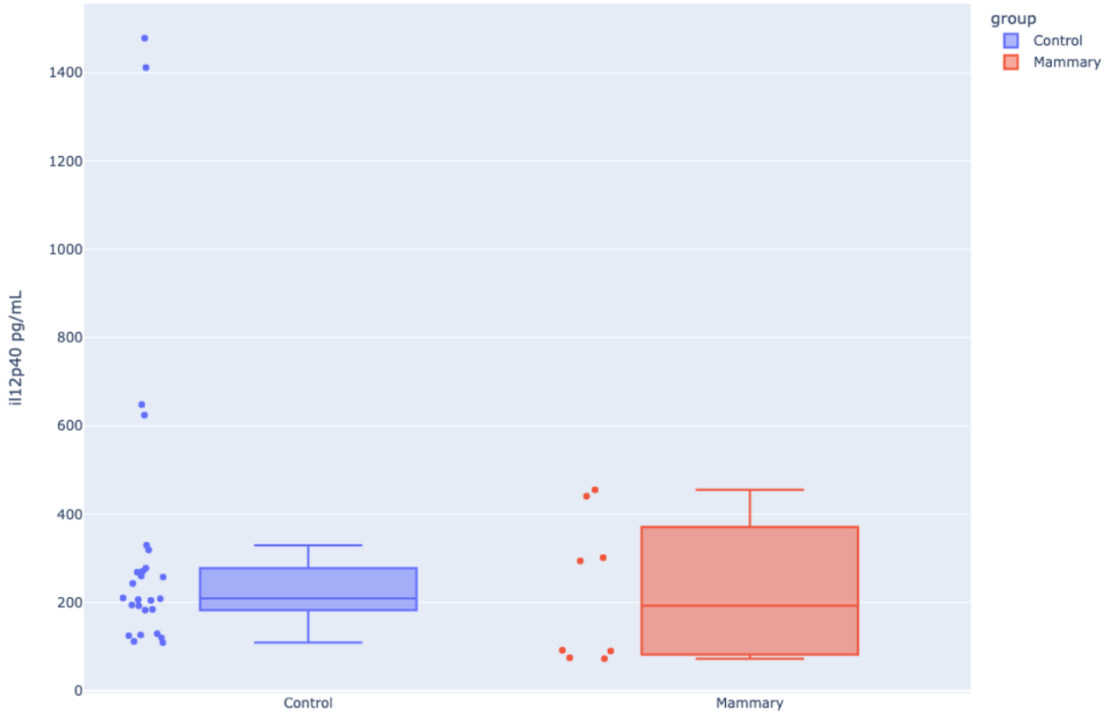


Figure 3: Box plot presentation of serum concentration of IL-12p40 in control group and patients with mammary tumours. Data is presented using two separate measurements for each individual as sample size is small. A total o 4 dogs with mast cell tumours were analyzed and compared to 13 controls.

4.5 Difference in IL-12p40 between tumour groups

There was a statistically significant difference between the lymphoma group compared to the other two groups respectively. The concentration of IL-12p40 in dogs with lymphoma was markedly increased compared to mammary tumours ($p = 0.0005$) and mast-cell tumours ($p = 0.0001$). No significant difference was found when comparing mammary tumours to mast-cell tumours ($p = 0.6$).

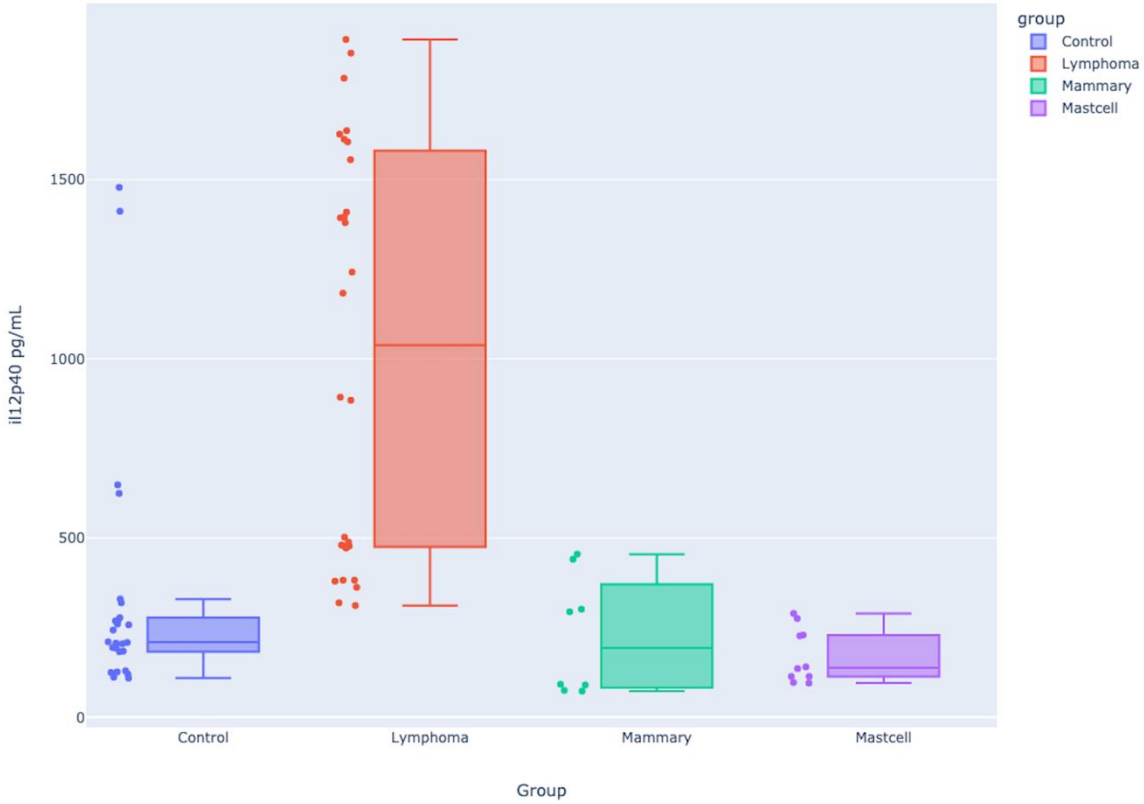


Figure 4: Box plot comparing representing the difference in concentration of IL-12p40 in all groups. Data is presented using two separate measurements for each individual as sample size is small. A total of 23 dogs with cancer were analyzed and compared to 13 controls.

