Chromogranin A Epitopes Catestatin and Vasostatin

Evaluation of their Potential Use as Clinical Biomarkers for Psychological and Pain-induced Stress in Dogs

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Cover: The illustration represents the author's dogs showing the veterinary instruments and assessment methods for stress and pain used in this thesis (drawing by Nuntarwat Wipoosak).

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Abstract

Psychological and physiological factors, including pain, can induce a stress response. All today's available methods for evaluating stress in dogs have shortcomings and it is therefore necessary to identify and evaluate new biomarkers. Chromogranin A (CgA) is a useful biomarker for stress assessment in humans. In dogs, the CgA epitopes catestatin (CST) and vasostatin (VS) can be measured. This thesis aimed to evaluate the potential use of the CgA epitopes CST and VS as biomarkers for psychological and pain-induced stress in dogs in a clinical setting.

Reference ranges of plasma CST, VS, and saliva CST concentrations in healthy low-stressed dogs were determined. Age, gender, breed, and time of day did not significantly affect CST and VS concentrations. CST and VS were evaluated as biomarkers for psychological stress. CST and VS in associated with other stress evaluation methods were compared in healthy dogs where one group was stressed and the other was not. In the stress group, saliva CST, serum cortisol, and stress scores increased significantly and saliva CST did not overlap with the reference range. Plasma CST and VS did not change significantly. CST and VS were further tested as biomarkers for pain-induced stress by comparing the concentrations in different surgical settings. CST, VS, and other pain and stress monitoring methods were investigated in healthy dogs that received analgesia and were subjected to elective ovariohysterectomy. Compared with before surgery, plasma CST decreased significantly during anesthetic recovery and at recall for suture removal and serum cortisol decreased significantly at recall, suggesting that CST may be a possible pain-induced stress biomarker. CST and VS were measured in dogs with fractures prior to and after morphine treatment and evaluated in association with other pain and stress evaluation methods and compared with control dogs. Circulating CST and cortisol decreased significantly in dogs with fractures, but did not differ significantly between before and after morphine analgesia. Plasma CST overlapped with the reference ranges but plasma VS did not differ significantly throughout the studies.

In conclusion, saliva CST may have potential as a psychological stress biomarker in dogs. Repeated sampling of plasma CST may be of interest for evaluating pain within the same patient. Plasma VS has no potential as a stress biomarker in dogs.

Keywords: Biomarker, Bone fracture, Catestatin, Dog, Pain, Stress, Surgery, Vasostatin

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Abstract

Syftet med denna avhandling var att utvärdera om catestatin (CST) och vasostatin (VS) kan användas som biomarkörer för psykisk och smärtinducerad stress hos hundar. Referensvärden för CST och VS i plasma och CST i saliv hos friska hundar med låg stressnivå bestämdes. Resultatet visade att ålder, kön, ras, och tid på dagen för provinsamlingen inte hade någon signifikant påverkan på CST och VS vare sig i plasma eller saliv. CST och VS potential som biomarkörer för psykisk stress utvärderades genom att jämföra koncentrationerna hos friska hundar med låg stressprofil med friska hundar med hög stressprofil. Hos hundarna med hög stressprofil uppmättes signifikant högre koncentrationer av CST i saliv och kortisol i serum jämfört med hundarna med låg stressprofil. CST i saliv var också högre än fastställda referensvärden, men ingen signifikant skillnad kunde påvisas a VS eende koncentrationerna av CST och VS i plasma hos hundar med låg och hög stressprofil. CST och VS testades ytterligare som möjliga biomarkörer för smärt-inducerad stress genom att jämföra koncentrationer vid olika kirurgiska ingrepp. Koncentrationerna av CST och VS tillsammans med andra metoder för monitorering av smärta och stress utvärderades hos friska hundar före och efter ovariohysterektomi. Alla hundar fick smärtlindring i samband med operationerna. Resultaten visade att koncentrationen av CST i plasma var signifikant lägre efter avslutad operation jämfört med innan operationen. Koncentrationen var också fortsatt låg vid återbesöken för stygntagning liksom kortisol. Resultaten antyder att CST möjligen kan ha potential som en biomarkör för smärtinducerad stress hos hund. För att vidare utvärdera om smärta påverkar koncentrationerna av CST och VS mättes nivåerna hos en grupp friska hundar och en grupp hundar som drabbats av benfrakturer. Prover togs före och efter smärtlindring med morfin. Resultatet visade att cirkulerande koncentrationer av CST och kortisol var signifikant lägre hos hundar med benfrakturer jämfört med friska hundar, men ingen signifikant skillnad kunde ses i prover tagna före jämfört med prover tagna efter morfingivan. Ingen signifikant förändring av koncentrationerna av VS kunde uppmätas vid någon tidpunkt i studien. Sammanfattningsvis tyder resultaten på att CST i saliv kan ha en viss potential som en biomarkör för psykisk stress hos hund. Upprepade mätningar av plasma CST kan möjligen användas för att evaluera smärtutveckling hos en enskild hund.

Keywords: Biomarker, Bone fracture, Catestatin, Dog, Pain, Stress, Surgery, Vasostatin

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Dedication

To King Bhumibol the Great, the ninth monarch of Thailand from the Chakri Dynasty

To my family, teachers, and all our dogs

Focus on the journey, not the destination. Joy is found not in finishing an activity but in doing it.

Greg Anderson

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Srithunyarat, T.*, Hagman, R., Höglund, O.V., Olsson, U., Stridsberg, M., Jitpean, S., Lagerstedt, A.S., Pettersson, A. (2017). Catestatin and vasostatin concentrations in healthy dogs. *Acta Veterinaria Scandinavica* 59 (1), p. 1.

II Srithunyarat, T.*, Höglund, O.V., Hagman, R., Olsson, U., Stridsberg, M., Lagerstedt, A.S., Pettersson, A. (2016). Catestatin, vasostatin, cortisol, temperature, heart rate, respiratory rate, scores of the short form of Glasgow composite measure pain scale and visual analog scale for stress and pain behavior in dogs before and after ovariohysterectomy. *BMC Research Notes* 9 (1), p. 381.

III Srithunyarat, T.*, Hagman, R., Höglund, O.V., Stridsberg, M., Olsson, U., Hanson, J., Nonthakotr, C., Lagerstedt, A.S., Pettersson, A. (2017). Catestatin, vasostatin, cortisol, and pain assessments in dogs suffering from traumatic bone fractures. *BMC Research Notes* 10 (1), p. 129.

IV Srithunyarat, T.*, Hagman, R., Höglund, O.V., Stridsberg, M., Hanson, J., Lagerstedt, A.S., Pettersson, A. Catestatin, vasostatin, cortisol, and visual analog scale scoring for stress assessment in healthy dogs (submitted)

Papers I–III are reproduced with the permission of the publishers.

* Corresponding author.

The contribution of Thanikul Srithunyarat to the papers included in this thesis was as follows:

I Conception and design of the study, sample collection, analysis and interpretation of data, drafting the article, and critical revision of the article.

II Conception and design of the study, sample collection, analysis and interpretation of data, drafting the article, and critical revision of the article.

III Conception and design of the study, sample collection, analysis and interpretation of data, drafting the article, and critical revision of the article.

IV Conception and design of the study, sample collection, analysis and interpretation of data, statistical analysis, drafting the article, and critical revision of the article.

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after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$) 57

Abbreviations

ASA	American Society of Anesthesiologists
ANP	Atrial Natriuretic Peptide
BNP	Brain Natriuretic Peptide
CgA	Chromogranin A
CMPS	Glasgow Composite Measure Pain Scale
CMPS-SF	Short Form of Glasgow Composite Measure Pain Scale
CST	Catestatin
CV	Coefficient of Variation
DEA	Dog Erythrocyte Antigen
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
HPA	Hypothalamic-pituitary-adrenal
KKU	Khon Kaen University
NRS	Numeric Rating Scale
OHE	Ovariohysterectomy
OP-VAS	Overall Pain Behavior Visual Analog Scale
RIA	Radioimmunoassay
RPM	Revolution per minute
SAM	Sympatho-adreno-medullary
SD	Standard Deviation
SDS	Simple Descriptive Scale
SLU	Swedish University of Agricultural Sciences
S-VAS	Stress Behavior Visual Analog Scale
UDS	University Animal Hospital at SLU
VAS	Visual Analog Scale
VS	Vasostatin

1 Introduction

1.1 Background

Stress is essential for coping with acute changes in the body's homeostasis, but stress and particularly prolonged stress reactions can also be detrimental (Hekman *et al.*, 2014; Goldstein, 2003; Sapolsky *et al.*, 2000; Roizen, 1988). Psychological and physiological factors including pain can induce a similar stress response in humans and in animals (Tranquilli *et al.*, 2007; Grant, 2006). Early detection of psychological and pain-induced stress is essential for animal welfare reasons, and for minimizing recovery time and duration of hospitalization in animals undergoing surgery or intensive care (Phillips, 2000). Stress is, however, difficult to evaluate in animals and therefore reduces the possibility of proper identification, prevention, and treatment. All documented methods for stress assessment in dogs have shortcomings (Rialland *et al.*, 2012; Fink, 2010; Tranquilli *et al.*, 2007; Mathews, 2000), and new reliable objective biomarkers for stress evaluation in dogs, suitable for use in a clinical setting, are needed.

1.2 Stress and pain

1.2.1 Stress and stress response

Stress, a normal physiological response essential for survival, is modulated by the sympatho-adreno-medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis. Stress and stress response are, however, integrated and complex (Fink, 2010; Goldstein, 2003). Stress can be classified as acute or chronic depending on its duration (Hansel *et al.*, 2010; Dhabhar, 2009; Dhabhar

& McEwen, 1997), and as physiological, psychological, or the combination of both depending on the cause (Eiden, 2013; Moberg & Mench, 2000).

The SAM and HPA axes have important roles on stress response. The SAM axis is promptly activated leading to the secretion of catecholamines from chromaffin granules (Hekman *et al.*, 2014; Goldstein, 2003; Derbyshire & Smith, 1984; Blaschko *et al.*, 1967), followed by activation of the HPA axis leading to changes in cortisol secretion. The physiological stress response, necessary for coping with different stressful situations, is characterized by changes in the body's homeostasis such as increased heart rate, vascular resistance, oxygen consumption, catabolism of glucose and lipid, and decreased anabolism. In addition to the physiological responses, stress induces psychological reactions leading to behavioral changes in dogs such as restlessness, lethargy, anorexia, sleeplessness, avoidance, aggression, shaking, and growling (Reid *et al.*, 2007; Holton *et al.*, 2001; Moberg & Mench, 2000).

1.2.2 Pain-induced stress response

Pain has been defined by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (McKelvey *et al.*, 2003). Pain pathways are similar in humans and dogs suggesting that all causes of pain in humans can be applied to dogs (Tranquilli *et al.*, 2007; McKelvey *et al.*, 2003). However, because pain perception is individual, each patient must be assessed individually (Epstein *et al.*, 2015; Tranquilli *et al.*, 2007; McKelvey *et al.*, 2003; Moberg & Mench, 2000).