4.6 Association between serum concentration of IL-12p40, C-reactive protein and tumour stage

4.6.1 C-reactive protein

All but one patient in the lymphoma group also had a blood-test result for their serum C-reactive protein (CRP), 3 out of 4 in the mammary tumour group and 4 out of 5 patients in the mast-cell tumour group. An average CRP value of 53.8 was found in the lymphoma group, compared to 3.7 in mammary tumours and 23.5 in mast cell tumours respectively. There was a statistically significant difference in the CRP value in the lymphoma group compared to the mammary tumour group ($p = 0.008$). There were no statistically significant differences in CRP between lymphoma and mast-cell tumours ($p = 0.1$) or mammary tumours and mast cell tumours ($p = 0.05$).

No statistically significant correlation between CRP and serum concentration of IL-12p40 were found in the lymphoma group, with an R^2 value of 0.02 ($p = 0.6$). The

same was true for mast-cell tumours, with an even lower R-value of 0.007 (p = 0.9). On the contrary, an R^2 value of 1.0 was found in the mammary tumour group, with a statistically significant correlation between serum level of IL-12p40 and CRP (p = 0.03).

Figure 5: Bubble diagram representing correlation between serum IL-12p40 and serum CRP levels with lines of best fit for each tumour group. Size of the bubble represents tumour stage,, the larger the size the higher the stage.

4.6.2 Tumour stage

As described earlier, every patient was staged in relation to tumour burden according to each type of tumour. Analysis of correlation between tumour stage and serum concentration of IL-12p40 revealed similar results as that of the correlation between CRP and IL-12p40.

No significant correlations between stage of tumour and IL-12p40 were found in the lymphoma or mast-cell tumour patients, with an R^2 value of 0.09 (p = 0.3) and 0.02 (p = 0.9) respectively. In the mammary tumour group however, statistically significant correlation between stage and IL-12p40 was found, with an R^2 value of 0.9 (p = 0.04). The data with the line of best fit for each group is plotted in figure x. The size of the circles represent CRP value, the larger the circle the higher the CRP. Patients with CRP <7 or where values were missing, which was the case for

one patient in each group, the values were set to 6 so that they would appear on the graph. This is because all tumours were staged, and the plot represents correlation between serum levels of IL-12p40 and tumour stage.

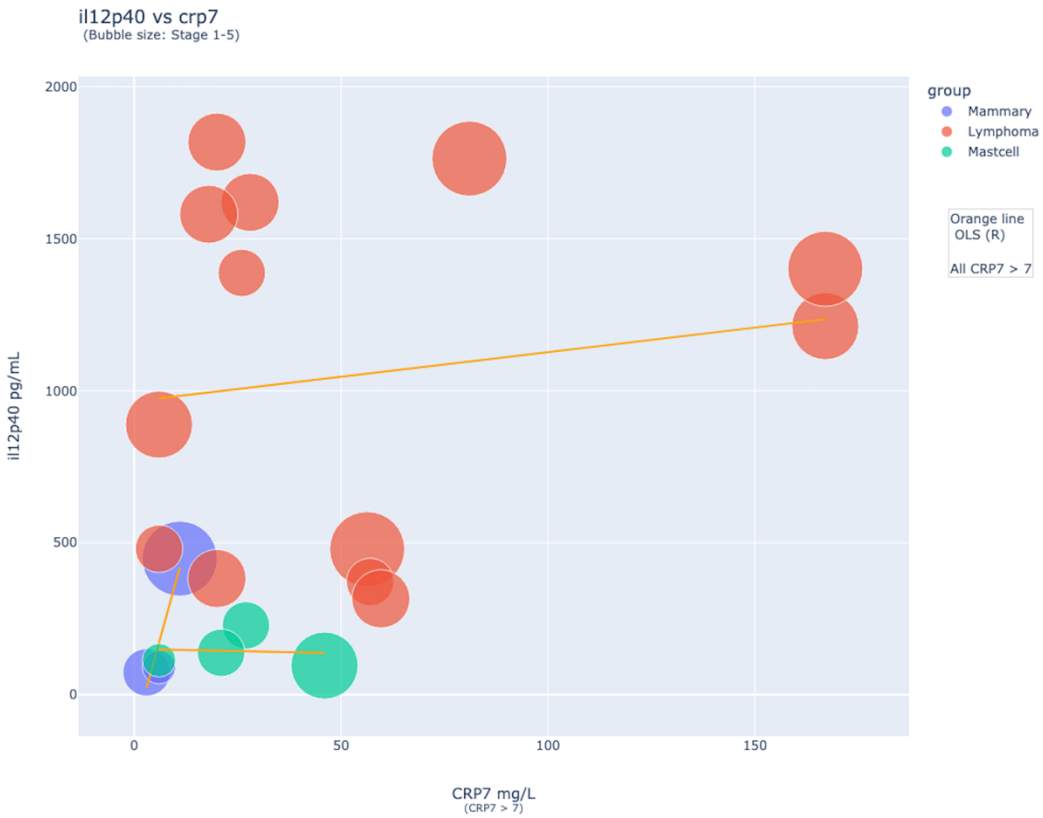


Figure 6: Bubble diagram representing correlation between serum IL-12p40 and tumour stage with lines of best fit for each tumour group. Size of the bubble represents CRP value, the larger the size the higher the CRP. Dogs with CRP <7 was set equal to 6 so that they would appear on the graph, and the same was done for dogs with no values.

5. Discussion

5.1 Differences in IL-12p40 levels among tumour groups and controls

In this study, a statistically significant increase in serum concentration of IL-12p40 was found in patients with lymphoma compared to controls as well as the other two tumour groups. There may be several potential explanations for this, depending on if the assumption is that there may be elevated levels of either IL-12 or IL-23, or potentially both. Because IL-12p40 is not only a component of IL-12, but also combines with the subunit p19 to form the IL-23 cytokine, measurement of IL-12p40 cannot distinguish between the two cytokines. Hence, it is not clear whether the patients in this study with elevated serum concentrations of IL-12p40 harboured predominantly high levels of IL-12 or high levels of IL-23.

The reason why a significant difference in the serum IL-12p40 levels in the lymphoma- group may be due to the larger sample size compared to the two small groups of mammary and mast cell tumours. The reason why the lymphoma-group was generally larger may be because lymphoma is the most common type of neoplasia currently diagnosed in canine patients (M 2016). A potential explanation for the increased serum-levels of IL-12p40 in these patients, regardless of group-size, may be that it is an aggressive type of cancer. Patients with intermediate- to high-grade lymphoma, such as those in this study, that do not receive treatment, will succumb to their disease within 4-6 weeks from diagnosis (Vail et al. 2019). When compared to the group with mammary carcinomas, even when these tumours are particularly aggressive, the median survival time is 11 months after surgical removal (Chocteau et al. 2021). Similarly with regards to mast cell tumours, a study on subcutaneous mast-cell tumours, which all of the patients in this group had in this study, the minimum mean survival time after surgery with incomplete excision in 66% of cases and metastasis in 6% of cases was reported as being around 3 years (Newman et al. 2007). Thus, both mammary and mast-cell tumours are generally considered less aggressive when compared to lymphoma.

On a prognostic perspective, chemotherapy, the standard line of treatment of high-grade lymphoma in dogs, lead to complete remission in 60-90% of cases with a median survival of 6-12 months (Vail et al. 2019). This is generally lower than the expected survival after surgical treatment of mammary and mast-cell tumours, or in some instances similar to very aggressive mammary carcinomas. Although survival time is definitively prolonged upon treatment of lymphoma, cures are rare and the disease is considered generally fatal. This again supports the claim that high-grade lymphoma is generally considered to be a more aggressive type of cancer in comparison to mammary tumours and mast cell tumours. This may provide some explanation as to why the concentration of IL-12p40 differs was significantly higher in lymphoma patients compared to the other tumour groups. It also suggests that serum IL-12p40 may play a role as a potential prognostic biomarker in canine cancer.

As discussed in the literature review, circulating levels of IL-12 in human colorectal cancer greatly decreased with disease progression. It may therefore be discussed that the increase in IL-12p40 in patients with lymphoma may represent an increase in IL-23 which has been shown to be overexpressed in several types of cancers in humans. To confirm this, following the patients and performing repeated measurements, preferably of IL-12 and IL-23 specifically, could be useful. Overall, the sample sizes in patients with mammary tumours and mast cell tumours were very small, and investigation of the levels of IL-12p40 in larger groups could provide more accurate data.

In summary, the results show that IL-12p40 may serve a role as a potential biomarker for canine lymphoma, as the levels were significantly higher in this group than that of controls. Even though two outliers were identified in the control group, possible reasons for this were found (with one patient having leucocytosis and the other eosinophilia). Thus, the values in the control-group was generally significantly lower than those in the lymphoma group. This suggests that serum IL-12p40 in lymphoma patients differ from that of the clinically healthy canine population, although larger groups of controls would be useful to draw further conclusions.

5.2 Correlation of serum IL-12p40 levels and C-reactive protein

No statistically significant correlations between serum CRP and IL-12p40 were found in the lymphoma group. The same was true for mast-cell tumours. In the mammary tumour group however, a significant correlation was found, which shall

be interpreted with caution since the sample size is very small. Despite the lack of linear correlation with IL-12p40 in the lymphoma group, the average serum CRP value was generally higher in this group, and when compared to mammary tumours it was significantly higher ($p < 0.05$). Therefore, it cannot be ruled out that the increase in IL-12p40 in this group may represent a systemic inflammation which may be attributed to a dysregulation of the immune system caused by the cancer itself. However, as in the study by von Euler et al. 2006, the levels of IL-6 in dogs with lymphoma are usually significantly higher. As IL-6 is well known to induce CRP production, the reason for the elevated CRP levels in the lymphoma group can most likely be attributed to IL-6 rather than elevated levels of IL-12 and/or IL-23.

Since tumours are striving to create a favorable TMT by shifting the cytokine-profile to one that promotes tumour survival, there may be higher levels of IL-23 in serum present. This may not cause such a significant increase in CRP, as the systemic inflammation is not as pronounced as if there were higher circulating levels of IL-12 - which is logical since the tumour attempts to avoid immune detection. This may explain why no definite correlation between IL-12p40 and CRP could be seen in the lymphoma or mast-cell tumour group, since the immune system becomes dysregulated to favor tumour-survival. This also touches on the difficulty to interpret the levels of IL-12p40 as either an increase in IL-12 or IL-23 or both. To investigate this phenomenon further, apart from repeating analysis with larger sample sizes, evaluating the correlation between CRP levels and IL-12 levels in serum could be useful.

5.3 Correlation of serum IL-12p40 levels and stage of disease/metastasis

Like the analysis of CRP, no statistically significant correlations between tumour stage in lymphoma or mast cell tumours and IL-12p40 were found. The lack of correlation may thus argue against its potential use as a prognostic marker in cancer. On the contrary, a strong correlation between IL-12p40 and stage of mammary tumour suggests that it may be useful in some cancers. However, it is important to note that the sample size is generally small throughout all groups, and definite conclusions cannot be drawn without analysing larger groups of patients as well as patients with other types of cancer.

Early studies have shown that IL-12p40 acts in a negative-feedback manner by down-regulating the effector functions of IL-12 through competitive binding to the IL-12Rβ1 receptor, the receptor subunit common to both IL-12 and IL-23 (Mattner et al. 1997). Because it binds to the shared receptor subunit, the action of IL-12 and

IL-23 on their respective receptors is limited and it is therefore considered having an antagonistic role. For example in mice, tumours engineered to produce IL-12p40 repressed IL-12 anti-tumour mediated immune response, as well as the anti-tumour specific IL-23 mediated responses in splenocytes (Shimozato et al. 2006). This might provide a possible explanation as to why patients in the lymphoma and mammary tumour groups, with more widespread/higher staged disease, also displayed higher serum levels of IL-12p40 - as it may represent a dysregulation of the immune system in these patients.