Pain can induce similar physiological, psychological, and behavioral responses as stress (Cremeans-Smith *et al.*, 2015; Epstein *et al.*, 2015; Tennant, 2013; Reid *et al.*, 2007; Holton *et al.*, 2001), leading to changes in catecholamine and cortisol concentrations as well as changes in behavioral expressions (Figure 1). In effect, pain can activate both the SAM and HPA axes (Giannoudis *et al.*, 2006; Tennant & Hermann, 2002; Desborough, 2000).

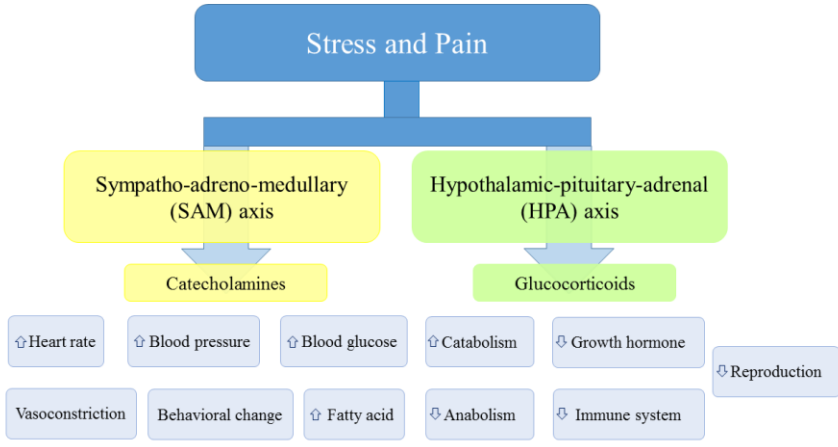

Figure 1. Stress and pain responses. Stress and pain induce similar responses through main axes of sympatho-adreno-medullary (SAM) and hypothalamic-pituitary-adrenal (HPA).

1.3 Assessment of stress and pain

Traditionally, both subjective parameters, such as changes in behaviors and objective parameters, such as measurable physiological responses, have been used for stress and pain assessment in humans and animals. However, as stated previously, no single method is completely reliable and all currently available methods have shortcomings (Rialland *et al.*, 2012).

1.3.1 Subjective assessment

Behavioral change is a common sign of stress and pain in animals and these changes can be monitored by observation (Epstein *et al.*, 2015; Tranquilli *et al.*, 2007; Grant, 2006). However, behavior can vary between species, breed, gender, age, and individuals. Further, different stimuli may lead to different behavioral changes (Epstein *et al.*, 2015).

Several methods and criteria have been used for monitoring stress and pain behavior. In human medicine, self reporting, where each individual rates their own experience using numeric rating or questionnaires, is widely used (Rapo-Pylkko *et al.*, 2016; Herr & Garand, 2001). However, self reporting has limited value for stress and pain assessment in infants, children, and animals due to impaired ability to describe their situation (Epstein *et al.*, 2015; Tranquilli *et al.*, 2007; Lee *et al.*, 2006).

In veterinary research, behavioral expression, monitored by an examiner, has been used for stress and pain assessment. Many scoring systems are available using different protocols and scales such as simple descriptive scale (SDS), numeric rating scale (NRS), visual analog scale (VAS), Glasgow composite measure pain scale (CMPS), short form of Glasgow composite measure pain scale (CMPS-SF), Melbourne Pain Scale, and Colorado State University canine acute pain scale (Epstein *et al.*, 2015; Reid *et al.*, 2007; Holton *et al.*, 2001; Firth & Haldane, 1999; Holton *et al.*, 1998a; Holton *et al.*, 1998b). The VAS, SDS, and NRS are simple methods for assessing pain using a linear scale and has been widely used in humans and dogs (Rapo-Pylkko *et al.*, 2016; Holton *et al.*, 1998b). The CMPS and CMPS-SF are validated for acute pain assessment e.g. postoperative pain in dogs (Reid *et al.*, 2007; Holton *et al.*, 2001). Different methods for monitoring pain that combine observations with computer technology such as analysis of facial expression, gait analysis, and intensive of activity may be found to be useful in the future. However, each protocol and criteria may only be suitable for specific situations. The advantages of behavioral assessment are speed and simplicity, but the limitations are that the method is examiner sensitive and many subjective assessment methods are not yet validated (Holton *et al.*, 2001). Monitoring behavior as a sole method may therefore be insufficient.

1.3.2 Objective assessment

Stress and pain-induced stress can be evaluated objectively by measuring physiological parameters and neuroendocrine biomarkers. In essence, physiological parameters are measured to assess sympathetic tone as a surrogate measure of stress. Although measurement and evaluation of objective responses are generally observer independent, they may be affected by the animal's perception of the monitoring procedure. This perception can in itself elicit a stress response, often referred to as the white coat effect, and must therefore be interpreted carefully.

Physiological parameters

The stress response causes changes in physiological parameters, which can be objectively measured such as heart rate, respiratory rate, blood pressure, and blood glucose concentrations (Bragg *et al.*, 2015; Höglund *et al.*, 2012; Marino *et al.*, 2011). The values, however, can also be altered because of various reasons, e.g., exercise, acute pain, heart disease, lung disease, kidney disease, and also by the white coat effect from psychological stress of the visit to the animal hospital.

Neuroendocrine biomarkers

Stress stimulates SAM and HPA axes, leading to the release of several neuroendocrine biomarkers such as catecholamines, cortisol, serotonin, vasopressin, and chromogranin A. These neuroendocrine biomarkers can also be used to objectively monitor stress.

Catecholamines (adrenaline and noradrenaline), are secreted from the sympathetic system and the adrenal medulla. The adrenaline and noradrenaline response is rapid when SAM is activated. Catecholamines are useful for monitoring the initial stage of stress and pain-induced stress. However, the degradation of circulating catecholamines is rapid, the half-life is short, and requires special handling procedures which limit the usefulness of catecholamines in a clinical setting (Goldstein, 2003; Crout, 1968).

Cortisol has traditionally been used as a sensitive biomarker for stress in humans and animals. Cortisol can be measured in blood, saliva, feces, hair and urine (Giannetto *et al.*, 2014; Hekman *et al.*, 2014; Jung *et al.*, 2014; Russell *et al.*, 2012; Dreschel & Granger, 2009; Schatz & Palme, 2001). Plasma cortisol will react in 4–30 minutes after stimulation of the HPA axis (Jung *et al.*, 2014). Circulating cortisol passively infiltrates into saliva, and saliva cortisol concentrations correlate with concentrations in blood (Vincent & Michell, 1992). Cortisol concentrations in both saliva and plasma vary during the day because of pulsatile secretion and circadian rhythms (Giannetto *et al.*, 2014; Fink, 2010; Hanson *et al.*, 2006; Kemppainen & Sartin, 1984). Daily variation have less effect on cortisol concentrations in feces, hair, and urine and rather more reflect secretion of cortisol over time (Hekman *et al.*, 2014). Cortisol concentrations can differ between species, individuals, gender, and age (Fink, 2010). Because concentrations of circulating cortisol can vary both within and between individuals, cortisol is unspecific and difficult to interpret for stress assessment. Further, the concentrations of cortisol can be influenced by many factors such as the white coat effect, pain, illness, or physiological and psychological changes further limiting the usefulness of cortisol as a stress and pain biomarker in a clinical setting (Höglund *et al.*, 2015; Bovens *et al.*, 2014; Jung *et al.*, 2014; Perego *et al.*, 2014; Muhtz *et al.*, 2013; Tennant, 2013; Haverbeke *et al.*, 2008; Hanson *et al.*, 2006; Fries *et al.*, 2005). However, due to the lack of better alternatives, cortisol is still used as a biomarker for stress and pain-induced stress responses.

1.3.3 Multimodal assessment

Multimodal assessments such as the Melbourne Pain Scale, combines results from both subjective and objective methods to assess acute pain in dogs (Firth & Haldane, 1999). No multimodal assessment protocol have yet been validated for stress and pain monitoring in dogs (Epstein *et al.*, 2015).

1.4 Chromogranin A

1.4.1 Background

Chromogranin A (CgA) is an acidic glycoprotein belonging to the Granin family. Its molecular weight is about 48–52 kDa and it consists of 431–439 amino acids depending on the species (Metz-Boutigue *et al.*, 1993). CgA is stored and released together with catecholamines from chromaffin granules (O'Connor & Bernstein, 1984; Blaschko *et al.*, 1967). Although distribution of CgA is widespread in several organs, CgA is largely found in and secreted from the adrenal medulla, neuroendocrine system, and sympathetic nerves (Winkler & Fischer-Colbrie, 1992; Smith & Winkler, 1967). CgA plays an important role in the formation of intracellular secretory granules and, when SAM is activated, exocytosis of secretory granules occurs leading to CgA being extracellularly coreleased with catecholamines (D'Amico M *et al.*, 2014; O'Connor & Bernstein, 1984; Blaschko *et al.*, 1967). Plasma norepinephrine and CgA concentrations correlate when the sympathochromaffin system is intensively stimulated. This suggests that CgA secretion may depend on the intensity of stimuli (Mahata *et al.*, 2004; Kanno *et al.*, 1999; Mahata *et al.*, 1997; Cryer *et al.*, 1991; O'Connor & Bernstein, 1984).

In mammals, CgA has also been found in exocrine tissues such as saliva glands, i.e. parotid, submandibular, and sublingual glands. However, the concentrations are much lower than found in endocrine cells particularly when compared to adrenal medulla (Saruta *et al.*, 2005; Sato *et al.*, 2002; Kanno *et al.*, 1999). Although the mechanism can differ, active secretion of saliva CgA has been demonstrated in different species of mammals. In humans, saliva CgA is produced in acinar cells of the submandibular gland, mainly in serous and ductal cells and secreted into ductal cavity (Saruta *et al.*, 2005) whereas, in rats, saliva CgA is produced in the exocrine cells of the granular convoluted tube (Saruta *et al.*, 2005; Sato *et al.*, 2002; Kanno *et al.*, 1999). No studies of saliva CgA production, however, have so far been performed in dogs.

The intact CgA molecule includes N- and C-terminal parts (Figure 2). Several factors may stimulate the biosynthesis and proteolytic processes in both N- and

C-terminals either by intragranular or extracellular mechanisms (Hendy *et al.*, 1995; Metz-Boutigue *et al.*, 1993; Tatemoto *et al.*, 1986). In general, CgA influences and modulates homeostasis of several systems such as the endocrine, cardiovascular, immunological, and neurological systems (D'Amico M *et al.*, 2014; Mahata *et al.*, 2010; Taupenot *et al.*, 2003; Jiang *et al.*, 2001; O'Connor & Bernstein, 1984).