A possible reason for the lack of association between stage of tumour and IL-12p40 in these groups may be an imbalance between the levels of IL-12 and IL-23. This balance can differ between tumour types as explained in the literature, since it is not always that high levels of IL-23 is found in concurrence with low levels of IL-12. Since we cannot distinguish between IL-12 and IL-23 in this study, it is difficult to determine the exact reason for this.

5.4 Potential independent role of IL-12p40

Recent studies have proposed that IL-12p40 may have an independent agonistic role in initiating an early immune response, in contrast to the generally accepted concept of its antagonistic function on the IL-12Rβ1 receptor subunit (Cooper & Khader 2007). Interestingly, early data has shown that IL-12p40 is secreted in a wide range of inflammatory conditions and is present in excess in both Th1 and Th2 type responses in both mice and humans (Abdi 2002). It was suggested that IL-12p40 secreted from APCs may dimerize with other molecules in the local inflammatory environment, or as in the case of cancer in the TMT. These dimers then communicate with T cells to drive the appropriate immune response depending on the condition, for example which pathogen the host is infected with or if there is an ongoing autoimmune response.

Thus, as discussed previously, this may explain why many of the patients in this study with concurrently elevated CRP levels displayed higher serum levels of IL-12p40 independent of tumour type. Regardless of whether the IL-12p40 levels represented either an abundance IL-12 or IL-23, it is rather the inflammation itself in cancer and the dysregulation of the immune response that may be the reason for the excess serum levels of IL-12p40.

5.5 Role of IL-12 in lymphoma

Although the anti-tumour immunity effect of IL-12 is well established, studies have shown that the role of IL-12 in lymphoma may deviate from that of other types of tumour (Charbonneau et al. 2012; Yang et al. 2012). In human follicular lymphoma, an extended exposure to IL-12 led to an exhaustion of T cells leading to poorer prognosis. This is a dysfunction T-cells due to expression of receptors coding for inhibitory signals and down-regulated effector function, which is seen not only in cancer but also in in infections (Wherry 2011). This leads to a suboptimal immune response and prevents the immune system from gaining control over tumours and cancer cells. Therefore, there is also a possibility that the elevated levels of IL-12p40 in dogs with lymphoma in this study could represent an increase in IL-12.

It should be noted that the mechanism by which long term IL-12 exposure induces T-cell exhaustion in lymphoma is not through IFN- γ production, but seem to occur independently through a STAT4 mediated mechanism (Yang et al. 2012). This is important because, as discussed, IFN- γ is strongly correlated to anti-tumour immunity regardless of tumour type and is an important component in the otherwise tumoricidal effect of IL-12. However, in canine large B-cell lymphoma, factors within the TMT inhibited the tumoricidal effect of CD8+ cytotoxic T lymphocytes via secretion of immunosuppressive cytokines such as IL-10 (Weng et al. 2023). Thus, the effector function of T-cells in lymphoma seems to be down-regulated in both human and canine patients. However, the long-term exposure of IL-12 in canine patients with lymphoma remains to be investigated.

6. Conclusion

A statistically significant increase in serum levels of IL-12p40 was found in patients with lymphoma compared to controls, which may suggest it could be of potential use as a biomarker for lymphoma. However, measuring the serum levels of IL-12p40 both before and after initiating for lymphoma-treatment protocols may be useful to evaluate the prognostic value of this marker, and thus no conclusion could be drawn in this regard. Since a great overlap in levels of IL-12p40 was observed between controls, mammary tumours and mast cell tumours, no conclusion can be drawn in this study as to if the levels may differ between these groups. Therefore, evaluation of its potential use as a biomarker for disease detection, prognosis and treatment remains unclear.

References

Abdi, K. (2002). IL-12: The role of p40 versus p75. *Scandinavian Journal of Immunology*, 56 (1), 1–11. https://doi.org/10.1046/j.1365-3083.2002.01101.x

Aggarwal, S., Ghilardi, N., Xie, M.-H., Sauvage, F.J. de & Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T Cell activation state characterized by the production of interleukin-17. *Journal of Biological Chemistry*, 278 (3), 1910–1914. https://doi.org/10.1074/jbc.M207577200

Baba, O.K., Sood, N.K. & Gupta, K. (2019). Clinical evaluation of glycoproteins and inflammatory cytokines in the serum of dogs affected with canine mammary cancer. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, 89 (4), 1465–1469. https://doi.org/10.1007/s40011-018-01073-7

Baghban, R., Roshangar, L., Jahanban-Esfahlan, R., Seidi, K., Ebrahimi-Kalan, A., Jaymand, M., Kolahian, S., Javaheri, T. & Zare, P. (2020). Tumor microenvironment complexity and therapeutic implications at a glance. *Cell Communication and Signaling* : CCS, 18, 59. https://doi.org/10.1186/s12964-020-0530-4

Bi, L., Wu, J., Ye, A., Wu, J., Yu, K., Zhang, S. & Han, Y. (2016). Increased Th17 cells and IL-17A exist in patients with B cell acute lymphoblastic leukemia and promote proliferation and resistance to daunorubicin through activation of Akt signaling. *Journal of Translational Medicine*, 14 (1), 132. https://doi.org/10.1186/s12967-016-0894-9

Chalmin, F., Mignot, G., Bruchard, M., Chevriaux, A., Végran, F., Hichami, A., Ladoire, S., Derangère, V., Vincent, J., Masson, D., Robson, S.C., Eberl, G., Pallandre, J.R., Borg, C., Ryffel, B., Apetoh, L., Rébé, C. & Ghiringhelli, F. (2012). Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity*, 36 (3), 362–373. https://doi.org/10.1016/j.immuni.2011.12.019

Charbonneau, B., Maurer, M.J., Ansell, S.M., Slager, S.L., Fredericksen, Z.S., Ziesmer, S.C., Macon, W.R., Habermann, T.M., Witzig, T.E., Link, B.K., Cerhan, J.R. & Novak, A.J. (2012). Pretreatment circulating serum cytokines associated with follicular and diffuse large B-cell lymphoma: A clinic-based case-control study. *Cytokine*, 60 (3), 882–889. https://doi.org/10.1016/j.cyto.2012.08.028

Chiocca, E.A., Yu, J.S., Lukas, R.V., Solomon, I.H., Ligon, K.L., Nakashima, H., Triggs, D.A., Reardon, D.A., Wen, P., Stopa, B.M., Naik, A., Rudnick, J., Hu, J.L., Kumthekar, P., Yamini, B., Buck, J.Y., Demars, N., Barrett, J.A., Gelb, A.B., Zhou, J., Lebel, F. & Cooper, L.J.N. (2019). Regulatable interleukin-12 gene therapy in patients with recurrent high-grade glioma: Results of a phase 1 trial. *Science Translational Medicine*, 11 (505), eaaw5680. https://doi.org/10.1126/scitranslmed.aaw5680

Chocteau, F., Mordelet, V., Dagher, E., Loussouarn, D., Abadie, J. & Nguyen, F. (2021). One-year conditional survival of dogs and cats with invasive mammary carcinomas: A concept inspired from human breast cancer. *Veterinary and Comparative Oncology*, 19 (1), 140–151. https://doi.org/10.1111/vco.12655

Colombe, P., Béguin, J., Benchekroun, G. & Le Roux, D. (2022). Blood biomarkers for canine cancer, from human to veterinary oncology. *Veterinary and Comparative Oncology*, 20 (4), 767–777. https://doi.org/10.1111/vco.12848

Cooper, A.M. & Khader, S.A. (2007). IL-12p40: an inherently agonistic cytokine. *Trends in Immunology*, 28 (1), 33–38. https://doi.org/10.1016/j.it.2006.11.002

Croxford, A.L., Kulig, P. & Becher, B. (2014). IL-12-and IL-23 in health and disease. *Cytokine & Growth Factor Reviews*, 25 (4), 415–421. https://doi.org/10.1016/j.cytogfr.2014.07.017

Demaria, S., Pikarsky, E., Karin, M., Coussens, L.M., Chen, Y.-C., El-Omar, E.M., Trinchieri, G., Dubinett, S.M., Mao, J.T., Szabo, E., Krieg, A., Weiner, G.J., Fox, B.A., Coukos, G., Wang, E., Abraham, R.T., Carbone, M. & Lotze, M.T. (2010). Cancer and inflammation: promise for biological therapy. *Journal of Immunotherapy* (Hagerstown, Md. : 1997), 33 (4), 335–351. https://doi.org/10.1097/CJI.0b013e3181d32e74

Díaz-Montero, C.M., Naga, O., Zidan, A.-A.A., Salem, M.L., Pallin, M., Parmigiani, A., Walker, G., Wieder, E., Komanduri, K., Cole, D.J., Montero, A.J. & Lichtenheld, M.G. (2011). Synergy of brief activation of CD8 T-cells in the presence of IL-12 and adoptive transfer into lymphopenic hosts promotes tumor clearance and anti-tumor memory. *American Journal of Cancer Research*, 1 (7), 882–896

Ebersbach, C., Beier, A.-M.K., Thomas, C. & Erb, H.H.H. (2021). Impact of STAT proteins in tumor progress and therapy resistance in advanced and metastasized prostate cancer. *Cancers*, 13 (19), 4854. https://doi.org/10.3390/cancers13194854

Eisenring, M., vom Berg, J., Kristiansen, G., Saller, E. & Becher, B. (2010). IL-12 initiates tumor rejection via lymphoid tissue–inducer cells bearing the natural cytotoxicity receptor NKp46. *Nature Immunology*, 11 (11), 1030–1038. https://doi.org/10.1038/ni.1947

von Euler, H.P., Öhrvik, A.B. & Eriksson, S.K. (2006). A non-radiometric method for measuring serum thymidine kinase activity in malignant lymphoma in dogs. *Research in Veterinary Science*, 80 (1), 17–24. https://doi.org/10.1016/j.rvsc.2005.05.001

Floss, D.M., Moll, J.M. & Scheller, J. (2020). IL-12 and IL-23—close relatives with structural homologies but distinct immunological functions. *Cells*, 9 (10), 2184. https://doi.org/10.3390/cells9102184

Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pagès, C., Tosolini, M., Camus, M., Berger, A., Wind, P., Zinzindohoué, F., Bruneval, P., Cugnenc, P.-H., Trajanoski, Z., Fridman, W.-H. & Pagès, F. (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*, 313 (5795), 1960–1964. https://doi.org/10.1126/science.1129139

Grivennikov, S.I., Greten, F.R. & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell*, 140 (6), 883–899. https://doi.org/10.1016/j.cell.2010.01.025

Gudbrandsdottir, G., Aarstad, H.H., Bostad, L., Hjelle, K.M., Aarstad, H.J., Bruserud, Ø., Tvedt, T.H.A. & Beisland, C. (2021). Serum levels of the IL-6 family of cytokines predict prognosis in renal cell carcinoma (RCC). *Cancer Immunology, Immunotherapy*, 70 (1), 19–30. https://doi.org/10.1007/s00262-020-02655-z

Guéry, L. & Hugues, S. (2015). Th17 cell plasticity and functions in cancer immunity. *BioMed Research International*, 2015, 314620. https://doi.org/10.1155/2015/314620

Haabeth, O.A.W., Lorvik, K.B., Hammarström, C., Donaldson, I.M., Haraldsen, G., Bogen, B. & Corthay, A. (2011). Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer. *Nature Communications*, 2, 240. https://doi.org/10.1038/ncomms1239

Hanahan, D. (2022). Hallmarks of cancer: new dimensions. *Cancer Discovery*, 12 (1), 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