CgA and its derived peptides have been suggested as sensitive biomarkers for stress in humans as well as in different animal species (Escribano *et al.*, 2013; Akiyoshi *et al.*, 2005; Nakane H, 1998). CgA can be proteolytically cleaved into several peptides with different biological activities including vasostatin, pancreastatin, catestatin, parastatin, and serpinin (Bandyopadhyay *et al.*, 2015; D'Amico M *et al.*, 2014; Tota *et al.*, 2012; Mahata *et al.*, 2010; Sanchez-Margalet *et al.*, 2010; Gayen *et al.*, 2009; Mahata *et al.*, 2004; Wen *et al.*, 2004; Mahata *et al.*, 2003; Jiang *et al.*, 2001; Fasciotto *et al.*, 2000; Metz-Boutigue *et al.*, 1998; Corti *et al.*, 1997; Mahata *et al.*, 1997; Hendy *et al.*, 1995; Aardal *et al.*, 1993; Helle *et al.*, 1993; Metz-Boutigue *et al.*, 1993; Aardal & Helle, 1992; Fasciotto *et al.*, 1992; Tatemoto *et al.*, 1986).

Figure 2. Schematic model of chromogranin A epitopes catestatin and vasostatin. Abbreviations: CgA: chromogranin A; VS: vasostatin; CST: catestatin; Number: amino acid sequence.

1.4.2 Chromogranin A derived peptides

The CgA derived active peptides contain different amino acid sequences and have various bioactivity, e.g. vasorelaxant and cardiosuppressive effects, inhibition of glucose-induced insulin secretion, inhibition of hypertension, inhibition of catecholamines secretion, and modulation of calcium and parathormone secretion (D'Amico M *et al.*, 2014; Helle, 2010; Mahata *et al.*, 2010; Hendy *et al.*, 1995). Catestatin and vasostatin, the two CgA-derived peptides (Figure 2), have multifunctional roles in a wide range of tissue systems; however, little is known about their degradation, secretion, function, and clearance rate in different species (Helle, 2010).

Catestatin

Catestatin (CST) modulates catecholamine secretion via a negative feedback mechanism and has been shown to have antihypertensive, antimicrobial, and cardiosuppressive effects (Imbrogno *et al.*, 2010; Mahata *et al.*, 2010; Radek *et al.*, 2008; Rangon *et al.*, 2003; Mahata *et al.*, 1999; Kennedy *et al.*, 1998; Mahata *et al.*, 1997). CST acts noncompetitively on nicotinic cholinergic receptors in inhibiting the secretion of catecholamines. The response, however, depends on the degree of stimuli (Mahata *et al.*, 2004; Mahata *et al.*, 1997). Concentrations of CST are decreased in the early stage of hypertension in human patients and in hypertensive patients (O'Connor *et al.*, 2002). Increased blood pressure in rats after target ablation of the CgA gene as well as in stress situations were cured by CST administration (Mahapatra *et al.*, 2005). CST regulates peripheral and baroreceptors for hypertension and stimulates histamine release from mast cells, which leads to vasodilation and relief of hypertension (Mahapatra, 2008; Rao *et al.*, 2007; Kruger *et al.*, 2003). In addition, in *in vitro* studies, CST has been shown to have an antimicrobial effect against Gram-positive and Gram-negative bacteria, fungi, and yeast (Mahata *et al.*, 2010; Briolat *et al.*, 2005; Metz-Boutigue *et al.*, 1998; Takiyyuddin *et al.*, 1993).

Vasostatin

Vasostatin (VS) has been shown to influence plasma calcium secretion, vasodilation, and have cardiosuppressive effects (Helle, 2010; Zhang *et al.*, 2009; Imbrogno *et al.*, 2004; Brekke *et al.*, 2002; Corti *et al.*, 2002; Aardal *et al.*, 1993). VS is the N-terminal fragment of the CgA molecule and has an *in vitro* antimicrobial effect on Gram-positive bacteria, fungi, and yeast (Helle, 2010; Lugardon *et al.*, 2000). VS has shown promise as a prognostic biomarker in critically ill patients where an increased concentration in the circulation indicated poor outcome (Schneider *et al.*, 2012).

1.4.3 Measurement of chromogranin A

Concentrations of CgA can be quantitatively measured in blood and saliva (Saruta *et al.*, 2005; Sato *et al.*, 2002; Yanaihara *et al.*, 1999; Nakane H, 1998; Winkler & Fischer-Colbrie, 1992; O'Connor & Bernstein, 1984). The measurement of CgA can be performed using immunoassays such as radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), and enzyme immunoassay (EIA) (Stridsberg *et al.*, 2004; Yanaihara *et al.*, 1999; O'Connor & Bernstein, 1984). However, the method for analyzing CgA and the measured sequences of CgA differ between studies, which needs to be

considered when comparing results. In effect, CgA is proteolytically cleaved into several biological peptides both before and after release to circulation. The measurement by a region specific RIA allows for assessment of different CgA epitopes by measuring their specific amino acid sequences both in the intact molecule and the biological peptides (Stridsberg *et al.*, 2004; Yanaihara *et al.*, 1999).

CgA and its active peptides contain amino acid sequences which differ between species. The interspecies cross reactivity of intact CgA has been compared between humans, cattle, sheep, goats, pigs, and horses showing that human intact CgA assay is not suitable for measuring CgA in these species whereas the VS epitopes of CgA molecule have a highly conserved amino acid sequence and can be analyzed using RIA (Stridsberg *et al.*, 2000). A study on interspecies cross reactivity between human and dog showed that CST (CgA 361–372) and VS (CgA 17–38), but not the intact CgA molecule, could be measured in dogs using region specific RIA (Stridsberg *et al.*, 2014). In a previous study in dogs, saliva CgA sequence 344–374 was measured by use of a human ELISA kit (Human chromogranin A ELISA, Yanaihara, Tokyo, Japan) (Kanai *et al.*, 2008). This region includes the sequence of CST (CgA 361–372) which can be measured using region specific RIA (Stridsberg *et al.*, 2014; Kanai *et al.*, 2008). The measurement of the region specific RIA reflects both the intact CgA molecule and the peptide.

1.4.4 Usefulness of chromogranin A in humans

Evaluation of CgA concentrations in blood and saliva has shown promise as a biomarker for stress in addition to diagnosis of neuroendocrine tumors, cardiovascular disease, periodontal disease, critical illness, gastritis, and organ failure (D'Amico M *et al.*, 2014; Lindahl *et al.*, 2013; Reshma *et al.*, 2013; Schneider *et al.*, 2012; Zhang *et al.*, 2008; Campana *et al.*, 2007; Ferrari *et al.*, 2004; Ferrari *et al.*, 1998; Nakane H, 1998).

Circulating CgA is the most reliable diagnostic and prognostic biomarker for neuroendocrine tumors such as pheochromocytoma. In these cases, the tumor itself produces CgA. CgA concentrations have been used for evaluation of treatment effectiveness, metastasis, and prognosis (Schneider *et al.*, 2012; Ferrari *et al.*, 2004; Ferrari *et al.*, 1998).

CgA has shown promise as a biomarker for cardiovascular diseases such as hypertension, hypertrophic cardiomyopathy, and dilated cardiomyopathy in humans (D'Amico M *et al.*, 2014; Pieroni *et al.*, 2007; Taupenot *et al.*, 2003). CgA can be produced by myocardium in rats colocalized with atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (Angelone *et al.*, 2012; Pieroni

et al., 2007). Increased levels of circulating CgA have been measured in human patients with cardiovascular diseases and patients undergoing cardiac arrest (D'Amico M *et al.*, 2014; Pieroni *et al.*, 2007; Taupenot *et al.*, 2003). The increased levels in cardiac arrest patients are probably due to a combination of SAM stimulation and release from the cells that produce ANP and BNP.

CgA concentrations are increased in patients with enterochromaffin-like cell hyperplasia in the gastric mucosa which can be seen in atrophic gastritis with *Helicobacter pylori* infection, and in conjunction with proton pump inhibitor treatment (D'Amico M *et al.*, 2014; Campana *et al.*, 2007; Kleveland *et al.*, 2001). In a patient with organ failure, CgA is increased due to renal and hepatic dysfunction (O'Connor *et al.*, 1989), which suggests that CgA may be metabolized in the liver and excrete into urine. CgA concentrations may therefore be unreliable as stress biomarkers in patients with renal and hepatic dysfunction, atrophic gastritis, as well as in those who have received medication with proton pump inhibitor (Gut *et al.*, 2016; D'Amico M *et al.*, 2014).

Whether there is a circadian variation in CgA secretion is still controversial and different studies have shown conflicting results (Den *et al.*, 2011; Den *et al.*, 2007; Takiyyuddin *et al.*, 1991). Although an active secretion of CgA into saliva has been demonstrated, the correlation between CgA in plasma and saliva is still unclear (Den *et al.*, 2011; Den *et al.*, 2007; Kanamaru *et al.*, 2006; Toda *et al.*, 2005; Giampaolo *et al.*, 2002; Takiyyuddin *et al.*, 1991).

1.4.5 Chromogranin A as stress biomarker in humans

Although catecholamines are indicators of sympathoadrenal activity, rapid degradation and circadian rhythm limit their usefulness as biomarkers in a clinical setting (Derbyshire & Smith, 1984; Crout, 1968). CgA is coreleased with catecholamines and CgA concentrations have been found to correlate with catecholamines when SAM is activated (Akiyoshi *et al.*, 2005; Nakane H, 1998; Cryer *et al.*, 1991; Takiyyuddin *et al.*, 1990). CgA is distributed in several tissues and measurable from both blood and saliva samples. Moreover, CgA is heat stable and has a longer half-life than catecholamines suggesting that CgA may be advantageous over catecholamines in a clinical setting (D'Amico M *et al.*, 2014; Hendy *et al.*, 1995; Winkler & Fischer-Colbrie, 1992; Takiyyuddin *et al.*, 1990; O'Connor *et al.*, 1989; O'Connor & Bernstein, 1984). Further, CgA concentrations are stable during storage and tolerate several freeze-thaw cycles (Escribano *et al.*, 2014; O'Connor *et al.*, 1989), and the concentration is unaffected by age (Toda *et al.*, 2005).

Blood sampling may induce a stress reaction because of anticipated pain or fear during the sampling procedure in humans. Saliva sampling has been

advocated because it is a noninvasive technique where samples are obtained by voluntarily spiting into a container without eliciting fear (D'Amico M et al., 2014; Kanno et al., 1999; Nakane H, 1998). Several studies have been performed evaluating saliva and plasma CgA in human; however, the results have been conflicting. Saliva CgA has been shown to increase in a response to acute psychological stress. Prior to saliva cortisol concentrations, an immediate increase in saliva CgA concentration can be measured in response to acute stress (Nakane H, 1998). In response to short-term psychological stress, such as experienced by children immediately after blood collection, in students before examination, and during cognitive tests, saliva CgA has been found to increase whereas saliva cortisol did not change significantly (Takatsuji et al., 2008; Kanamaru et al., 2006; Lee et al., 2006). However, saliva CgA did not change significantly during brief intensive physical stress (Nakane H, 1998). Plasma CgA increase significantly in response to short-term intensive physical exercise, but not to prolonged low intensive exercise (Takiyyuddin et al., 1990). Saliva CgA responded immediately and the changes were still present after 20–30 minutes (Yamakoshi et al., 2009; Kanamaru et al., 2006; Nakane H, 1998). Because saliva cortisol correlates with circulating cortisol, increased saliva cortisol may occur first after 15–30 minutes. However, normal reference range in humans with lower-stress profiles in relation to age, gender, and time of day are lacking.