Hanahan, D. & Weinberg, R.A. (2000). The hallmarks of cancer. *Cell*, 100 (1), 57–70. https://doi.org/10.1016/S0092-8674(00)81683-9

Hutchison, S., Sahay, B., de Mello, S.C., Sayour, E., Lejeune, A., Szivek, A., Livaccari, A., Fox-Alvarez, S., Salute, M., Powers, L. & Milner, R. (2019). Characterization of myeloid-derived suppressor cells and cytokines GM-CSF, IL-10 and MCP-1 in dogs with malignant melanoma receiving a GD3-based immunotherapy. *Veterinary Immunology and Immunopathology*, 216, 109912. https://doi.org/10.1016/j.vetimm.2019.109912

Irac, S.E., Oksa, A., Jackson, K., Herndon, A., Allavena, R. & Palmieri, C. (2019). Cytokine expression in canine lymphoma, osteosarcoma, mammary gland tumour and melanoma: comparative aspects. *Veterinary Sciences*, 6 (2), 37. https://doi.org/10.3390/vetsci6020037

Jiang, T., Zhou, C. & Ren, S. (2016). Role of IL-2 in cancer immunotherapy. *Oncoimmunology*, 5 (6), e1163462. https://doi.org/10.1080/2162402X.2016.1163462

Kartikasari, A.E.R., Huertas, C.S., Mitchell, A. & Plebanski, M. (2021). Tumor-induced inflammatory cytokines and the emerging diagnostic devices for cancer detection and prognosis. *Frontiers in Oncology*, 11, 692142. https://doi.org/10.3389/fonc.2021.692142

Koneru, M., Purdon, T.J., Spriggs, D., Koneru, S. & Brentjens, R.J. (2015). IL-12 secreting tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors in vivo. *Oncoimmunology*, 4 (3), e994446. https://doi.org/10.4161/2162402X.2014.994446

Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K. (2009). IL-17 and Th17 cells. *Annual Review of Immunology*, 27 (1), 485–517. https://doi.org/10.1146/annurev.immunol.021908.132710

Kryczek, I., Banerjee, M., Cheng, P., Vatan, L., Szeliga, W., Wei, S., Huang, E., Finlayson, E., Simeone, D., Welling, T.H., Chang, A., Coukos, G., Liu, R. & Zou, W. (2009). Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood*, 114 (6), 1141–1149. https://doi.org/10.1182/blood-2009-03-208249

Kueberuwa, G., Kalaitsidou, M., Cheadle, E., Hawkins, R.E. & Gilham, D.E. (2017). CD19 CAR T cells expressing IL-12 eradicate lymphoma in fully lymphoreplete mice through induction of host immunity. *Molecular Therapy Oncolytics*, 8, 41–51. https://doi.org/10.1016/j.omto.2017.12.003

Kusaba, T., Nakayama, T., Yamazumi, K., Yakata, Y., Yoshizaki, A., Inoue, K., Nagayasu, T. & Sekine, I. (2006). Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. *Oncology Reports*, 15 (6), 1445–1451. https://doi.org/10.3892/or.15.6.1445

Langowski, J.L., Zhang, X., Wu, L., Mattson, J.D., Chen, T., Smith, K., Basham, B., McClanahan, T., Kastelein, R.A. & Oft, M. (2006). IL-23 promotes tumour incidence and growth. *Nature*, 442 (7101), 461–465. https://doi.org/10.1038/nature04808

Latchman, D.S. (1993). Transcription factors: an overview. *International Journal of Experimental Pathology*, 74 (5), 417–422

Liu, Y., Di, S., Shi, B., Zhang, H., Wang, Y., Wu, X., Luo, H., Wang, H., Li, Z. & Jiang, H. (2019). Armored inducible expression of IL-12 enhances antitumor activity of glypican-3-targeted chimeric antigen receptor-engineered T cells in hepatocellular carcinoma. *Journal of Immunology* (Baltimore, Md.: 1950), 203 (1), 198–207. https://doi.org/10.4049/jimmunol.1800033

M, Z. (2016). Canine lymphoma: a review. *The Veterinary Quarterly*, 36 (2). https://doi.org/10.1080/01652176.2016.1152633

Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. (2008). Cancer-related inflammation. *Nature*, 454 (7203), 436–444. https://doi.org/10.1038/nature07205

Margueron, R. & Reinberg, D. (2010). Chromatin structure and the inheritance of epigenetic information. *Nature Reviews.* Genetics, 11 (4), 285–296. https://doi.org/10.1038/nrg2752

Mattner, F., Ozmen, L., Podlaski, F.J., Wilkinson, V.L., Presky, D.H., Gately, M.K. & Alber, G. (1997). Treatment with homodimeric interleukin-12 (IL-12) p40 protects mice from IL-12-dependent shock but not from tumor necrosis factor alpha-dependent

shock. *Infection and Immunity*, 65 (11), 4734–4737. https://doi.org/10.1128/iai.65.11.4734-4737.1997

Mirlekar, B. & Pylayeva-Gupta, Y. (2021). IL-12 family cytokines in cancer and immunotherapy. *Cancers*, 13 (2), 167. https://doi.org/10.3390/cancers13020167

Mondal, S., Kundu, M., Jana, M., Roy, A., Rangasamy, S.B., Modi, K.K., Wallace, J., Albalawi, Y.A., Balabanov, R. & Pahan, K. (2020). IL-12 p40 monomer is different from other IL-12 family members to selectively inhibit IL-12Rβ1 internalization and suppress EAE. *Proceedings of the National Academy of Sciences*, 117 (35), 21557–21567. https://doi.org/10.1073/pnas.2000653117

Newman, S.J., Mrkonjich, L., Walker, K.K. & Rohrbach, B.W. (2007). Canine subcutaneous mast cell tumour: diagnosis and prognosis. *Journal of Comparative Pathology*, 136 (4), 231–239. https://doi.org/10.1016/j.jcpa.2007.02.003

Nguyen, K.G., Vrabel, M.R., Mantooth, S.M., Hopkins, J.J., Wagner, E.S., Gabaldon, T.A. & Zaharoff, D.A. (2020). Localized interleukin-12 for cancer immunotherapy. *Frontiers in Immunology*, 11, 575597. https://doi.org/10.3389/fimmu.2020.575597

Nishi, M., Batsaikhan, B.-E., Yoshikawa, K., Higashijima, J., Tokunaga, T., Takasu, C., Kashihara, H., Ishikawa, D. & Shimada, M. (2017). High STAT4 expression indicates better disease-free survival in patients with gastric cancer. *Anticancer Research*, 37 (12), 6723–6729. https://doi.org/10.21873/anticanres.12131

Numasaki, M., Fukushi, J., Ono, M., Narula, S.K., Zavodny, P.J., Kudo, T., Robbins, P.D., Tahara, H. & Lotze, M.T. (2003). Interleukin-17 promotes angiogenesis and tumor growth. *Blood*, 101 (7), 2620–2627. https://doi.org/10.1182/blood-2002-05-1461

Ostrand-Rosenberg, S. & Sinha, P. (2009). Myeloid-derived suppressor cells: linking inflammation and cancer. *Journal of Immunology* (Baltimore, Md. : 1950), 182 (8), 4499–4506. https://doi.org/10.4049/jimmunol.0802740

Pardoll, D.M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews*. Cancer, 12 (4), 252–264. https://doi.org/10.1038/nrc3239

Pavlin, D., Cemazar, M., Sersa, G. & Tozon, N. (2012). IL-12 based gene therapy in veterinary medicine. *Journal of Translational Medicine*, 10, 234. https://doi.org/10.1186/1479-5876-10-234

Portela, R.F., Fadl-Alla, B.A., Pondenis, H.C., Byrum, M.L., Garrett, L.D., Wycislo, K.L., Borst, L.B. & Fan, T.M. (2014). Pro-tumorigenic effects of transforming growth factor beta 1 in canine osteosarcoma. *Journal of Veterinary Internal Medicine*, 28 (3), 894–904. https://doi.org/10.1111/jvim.12348

Read, K.A., Powell, M.D., Sreekumar, B.K. & Oestreich, K.J. (2019). In vitro differentiation of effector CD4+ T helper cell subsets. In: Allen, I.C. (ed.) *Mouse Models of Innate Immunity*: *Methods and Protocols*. Springer. 75–84. https://doi.org/10.1007/978-1-4939-9167-9_6

Rébé, C. & Ghiringhelli, F. (2019). STAT3, a master regulator of anti-tumor immune response. *Cancers*, 11 (9). https://doi.org/10.3390/cancers11091280

Sakaguchi, S. (2004). Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology*, 22 (1), 531–562. https://doi.org/10.1146/annurev.immunol.21.120601.141122

Shimozato, O., Ugai, S., Chiyo, M., Takenobu, H., Nagakawa, H., Wada, A., Kawamura, K., Yamamoto, H. & Tagawa, M. (2006). The secreted form of the p40 subunit of interleukin (IL)-12 inhibits IL-23 functions and abrogates IL-23-mediated antitumour effects. *Immunology*, 117 (1), 22–28. https://doi.org/10.1111/j.1365-2567.2005.02257.x

Stanilov, N., Miteva, L., Deliysky, T., Jovchev, J. & Stanilova, S. (2010). Advanced colorectal cancer is associated with enhanced IL-23 and IL-10 serum levels. *Laboratory Medicine*, 41 (3), 159–163. https://doi.org/10.1309/LM7T43AQZIUPIOWZ

Stanilov, N., Miteva, L., Jovchev, J., Cirovski, G. & Stanilova, S. (2014). The prognostic value of preoperative serum levels of IL-12p40 and IL-23 for survival of patients with colorectal cancer. *APMIS*, 122 (12), 1223–1229. https://doi.org/10.1111/apm.12288

Thamm, D.H., Kamstock, D.A., Sharp, C.R., Johnson, S.I., Mazzaferro, E., Herold, L.V., Barnes, S.M., Winkler, K. & Selting, K.A. (2012). Elevated serum thymidine kinase activity in canine splenic hemangiosarcoma. *Veterinary and Comparative Oncology*, 10 (4), 292–302. https://doi.org/10.1111/j.1476-5829.2011.00298.x

Thierfelder, W.E., van Deursen, J.M., Yamamoto, K., Tripp, R.A., Sarawar, S.R., Carson, R.T., Sangster, M.Y., Vignali, D.A.A., Doherty, P.C., Grosveld, G.C. & Ihle, J.N. (1996). Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature*, 382 (6587), 171–174. https://doi.org/10.1038/382171a0

Tosolini, M., Kirilovsky, A., Mlecnik, B., Fredriksen, T., Mauger, S., Bindea, G., Berger, A., Bruneval, P., Fridman, W.-H., Pagès, F. & Galon, J. (2011). Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer. *Cancer Research*, 71 (4), 1263–1271. https://doi.org/10.1158/0008-5472.CAN-10-2907

Tugues, S., Burkhard, S.H., Ohs, I., Vrohlings, M., Nussbaum, K., vom Berg, J., Kulig, P. & Becher, B. (2015). New insights into IL-12-mediated tumor suppression. *Cell Death and Differentiation*, 22 (2), 237–246. https://doi.org/10.1038/cdd.2014.134

Vail, D.M., Thamm, D.H. & Liptak, J.M. (2019). 33 – Hematopoietic tumors. In: *Withrow and MacEwen's Small Animal Clinical Oncology*. 6th ed., Saunders. 688. https://doi.org/10.1016/B978-0-323-59496-7.00033-5

Wang, H. & Yang, X. (2017). Association between serum cytokines and progression of breast cancer in Chinese population. *Medicine*, 96 (49), e8840. https://doi.org/10.1097/MD.0000000000008840

Wang, W., Liu, S., Lu, F., Yang, B., Zhuang, X., Yin, J., Chen, G. & Sun, C. (2023). STAT4, a potential predictor of prognosis, promotes CD8 T-cell infiltration in ovarian serous carcinoma by inducing CCL5 secretion. *Oncology Reports*, 50 (1), 140. https://doi.org/10.3892/or.2023.8577