1.4.6 Chromogranin A in dogs

Although few studies on CgA, prior to those studies included in this thesis, have been reported in dogs, CgA and its derived peptides have been presented as potential biomarkers for neuroendocrine tumors, sepsis, and stress (Jitpean *et al.*, 2015; Srithunyarat *et al.*, 2015; Byström, 2014; Srithunyarat, 2014; Stridsberg *et al.*, 2014; Kanai *et al.*, 2008; Akiyoshi *et al.*, 2005; Myers *et al.*, 1997). CgA has been found in endocrine tissue, endocrine tumors, and pancreatic tumors in both the original tissues and metastasis in dogs. The high concentrations of plasma CgA found in dogs with insulinoma shows that CgA can be useful in diagnosing of neuroendocrine tumors (Myers *et al.*, 1997). In one study, CST concentrations were significantly decreased in dogs with pyometra compared to a healthy control group whereas no changes were found in VS concentrations (Jitpean *et al.*, 2015). This study suggested a possible role for CST as a biomarker for sepsis. Moreover, in dogs exhibiting severe stress by experimentally insulin-induced hypoglycemia, plasma CgA significantly increased and correlated with plasma cortisol and catecholamine (Akiyoshi *et al.*, 2005). In a study evaluating surgical stress in dogs undergoing ovariohysterectomy, plasma VS did not

change in response to surgical stress induced by ovarian removal (Höglund *et al.*, 2015). No circadian variation in canine saliva CgA has been demonstrated and saliva CgA seems unaffected by gender (Kanai *et al.*, 2008).

2 Aims and hypothesis of the thesis

The general aims of this thesis were to evaluate the potential use of the CgA epitopes CST and VS as biomarkers for psychological and pain-induced stress in dogs in a clinical setting. We hypothesized that the concentrations of CST and VS would differ in dogs during psychological and pain-induced stress compared with low-stressed healthy dogs. Furthermore, we hypothesized that the changes in CST and VS concentration would agree with other stress and pain assessments.

The specific aims were to:

- Investigate concentrations of and establish reference ranges for the CgA epitopes CST and VS in healthy dogs accustomed to sampling procedures (Paper I).
- Investigate and compare concentrations of the CgA epitopes CST and VS, cortisol, VAS, CMPS-SF, and physiological assessment parameters in dogs before and after a standardized ovariohysterectomy procedure receiving analgesia (Paper II).
- Investigate and compare concentrations of the CgA epitopes CST and VS, cortisol, VAS, and CMPS-SF before and after morphine analgesia in dogs suffering from traumatic bone fractures, and to compare the results to healthy control dogs to evaluate CST and VS potential as biomarkers for pain-induced stress (Paper III).
- Investigate and compare concentrations of the CgA epitopes CST and VS, cortisol, and stress VAS between two groups of healthy dogs of which one group was accustomed and the other unaccustomed to being handled in an animal hospital environment to evaluate the potential use of CST and VS as psychological stress biomarker (Paper IV).

3 Materials and methods

An overview of the general materials and methods are described. For more information see Paper I–IV.

3.1 Study design and ethical permission

The studies included in this thesis were performed in a clinical environment. Studies occurred at two locations: Swedish University of Agricultural Sciences (SLU) and Khon Kaen University (KKU). All studies were ethically approved by either the Uppsala Ethical Committee (C301/12) or the KKU Ethical Legislation (AEKKU 26/2557) depending on the location for the study. All dog owners were informed and gave their consent prior to participation of the dogs.

3.2 Animals

Four groups of dogs were included in this thesis: research Beagle dogs, blood donor dogs, dogs undergoing elective ovariohysterectomy (OHE), and dogs with traumatic bone fractures. No dogs had other concurrent diseases or a history of receiving steroidal or proton pump inhibitor drugs prior to inclusion.

Research Beagle dogs: Ten dogs, three males and seven females, were included. All dogs were familiar with and trained for sampling procedures. They were fed twice daily with *ad libitum* water access and were housed at the research division at the Department of Clinical Sciences, SLU. All dogs were examined and deemed healthy and classified in accordance with the American Society of Anesthesiologists (ASA) physical status classification system as ASA I.

Blood donor dogs: Thirty three privately-owned dogs, twenty four males and nine females, which routinely donated blood during April 2014 and from September 2014 to February 2015 at the University Animal Hospital (UDS), SLU,

were included. All dogs were familiar to the sampling procedures and the animal hospital environment. They were healthy and classified as ASA I based on physical examination and blood screening tests prior to blood donation in accordance with the routines for blood donation at UDS as described in Paper I.

Dogs undergoing elective OHE: Thirty privately-owned intact female dogs that underwent elective OHE at KKU Veterinary Teaching Hospital during March to June 2015 were included. The dogs were healthy and classified as ASA I before inclusion based on a complete physical examination and blood screening tests prior to the surgery as described in Paper II.

Dogs with traumatic bone fractures: Fourteen privately-owned dogs, nine males and five females, suffering from traumatic bone fractures limited to the hind limb or pelvis, admitted to KKU Veterinary Teaching Hospital during March to June 2015, were included. All dogs underwent a complete physical examination, blood screening tests, and radiological examination as described in Paper III. Only dogs with ASA I–II and, based on history taking at the time of admission, had bone fractures within a 4-day period and an analgesic withdrawal period over 6 hours, were included. On admission, all dogs with bone fractures received a prompt intramuscular injection with 0.5 mg/kg morphine sulfate (Morphine Sulfate injection, M & H manufacturing, Samutprakan, Thailand). Dogs were also categorized based on cause of trauma, duration of injury, fracture site and number of bone fractures, prior analgesic treatments, and withdrawal period from previous analgesia.

3.3 Study protocol

The study procedures of each group of dogs are illustrated in Figure 3.

Paper I: In this study, saliva and blood were collected from both research Beagle dogs (n = 10) and blood donor dogs (n = 33). In the research Beagle dogs, samples were collected twice daily (6:30 a.m. to 7:30 a.m. and 1:00 p.m. to 2:00 p.m.) for a five-day period. These time points were selected based on the results from a previous pilot study (Srithunyarat, 2014). Blood donor dogs, in association with routine blood donation, were sampled at one time point between 8:00 a.m. to 3:00 p.m. During the study period, dogs donated blood between 1–3 times, leading to a total of 50 collection occasions. In association with each saliva and blood collection, stress behavior was scored using visual analog scale (S-VAS). Blood was analyzed for plasma CST and VS and serum cortisol, and saliva for CST.

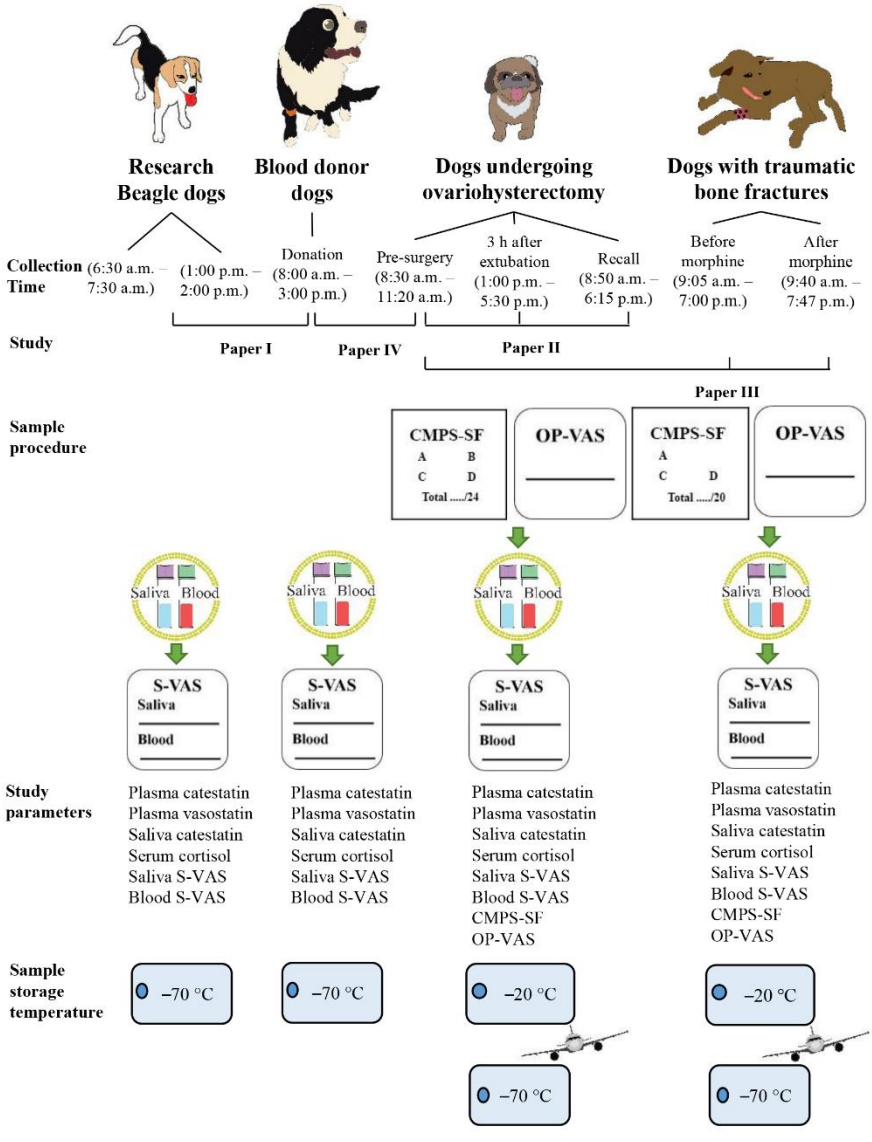

Figure 3. Study protocols of the four dog groups. Abbreviations: CMPS-SF: the short form of Glasgow composite measure pain scale; OP-VAS: overall pain behavior visual analog scale; S-VAS: stress behavior visual analog scale; Airplane: sample transportation with temperature control (−20 °C).

Paper II: Healthy dogs undergoing elective OHE (ASA I) were included (n = 30). Complete physical examination, pain behavior assessments using the short form of the Glasgow Composite Measure Scale (CMPS-SF) (Figure 4) and overall pain behavior VAS (OP-VAS) (Figure 5), and saliva and blood sample collection were performed before surgery (before premedication), 3 hours after extubation, and once at recall for removal of external stitches and control of wound healing (day 7 to 15 after surgery). Stress behavior using VAS scores (S-VAS) assessed at each saliva and blood sampling occasion (Figure 6) were also recorded. Blood was analyzed for plasma CST, plasma VS, and serum cortisol, and saliva for CST.

Paper III: Previously healthy dogs with traumatic bone fractures (ASA I–II) (n = 14) and healthy dogs before elective OHE (ASA I) (Paper II) (n = 30) were included. Complete physical examination, pain behavior assessments using CMPS-SF and OP-VAS, and saliva and blood sample collection were performed immediately before and 35–70 minutes after morphine administration in dogs suffering from traumatic bone fractures. Stress behavior was scored using S-VAS in all dogs. Blood was analyzed for plasma CST, plasma VS, and serum cortisol, and saliva for CST.

Paper IV: Blood donor dogs (Paper I) (n = 33) and healthy dogs before elective OHE (Paper II) (n = 30) were included in the study. The control group consisted of the blood donor dogs that were familiar with the animal hospital environment and sampling collection procedures. The stress group consisted of healthy dogs unfamiliar with the animal hospital environment and sampling procedure that were admitted to the animal hospital for elective OHE. For the blood donor dogs, one sampling occasion was included. For dogs that donated blood on repeated occasions during the study period, one sample was randomly selected. For the stress group, preoperative data were used as a reflection of dogs subjected to psychological stress. Saliva and blood samples, saliva and blood S-VAS scores were included in this study. Blood was analyzed for plasma CST, plasma VS, and serum cortisol, and saliva for CST.

SHORT FORM OF THE GLASGOW COMPOSITE PAIN SCALE

Dog's name _____

Hospital Number _____ **Date** / / **Time**

Surgery Yes/No (delete as appropriate)

Procedure or Condition_____

_____

In the sections below please circle the appropriate score in each list and sum these to give the total score.

A. Look at dog in Kennel

Is the dog?

(i)		(ii)	
Quiet	0	Ignoring any wound or painful area	0
Crying or whimpering	1	Looking at wound or painful area	1
Groaning	2	Licking wound or painful area	2
Screaming	3	Rubbing wound or painful area	3
		Chewing wound or painful area	4

In the case of spinal, pelvic or multiple limb fractures, or where assistance is required to aid locomotion do not carry out section **B** and proceed to **C**
Please tick if this is the case ☐ then proceed to C.

B. Put lead on dog and lead out of the kennel.

When the dog rises/walks is it?

(iii)	
Normal	0
Lame	1
Slow or reluctant	2
Stiff	3
It refuses to move	4

C. If it has a wound or painful area including abdomen, apply gentle pressure 2 inches round the site.

Does it?

(iv)	
Do nothing	0
Look round	1
Flinch	2
Growl or guard area	3
Snap	4
Cry	5

D. Overall

Is the dog? *(v)*		*Is the dog?* *(vi)*	
Happy and content or happy and bouncy	0	Comfortable	0
Quiet	1	Unsettled	1
Indifferent or non-responsive to surroundings	2	Restless	2
Nervous or anxious or fearful	3	Hunched or tense	3
Depressed or non-responsive to stimulation	4	Rigid	4

Total Score (i+ii+iii+iv+v+vi) = _____

Figure 4. Short form of the Glasgow composite measure pain scale (CMPS-SF) (Reid *et al.*, 2007)

No pain ——————————— Worst possible pain

Figure 5. Overall pain behavior visual analog scale (OP-VAS)

Figure 6. Criteria for scoring subjective saliva and blood sampling stress behavior visual analog scale (S-VAS). A: criteria used during saliva sampling; B: criteria used during blood sampling.

3.3.1 Physical examination

Research Beagle dogs: The physical examination protocol included mental status, general attitude, appetite, mucus membrane appearance, capillary refill time, rectal temperature, body weight, body condition score, hydration status, auscultation of heart and respiratory rate and sounds, abdominal palpation, musculoskeletal system palpation, lymph node palpation, hair and skin condition, mouth, ear, and eye examination. All physical examinations were performed.

Blood donor dogs: Dogs were routinely physical examined in accordance with the UDS routines by a veterinarian prior to blood donation.

Dogs undergoing elective OHE and dogs with traumatic bone fractures dogs: A complete physical examination using a standardized protocol including mental status, general attitude, appetite, mucus membrane appearance, capillary refill time, rectal temperature, body weight, body condition score (9 scales), hydration status, auscultation of heart and respiratory rate and sounds, abdominal palpation, musculoskeletal system palpation, lymph node palpation, hair and skin condition, mouth, ear, and eye examination was performed (Figure 6).

3.3.2 Sample collection

Saliva was collected using a salimatrics swab (SalivaBio Children's swab, Salimetrics, PA, USA) placed into the buccal cavity of the dog's mouth for 60–90 seconds. The swab was then transferred into a swab storage tube (Swab storage tubes, Salimetrics, PA, USA) and centrifuged at 3000 RPM for 15 minutes. The deposited saliva was freeze stored until the analysis.

Blood was collected from the distal cephalic vein using a butterfly needle into lithium heparinized tubes and clot activator tubes (BD Vacutainer, Becton-Dickson, Plymouth, United Kingdom). Plasma and serum samples were obtained after centrifugation at 3300 RPM for 5 minutes. Samples were then frozen until analysis.

Saliva and blood samples were collected at time points and stored as follows:

Research Beagle dogs: Saliva samples were collected prior to blood collection by the same veterinarian (the author). Blood samples were collected by the same veterinarian. Saliva and plasma samples were directly freeze stored at –70 °C.

Blood donor dogs: Blood was collected by two certified veterinary nurses for routine health screening prior to blood donation and the remaining blood was used for Paper I and IV. Saliva samples were collected by the same veterinarian (the author). The order of blood and saliva sampling was randomized for practical reasons. Saliva, plasma, and serum samples were freeze stored at –70 °C.

Dogs undergoing elective OHE and dogs with traumatic bone fractures: Saliva and blood samples were collected by the same veterinarian (the author). Saliva, plasma, and serum samples were initially stored at −20 °C at KKU, then transported at a temperature below –20 °C to SLU (Temperature control, World Courier, Bangkok, Thailand) and freeze stored in –70 °C until analysis.

Dog name_____**Breed**_____**HN**_____**ID**_____
Age_____**Birthdate**_____**Gender Female / Male**
Owner_____**Address**_____
_____**Tel**_____
Date_____**Time**_____**Temp**_____**BW**_____**kg**
CC:_____
Hx:_____
_____
Disease history:_____
_____
Previous medication:_____
PE: Mental: responsive/depress/stupor/coma, General attitude: normal/mild/moderate/ severe, BCS_____/9, Membrane: white/pale/pink/red/icterus/cyanosis, _____ CRT_____sec, Hydration status_____, Appetite: Normal/Hungry/ Refuse/NPO/force feed,_____, Movement: Normal/stumbling/recumbent /paralyzed/lameness, _____, Palpation: Limb RtFL: crepitus/pain/_____ LtFL: crepitus/pain/ _____ RtHL: crepitus/pain/ _____ LtHL: crepitus/pain/ _____, Neck: crepitus/pain/ _____, Back crepitus/pain/ _____, Heart rate_____bpm, Heart sound: normal/murmur/_____, Heart rhythm: normal/arrhythmia, _____, Respiratory rate_____bpm, Respiratory sound: normal/increase/ decrease/ dull/ crackle/ wheeze/_____, Abdominal palpation: soft/ tense/ _____location: cranial, middle, caudal,_____ Urination: normal/ hematuria/ stranguria/anuria/_____, Lymph node palpation: normal/enlargement/_____, Hair and skin condition: normal/ alopecia/scale/dermatitis/pustule/ _____, Mouth: gingivitis/ calculus/ periodontitis/ tooth fracture/ _____, Ears: otitis/discharge/injury/ _____, Eyes: keratitis/ulcer/glaucoma/cataract/discharge/_____ Injuries: _____, Surgical wound: erythema/swelling/discharge/ stitch bite/dehiscence _____, Glasgow pain score_____ other_____
_____
Overall pain ——————————————————
Saliva stress ——————————————————
Blood stress ——————————————————
Blood test: CBC / BP / Crea / BUN / ALT /_____
_____
Sample collection: Saliva, Blood, Consent, Tube ID_____
Follow up date_____
Examiner_____Surgeon_____

Thanikul Srithunyarat

Figure 7. Physical examination chart. Abbreviations: HN: hospital number; BCS: body condition score; CRT: capillary refill time; NPO: nothing per oral; RtFL: right forelimb; LtFL: left forelimb; RtHL: right hind limb; LtHL: left hind limb; bpm: beats/breaths per minute; CBC: complete blood count; BP: blood parasites; Crea: creatinine; BUN: blood urine nitrogen; ALT: alanine aminotransferase.

3.3.3 Subjective stress and pain assessments

In all dogs, stress and pain behavior were scored by the same observer (the author).

Pain behavior was scored in dogs undergoing elective OHE and in dogs with traumatic bone fractures using the CMPS-SF and OP-VAS prior to sample collection. The CMPS-SF had a total score of 24 in dogs undergoing elective OHE. In accordance to the instructions for use of the CMPS-SF, a total score is 20 in dogs with traumatic bone fractures. Scores ≥ 6/24 and ≥ 5/20 indicate pain and that analgesic treatment is required (Reid *et al.*, 2007). The OP-VAS was scored using a 100-mm VAS line where one end indicated "no pain" and the other end "worst possible pain" (Figure 5).

Stress behavior was also scored shortly after each saliva and blood sampling occasion using a 100-mm line of visual analog scale (S-VAS) in all dogs. The criteria for saliva and blood sampling S-VAS, modified from a study by Norling (Norling *et al.*, 2012), are illustrated in Figure 6.

3.4 Laboratory analysis

3.4.1 Analysis of chromogranin A epitopes catestatin and vasostatin

CgA, in this study, was analyzed using rabbit antibodies to the human CgA sequence 17–38 for VS and 361–372 for CST, as previously reported (Stridsberg *et al.*, 2014). This method has been developed for both tissue and circulating concentrations. The limit of detection is 0.01 nmol/L for plasma CST and VS and 0.04 nmol/L for saliva CST and coefficient of variation (CV) was < 10% (Stridsberg *et al.*, 2004). Samples were analyzed for CST and VS in duplicate using region specific RIA at the Clinical Chemistry Laboratory, Uppsala University Hospital, Uppsala, Sweden. The overall CV in all studies was < 10%. A volume of 300 µL saliva and 100 µL plasma was required for each analysis. Saliva volumes were unpredictable and often insufficient for analyzing of CST and VS. When saliva was obtained, only saliva CST was analyzed.

3.4.2 Cortisol analysis

Serum samples were analyzed in duplicate for cortisol using solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000, Siemens, Erlangen, Germany) at the Clinical Chemistry Laboratory, UDS, SLU. The overall CV was < 5 %.

3.5 Statistical analysis

Data were first checked for normality and homoscedasticity and parameters with skewness were transformed by natural log. In Paper I, both CST and VS concentration were natural log transformed and, in Paper II–IV, only plasma VS concentrations were natural log transformed. All statistical analysis were performed using SAS package (SAS Institute, 2014) and the p value of < 0.05 was considered significant.

Paper I: Plasma CST, plasma VS, saliva CST, saliva S-VAS, and blood S-VAS scores were analyzed in the research Beagle dog group and blood donor dog group. Reference ranges for plasma CST, plasma VS, and saliva CST were calculated from natural log transformed data using percentile 2.5 to 97.5 and then back-transformed to the original scale. Age, gender, breed, and time of collection were calculated using Mixed Model procedure and Tukey adjustment for multiplicity (SAS Institute, 2014). Independent sample t-test was also used to analyze the difference between the dog groups. Correlation between parameters was analyzed using Proc Corr in SAS package. Serum cortisol concentration was presented as mean ± SD.