Weng, H.-P., Ke, C.-H., Tung, C.-W., Tani, A., Wang, C.-C., Yang, W.-Y., Wang, Y.-S., Han, W., Liao, C.-H., Tomiyasu, H. & Lin, C.-S. (2023). Canine diffuse large B-cell lymphoma downregulates the activity of CD8 + T-cells through tumor-derived extracellular vesicles. *Cancer Cell International*, 23 (1), 252. https://doi.org/10.1186/s12935-023-03104-4

Wherry, E.J. (2011). T cell exhaustion. *Nature Immunology*, 12 (6), 492–499. https://doi.org/10.1038/ni.2035

Wubetu, G.Y., Utsunomiya, T., Ishikawa, D., Yamada, S., Ikemoto, T., Morine, Y., Iwahashi, S., Saito, Y., Arakawa, Y., Imura, S., Kanamoto, M., Zhu, C., Bando, Y. & Shimada, M. (2014). High STAT4 expression is a better prognostic indicator in patients with hepatocellular carcinoma after hepatectomy. *Annals of Surgical Oncology*, 21 (4), 721–728. https://doi.org/10.1245/s10434-014-3861-9

Yamaguchi, T. & Sakaguchi, S. (2006). Regulatory T cells in immune surveillance and treatment of cancer. *Seminars in Cancer Biology*, 16 (2), 115–123. https://doi.org/10.1016/j.semcancer.2005.11.005

Yan, J., Smyth, M.J. & Teng, M.W.L. (2018). Interleukin (IL)-12 and IL-23 and their conflicting roles in cancer. *Cold Spring Harbor Perspectives in Biology*, 10 (7), a028530. https://doi.org/10.1101/cshperspect.a028530

Yang, X.O., Panopoulos, A.D., Nurieva, R., Chang, S.H., Wang, D., Watowich, S.S. & Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *The Journal of Biological Chemistry*, 282 (13), 9358–9363. https://doi.org/10.1074/jbc.C600321200

Yang, Z.-Z., Grote, D.M., Ziesmer, S.C., Niki, T., Hirashima, M., Novak, A.J., Witzig, T.E. & Ansell, S.M. (2012). IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *The Journal of Clinical Investigation*, 122 (4), 1271–1282. https://doi.org/10.1172/JCI59806

Zundler, S. & Neurath, M.F. (2015). Interleukin-12: Functional activities and implications for disease. *Cytokine & Growth Factor Reviews*, 26 (5), 559–568. https://doi.org/10.1016/j.cytogfr.2015.07.003

Popular science summary

Cancer is a widespread disease characterized by uncontrollable cell-division, it affects not only humans, but also our companion animals. For cancer to grow and spread to different parts of the body, it must avoid being detected and destroyed by the immune system. To do this, cancer cells can manipulate the nearby environment in the body to its advantage by creating a state of chronic inflammation that favours tumour survival.

For all cells to communicate, regardless of if they are cancer cells, immune cells or simply normal cells that constitute our organs, they need substances that carry information. These substances are usually proteins. One of the most common signalling substances in our body are called cytokines, generally referred to as "interleukins". The interleukins are usually assigned different numbers depending on their function, what cells they are produced by, and which type of cells they communicate with. Different cytokines convey different types of information and alters the physiological environment differently. As an example, some cytokines alert the immune system when we are infected by a pathogen to stimulate an immune response that eliminates the intruder, such as a virus. Some cytokines alert the body to know that it is time to stop attacking an infection once the virus or bacteria are defeated, and thus down-regulates the immune systems response.

By manipulating the nearby environment, cancer can hijack the immune system in its favour. It does so by stimulating the production of cytokines that attract immune cells that maintain inflammation, which is necessary for cancer to grow, but does not kill off the cancer cells. Studies have shown that certain cytokines, primarily those that down-regulate the immune system, are associated with worse outcome in patients with different types of cancer. Similarly, cytokines that are associated with stimulation of the immune system to recognize and kill cancer cells are linked to better outcome, and in some cases also better response to cancer treatment.

By recognizing cytokines that favour tumour development and those that stimulate tumour eradication, potential for developing new cancer treatments arise. Some cytokines, such as Interleukin-2, are already approved for treatment of kidney cancer and melanoma, a type of skin cancer, in humans.

In this study the levels of a certain subset of cytokines termed Interleukin-12 (IL-12) and Interleukin-23 (IL-23) were investigated. The purpose was to see whether the levels of these cytokines may be useful for detecting the presence of disease and if they differ between different types of cancer. Generally, several studies have indicated that IL-12 stimulates the recognition and killing of cancer cells while IL-23 can allow the tumour to hide from the immune system and is correlated with worse outcome.

Analysis of blood samples from dogs with lymphoma, mammary tumours and mast cell tumours were performed using ELISA, a type of analysis that utilize antibodies to detect proteins, such as cytokines. Analysis of healthy dogs, termed the control group were also performed, and the groups were compared to one another. Since the analysis was done measuring a protein that is a building block in both IL-12 and IL-23, it is uncertain whether the results indicate that IL-12, IL-23 or both cytokines are present.

This study concluded that the levels of these cytokines were significantly higher in dogs with lymphoma compared to the other groups. However, no definite conclusions could be drawn regarding the remaining groups as the number of patients in these groups were very small. Further studies, preferably with larger groups and multiple types of cancer, are required to investigate the usefulness of measuring these cytokines. For example, investigating if certain levels of these cytokines correlate with better or worse prognosis and treatment response could be useful to monitor cancer in both humans and animals.

Acknowledgements

I would like to thank my supervisor Henrik Rönnberg and my assistant supervisor Sara Saellström for guiding me and providing an excellent case-log of patients to analyse. A special thanks to Gabriella Hallbrink Ågren for analysing the samples and providing the data necessary for this study.

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file. If you are more than one author, the checked box will be applied to all authors. Read about SLU's publishing agreement here:

- https://www.slu.se/en/subweb/library/publish-and-analyse/register-and-publish/agreement-for-publishing/.

☒ YES, I hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.