Paper II: Plasma CST, plasma VS, saliva CST, serum cortisol, CMPS-SF, OP-VAS, saliva S-VAS, blood S-VAS score, temperature, respiratory rate, and heart rate were compared between three different time points including before surgery, 3 hours after extubation, and at recall using Mixed Model procedure, where dog was a random factor, and Tukey adjustment was used for multiplicity.

Paper III: Parameters including plasma CST, plasma VS, saliva CST, serum cortisol, CMPS-SF, OP-VAS, saliva S-VAS, blood S-VAS scores, temperature, respiratory rate, and heart rate in dogs with traumatic bone fractures were compared between before and after morphine administration using Mixed Model procedure with dog as a random factor. Parameters in dogs with traumatic bone fractures were also compared with dogs before elective OHE as a control group using two independent sample t-test. All assessed parameters were also analyzed based on cause of trauma (unknown or car accident), duration of injury (< 48 hours or ≥ 48 hours), fracture site (femur, tibia, both femur and tibia, or pelvis) and number of bone fractures (1–7), last analgesic drug received (no analgesia, unknown/not specified analgesia, carprofen, or morphine), and withdrawal period from previous analgesia (≤ 12 hours or > 12 hours) in dogs with traumatic bone fractures using Mixed Model procedure. Pairwise comparisons were adjusted for multiplicity using Tukey's method. However, saliva samples were insufficient, and saliva CST therefore could not be determined in this study.

CMPS-SF, OP-VAS, serum cortisol, and plasma CST concentrations were calculated for delta values by subtracting post treatment values from baseline values prior to morphine treatment. Absolute values were also defined for serum

cortisol, and plasma CST concentrations as the delta values without + or – signs. The correlation were calculated using Proc Corr between analgesia group, the delta values for CMPS-SF and OP-VAS scores, and the absolute delta values for serum cortisol and plasma CST concentrations.

Paper IV: Plasma CST, plasma VS, saliva CST, serum cortisol, saliva S-VAS, and blood S-VAS scores were compared between two dogs groups using independent sample t-test. Correlation of parameters were also calculated using Proc Corr.

4 Results

The results in Paper I–IV are summarized as follows.

4.1 Assessments in healthy dogs accustomed to the sampling procedures

Age, gender, body weight, and general information of included dogs and concentrations of plasma CST, plasma VS, and saliva CST and saliva and blood S-VAS scores in both research Beagle and blood donor dog groups are presented in Paper I.

4.1.1 Chromogranin A epitopes catestatin and vasostatin

Reference ranges of plasma CST, plasma VS, and saliva CST concentrations were established as the reference ranges measured in 33 healthy dogs accustomed to the sampling procedures and are shown in Table 1.

Table 1. *Reference ranges of Chromogranin A epitopes catestatin and vasostatin in 33 healthy dogs accustomed to sampling procedures*

Chromogranin A epitope	Reference range (nmol/L)
Plasma catestatin	0.53–0.98
Plasma vasostatin	0.11–1.30
Saliva catestatin	0.31–1.03

The mean ± SD plasma CST, plasma VS, and saliva CST concentrations were 0.81 ± 0.08, 0.57 ± 0.55, and 0.83 ± 0.12 nmol/L, respectively, in research Beagle dogs, and 0.76 ± 0.10, 0.44 ± 0.39, and 0.64 ± 0.21 nmol/L, respectively, in blood donor dogs. There was no significant correlation between plasma CST, plasma VS, and saliva CST. Furthermore, CST and VS differed significantly ($p <$

0.0001) in both dog groups. Concentrations of plasma CST, plasma VS, and saliva CST did not differ significantly between research Beagle and blood donor dog groups. Concentrations of plasma CST, plasma VS, and saliva CST did not differ significantly by age, gender, breed, or time of collection as well as between dogs with different DEA 1.1 or between dogs with and without antibodies against *Borrelia burgdorferi*.

4.1.2 Cortisol

The mean ± SD serum cortisol concentration was 39.9 ± 6.1 nmol/L in the research Beagle dog group and 65.8 ± 28.2 nmol/L in the blood donor dog group.

4.1.3 Visual analog scale

The mean ± SD saliva and blood sampling S-VAS scores in research Beagle dogs were 11.1 ± 7.8 and 8.9 ± 10.5 mm, respectively, and in blood donor dogs were 21.2 ± 16.7 and 19.1 ± 17.3 mm, respectively. In this study, the S-VAS scores in all dogs were low, which indicates minimal stress behavior (Paper I). No significant differences based on sampling method (saliva and blood sampling), age, gender, breed, time of collection or between research Beagle and blood donor dog groups were found.

4.2 Assessments in dogs undergoing elective ovariohysterectomy

Data on age, gender, body weight, and general information of included dogs and concentrations of plasma CST, plasma VS, and saliva CST, temperature, heart rate, respiratory rate, CMPS-SF, OP-VAS, saliva and blood S-VAS scores from each time point are presented in Paper II and Table 2.

4.2.1 Chromogranin A epitopes catestatin and vasostatin

Plasma CST concentrations were significantly decreased at 3 hours after extubation (p = 0.002) and at recall (p = 0.04) compared with before surgery whereas no significant difference between 3 hours after extubation and at recall (p = 0.56) was found (Figure 8). No significant changes in plasma VS and saliva CST were reported at any of the time points (Figure 9–10 and Table 2).

4.2.2 Cortisol

Serum cortisol concentrations at recall were significantly decreased compared with before surgery ($p = 0.009$). No significant differences were found between before surgery and 3 hours after extubation ($p = 0.73$) and between 3 hours after extubation and at recall ($p = 0.06$) (Figure 11 and Table 2).

4.2.3 Physiological assessments

Rectal temperature and respiratory rate were significantly lower at 3 hours after extubation compared with before surgery and at recall ($p < 0.0001$) while no significant difference was found between before surgery and at recall ($p = 0.9$) (Figure 12–13). Heart rate did not differ significantly between any time points (Figure 14).

4.2.4 The short form of Glasgow composite measure pain scale

The CMPS-SF scores were significantly higher at 3 hours after extubation than before surgery and at recall ($p < 0.0001$) (Figure 15 and Table 2). No significant differences were found between CMPS-SF scores before surgery and at recall ($p = 0.07$).

4.2.5 Visual analog scale

The OP-VAS results were in agreement with the CMPS-SF where scores at 3 hours after extubation were significantly higher than before surgery and at recall ($p < 0.0001$). Saliva and blood S-VAS scores were significantly lower than before surgery ($p < 0.0001$ for saliva and $p = 0.0001$ for blood S-VAS) and at recall ($p = 0.003$ for saliva and $p = 0.03$ for blood S-VAS). No significant difference was found between before surgery and at recall regarding OP-VAS, saliva and blood S-VAS results (Figure 16–18 and Table 2).

4.3 Stress and pain assessments in dogs with traumatic bone fractures

Data of the included dogs regarding the parameters age, gender, body weight, and general information of included dogs, concentrations of plasma CST, plasma VS, serum cortisol, temperature, heart rate, respiratory rate, CMPS-SF, OP-VAS, saliva and blood S-VAS scores from each time point, are presented in Paper III and Table 2. Cause of trauma, duration of injury, fracture site and number of bone fractures, and withdrawal period from previous analgesia in dogs with

traumatic bone fractures did not significantly affect any of the assessed parameters for all comparisons in this study.

4.3.1 Chromogranin A epitopes catestatin and vasostatin

In dogs with fractures before morphine administration, concentrations of plasma CST were significantly decreased (p = 0.009) compared with the healthy dogs prior to OHE (Figure 8 and Table 2). After morphine administration, plasma CST concentrations were still lower compared with the healthy dogs (p = 0.002) and no significant changes were found between before and after morphine administration (p = 0.30). No significant changes of plasma VS were detected between any of the sampling time points (Figure 9). Plasma CST and VS concentrations overlapped to a large degree with the reference ranges in low-stressed healthy dogs, as established in Paper I. The absolute delta values for plasma CST significantly correlated with the delta values for CMPS-SF (r = 0.31, p = 0.04). Plasma CST did not significantly differ between the different analgesia groups (p = 0.3) (Paper III). Saliva was not obtained in a sufficient volume to be able to analyze saliva CST.

4.3.2 Cortisol

Serum cortisol concentrations were significantly lower in dogs with bone fractures prior to morphine administration compared with the healthy dogs (p = 0.01) (Figure 11 and Table 2). No significant differences were found between before and after morphine treatment (p = 0.3) and between healthy dogs and dogs with fractures after morphine treatment (p = 0.1). Cortisol concentrations were significantly lower in dogs without prior analgesia treatment than in dogs that had received unknown, carprofen, or morphine analgesia more than 6 hours prior to inclusion in the study (p = 0.02) (Paper III).

4.3.3 Physiological assessments

The physiological parameters of temperature, respiratory rate, and heart rate did not differ significantly between healthy dogs and dogs with traumatic bone fractures (Figure 12–14). After morphine administration, temperature and heart rate (p = 0.001) decreased significantly, whereas no changes was found in respiratory rate, compared with before morphine treatment.

4.3.4 The short form of Glasgow composite measure pain scale

Scores of the CMPS-SF in dogs with bone fractures both before and after morphine administration were significantly higher than in the healthy dogs ($p < 0.0001$). After morphine administration, the CMPS-SF scores had decreased significantly ($p = 0.005$) compared with before the treatment (Figure 15 and Table 2).

4.3.5 Visual analog scale

The OP-VAS scores, similar to the CMPS-SF, were significantly higher in dogs with fractures both before and after morphine treatment compared with the healthy dogs ($p < 0.0001$). After morphine administration, OP-VAS scores were significantly decreased compared with before treatment ($p = 0.02$) (Figure 16 and Table 2).

The saliva and blood S-VAS scores were significantly lower in dogs with bone fractures before morphine administration than in healthy dogs ($p = 0.048$ for saliva and $p = 0.02$ for blood S-VAS, respectively). After morphine treatment, saliva and blood S-VAS scores were significantly lower than in healthy dogs ($p = 0.002$ for saliva and $p = 0.0005$ for blood S-VAS, respectively) and dogs with bone fractures before morphine administration ($p = 0.02$ for saliva and $p = 0.01$ for blood S-VAS, respectively) (Figure 17–18 and Table 2).

4.4 Assessments for psychological stress in healthy dogs

Data of the parameters age, gender, body weight, general information of included dogs, concentrations of plasma CST, plasma VS, serum cortisol, saliva and blood S-VAS scores are presented in Paper IV and Table 2.

4.4.1 Chromogranin A epitopes catestatin and vasostatin

Saliva CST concentrations were significantly higher in the stress group than in the control group ($p = 0.003$) and higher than the reference ranges for the low-stressed healthy dogs reported in Paper I (Figure 10 and Table 2). Plasma CST ($p = 0.88$) and VS ($p = 0.09$) concentrations did not differ significantly between dog groups (Figure 8–9 and Table 2).

Saliva and plasma CST correlated significantly with serum cortisol concentrations ($r = 0.34$, $p = 0.04$ for saliva CST and $r = 0.29$, $p = 0.03$ for plasma CST). Additionally, saliva CST also correlated significantly with saliva S-VAS score ($r = 0.47$, $p = 0.003$). No significant correlation was found between saliva and plasma CST.

4.4.2 Cortisol

Serum cortisol concentrations were significantly higher in the stress group compared with the healthy group ($p < 0.0001$) (Figure 11 and Table 2). Serum cortisol concentrations were significantly correlated to both saliva and plasma CST concentrations.

4.4.3 Visual analog scale

Saliva S-VAS and blood S-VAS scores were significantly higher in the stress group compared with the control group ($p = 0.0009$ for saliva and $p = 0.002$ for blood, respectively) (Figure 17–18 and Table 2). Saliva S-VAS scores significantly correlated with blood S-VAS scores (r = 0.86, $p < 0.0001$).

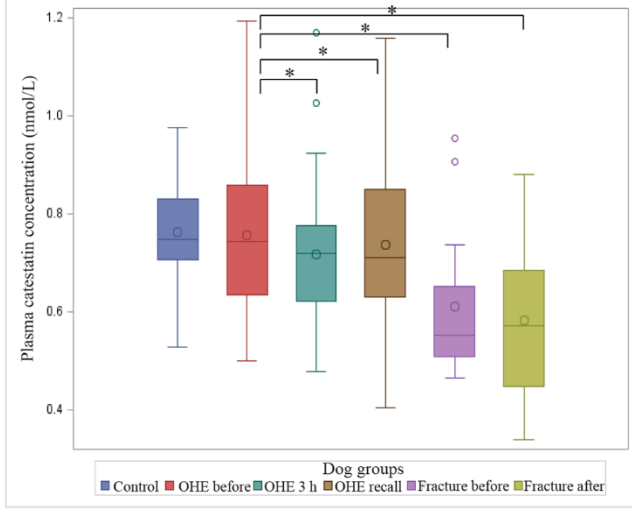

Figure 8. Boxplot of plasma catestatin concentrations in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

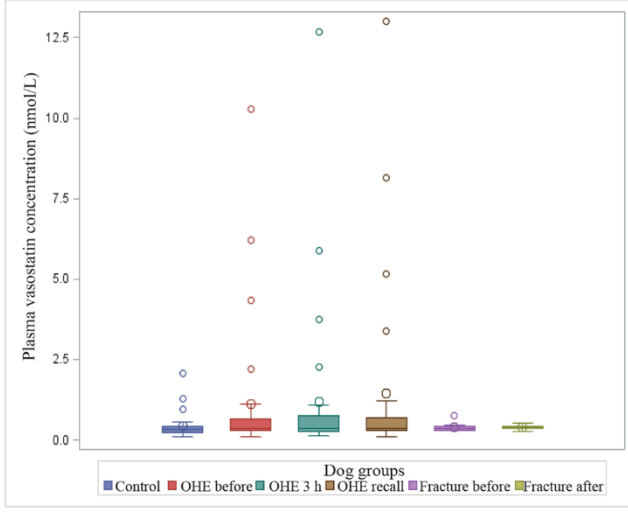

Figure 9. Boxplot of plasma vasostatin concentrations in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

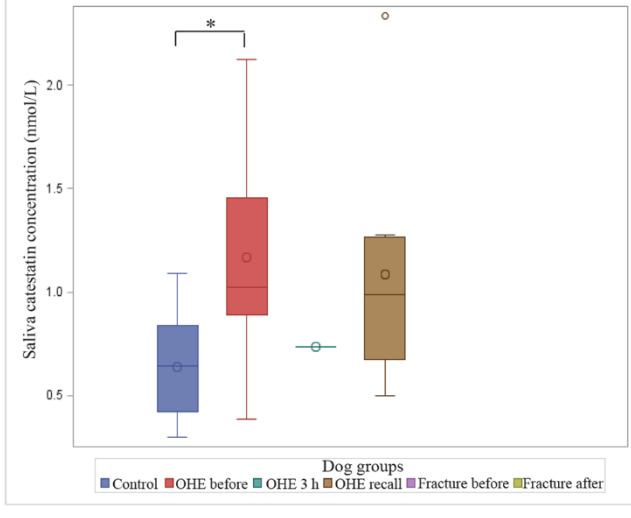

Figure 10. Boxplot of saliva catestatin concentrations in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

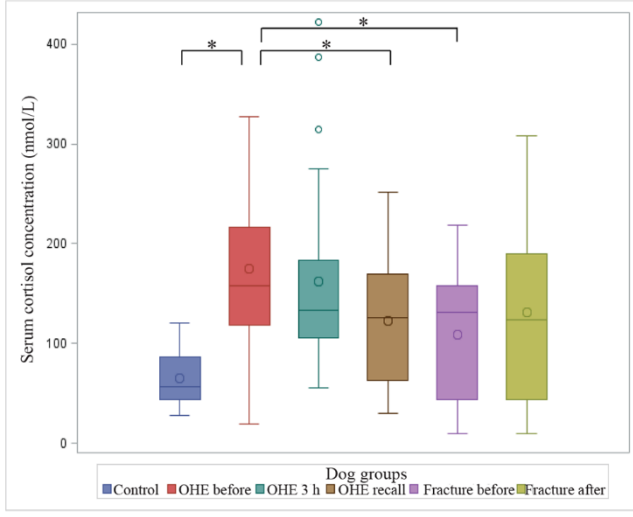

Figure 11. Boxplot of serum cortisol concentrations in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

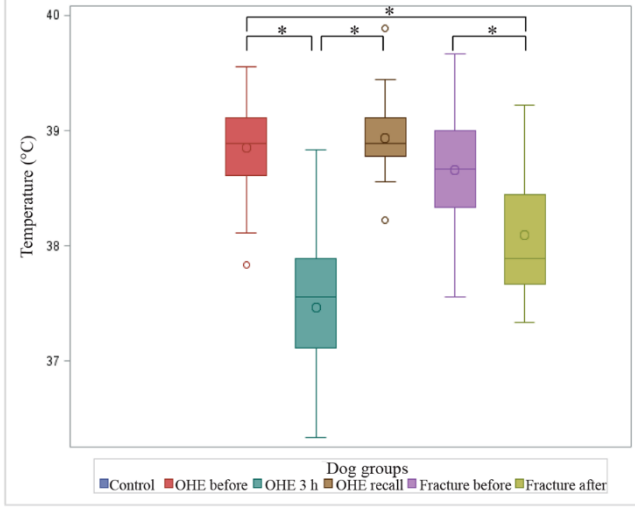

Figure 12. Boxplot of rectal temperature in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

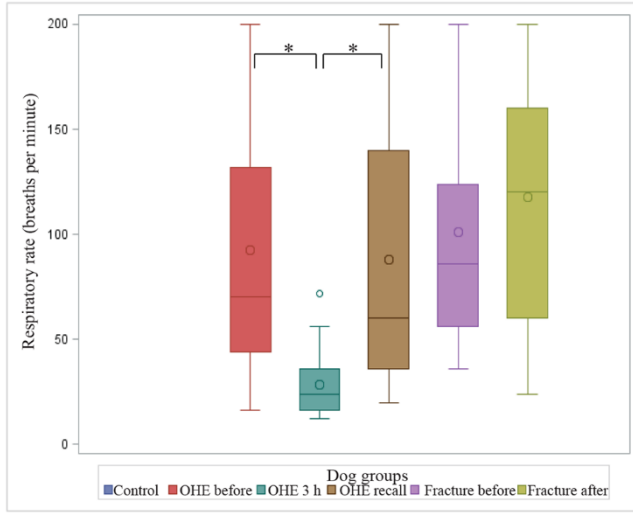

Figure 13. Boxplot of respiratory rate in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

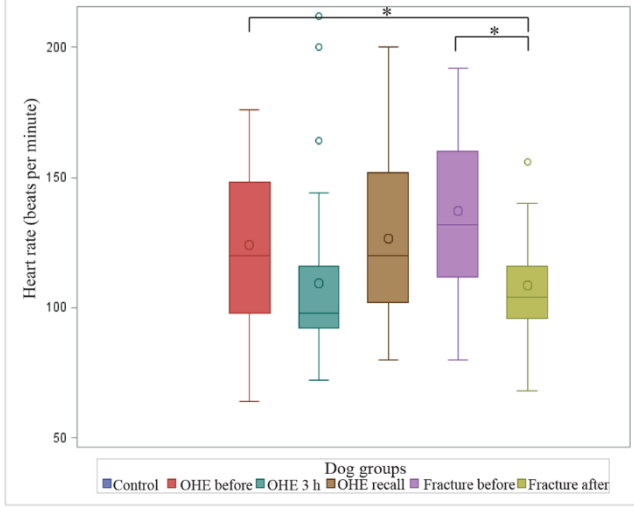

Figure 14. Boxplot of heart rate in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

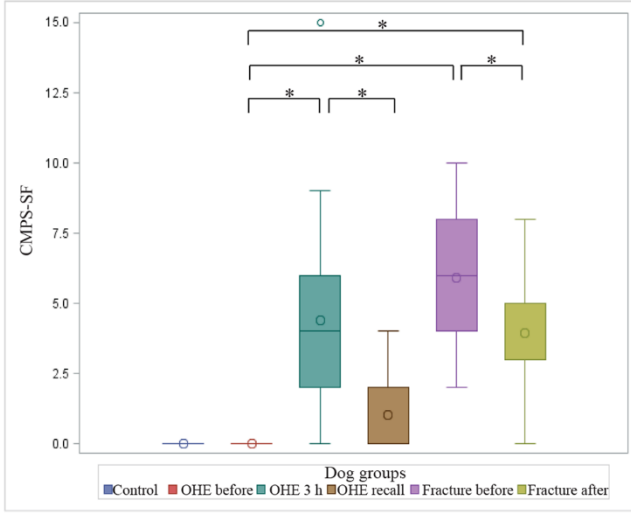

Figure 15. Boxplot of subjective assessments of the short form of Glasgow composite measure pain scale (CMPS-SF) in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

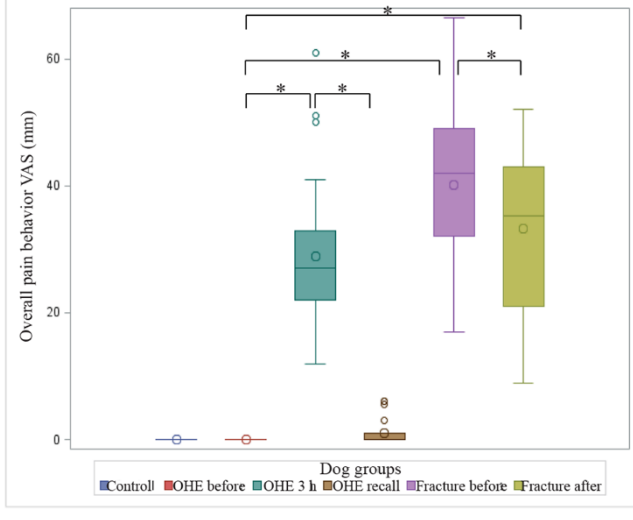

Figure 16. Boxplot of overall pain behavior visual analog scale (OP-VAS) in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

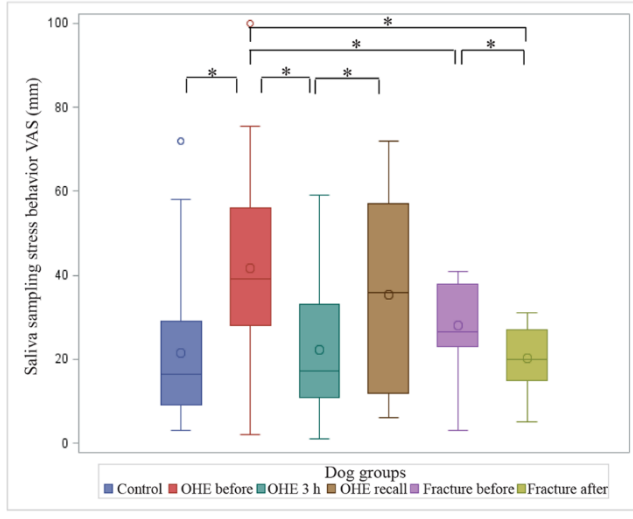

Figure 17. Boxplot of saliva sampling stress behavior visual analog scale (S-VAS) in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

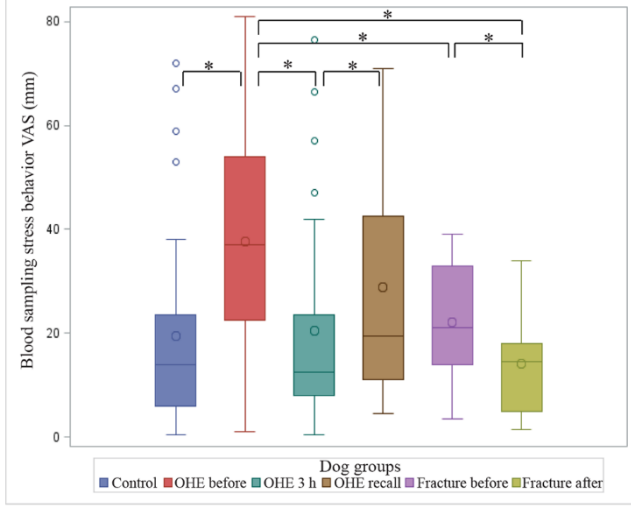

Figure 18. Boxplot of blood sampling stress behavior visual analog scale (S-VAS) in dogs. Dogs were grouped into healthy dogs with low stress (Control, n = 33), healthy dogs before ovariohysterectomy (OHE before, n = 30), after ovariohysterectomy at 3 hours after extubation (OHE 3 h, n = 30), and at recall (OHE recall, n = 27), dogs with fractures before morphine treatment (Fracture before, n = 14), and after morphine treatment (Fracture after, n = 14). * Significant difference between groups ($p < 0.05$)

Table 2. *Data (mean ± SD) of the assessed parameters in blood donor dogs, dogs undergoing elective ovariohysterectomy, and dogs with traumatic bone fractures*

Parameters	Blood donor dogs	Dogs undergoing elective ovariohysterectomy			Dogs with traumatic bone fractures	
Time point	Donation	Before surgery	3 h after extubation	Recall	Before morphine	After morphine
Time	8:00 a.m.–3:00 p.m.	8:30 a.m.–11:20 a.m.	1:00 p.m.–5:30 p.m.	8:50 a.m.–6:15 p.m.	9:05 a.m.–7:00 p.m.	9:40 a.m.–7:47 p.m.
n	33	30	30	27	14	14
Plasma catestatin (nmol/L)	0.76 ± 0.10	0.76 ± 0.17	0.72 ± 0.16	0.74 ± 0.17	0.61 ± 0.15	0.58 ± 0.16
Plasma vasostatin (nmol/L)	0.42 ± 0.39	1.12 ± 2.16	1.17 ± 2.49	1.45 ± 2.93	0.39 ± 0.12	0.39 ± 0.07
Saliva catestatin (nmol/L)	0.64 ± 0.24	1.17 ± 0.48	0.74	1.09 ± 0.59	NA	NA
Serum cortisol (nmol/L)	65 ± 28	175 ± 79	162 ± 88	123 ± 64	108 ± 69	131 ± 94
Temperature (°C)	-	38.9 ± 0.4	37.5 ± 0.6	38.9 ± 0.4	38.7 ± 0.5	38.1 ± 0.5
Respiratory rate (bpm)	-	93 ± 63	29 ± 15	88 ± 63	101 ± 60	118 ± 63
Heart rate (bpm)	-	124 ± 31	110 ± 33	126 ± 32	137 ± 33	109 ± 24
CMPS-SF	-	0	4 ± 3 (/24)	1 ± 1 (/24)	6 ± 3 (/20)	4 ± 2 (/20)
OP-VAS (mm)	-	0	29 ± 11	1 ± 2	40 ± 14	33 ± 13
Saliva S-VAS (mm)	22 ± 19	42 ± 24	22 ± 16	35 ± 22	28 ± 11	20 ± 8
Blood S-VAS (mm)	19 ± 20	38 ± 22	20 ± 20	29 ± 21	22 ± 12	14 ± 9

Data from dogs undergoing ovariohysterectomy at all time points (before surgery, 3 hours after extubation, and at recall) are compared in Paper II, dogs with traumatic bone fractures both before and after morphine treatment compared with dogs undergoing elective ovariohysterectomy before surgery are compared in Paper III, and blood donor dogs compared with dogs undergoing ovariohysterectomy before surgery in Paper IV. Abbreviations: NA: not assessed due to limited saliva volume; bpm: beats/breaths per minute; CMPS-SF: the short form of Glasgow composite measure pain scale; OP-VAS: overall pain behavior visual analog scale; S-VAS: stress behavior visual analog scale

5 Discussion

To evaluate their potentials, reference ranges were initially established in healthy low-stressed dogs. The potential and usefulness of CST and VS in different clinical settings were thereafter evaluated by comparing CST and VS concentrations, as well as other stress and pain assessments, in dogs experiencing psychological and pain-induced stress.

5.1 Chromogranin A epitopes catestatin and vasostatin in healthy dogs

5.1.1 Reference ranges of catestatin and vasostatin in low-stressed healthy dogs

Reference ranges of plasma CST, VS, and saliva CST concentration were established in dogs experiencing minimal stress. Complete avoidance of stress is difficult to achieve; it is however imperative to minimize when establishing reference ranges for further studies on stress in both animals and humans. In this study, only dogs that were accustomed to the sampling procedures and environment were included. The stress levels were deemed minimal based on low S-VAS scores and serum cortisol concentrations. Concentrations of stress biomarkers may be influenced by biological variations i.e. rapid degradation in catecholamines and confounding factors of age, gender, pulsatile secretion, and circadian variations in cortisol. Our findings showed that CST and VS concentrations were not significantly affected by age, gender, and breed in dogs. Further, by analyzing repeated samples within individuals over time, we showed that CST and VS did not vary depending on time at which the sample was obtained. These findings are in agreement with a study on saliva CgA in dogs over a 24-hour period where no circadian rhythm was found (Kanai *et al.*, 2008). The reference ranges of plasma CST, plasma VS, and saliva CST established in

this thesis, therefore, can be used as a baseline for low-stressed healthy dogs and will be important in further studies on CST and VS in dogs.

The range for plasma CST found in this study was lower than in a control group in a previous study using the same region specific RIA (Jitpean *et al.*, 2015). However, in the previous study, samples had been stored for over 5 years with repeated thawing cycles. Although CgA has been found to be stable during storage, temperature variations, and through thawing and freezing cycles in several species (Escribano *et al.*, 2014; Taupenot *et al.*, 2003; O'Connor *et al.*, 1989), no long-term studies have been performed in dogs. In addition, because the region specific RIA measured both the degradation peptide and the intact molecule, it is possible that the sensitivity to storage may vary between these two parameters.

Although both CST and VS are derived from CgA, CST and VS concentrations differed significantly and did not significantly correlate. This was unexpected and possibly the significant differences in concentrations seen in Paper I reflect different functions and clearance rates of the two epitopes.

An active secretion of CgA has been found in saliva glands in humans, rats, and horses (Saruta *et al.*, 2005; Sato *et al.*, 2002; Kanno *et al.*, 1999). In rats, saliva CgA concentration has been shown to depend on the intensity of stimuli and less likely to be affected by saliva flow (Mahata *et al.*, 2004; Kanno *et al.*, 1999; Mahata *et al.*, 1997; Cryer *et al.*, 1991; O'Connor & Bernstein, 1984). The lack of correlation between saliva CST and plasma CST in our studies suggests an active secretion of CST also from canine salivary glands. According to our findings, it is important to evaluate different CgA epitopes separately, whether from blood and saliva.

Saliva sampling is a well-known noninvasive sampling technique commonly used in humans because it induces less stress than other sampling techniques. Stress behavior during blood and saliva sampling was scored in all the included studies using an S-VAS. All the S-VAS scoring was performed by the same person. Blood sampling S-VAS scores were similar to saliva sampling S-VAS, and no significant difference was found between blood and saliva sampling S-VAS in any of the dog groups. Although saliva sampling is noninvasive, it may induce a similar degree of psychological stress as blood sampling in dogs. However, obtained saliva volumes were unpredictable and often insufficient for analyzing both CST and VS in dogs, making saliva sampling difficult to use in a clinical setting.

5.1.2 Catestatin and vasostatin in healthy dogs subjected to psychological stress

The potential of CST and VS as psychological stress biomarkers was further evaluated by comparing concentrations between healthy low-stressed and stressed dogs (Paper IV). Commonly, psychological stress, often referred to as the "white coat effect", is elicited when dogs are exposed to an environment and treated at a veterinary hospital. This psychological stress can stimulate SAM and HPA axes (Hekman *et al.*, 2014; Höglund *et al.*, 2012). In Paper IV, the control group was composed of dogs familiar with the sampling procedures and the animal hospital environment. The dogs perceived minimal stress based on their low S-VAS scores and serum cortisol concentrations. In contrast, dogs in the stress group were unfamiliar with the sampling procedures and the animal hospital environment and, based on the S-VAS and cortisol concentrations, experienced significantly higher psychological stress. In a comparison between the control and stress group, the mean saliva CST concentrations doubled in the stress group and significantly differed from the controls. Additionally, the overall saliva CST concentration in the stress group was over the reference range reported in healthy low-stressed dogs in Paper I.

In humans, several studies have indicated that saliva CgA shows promise as a sensitive biomarker for psychological stress (Takatsuji *et al.*, 2008; Kanamaru *et al.*, 2006; Lee *et al.*, 2006; Nakane H, 1998). Although it was difficult to obtain a sufficient saliva volume, our results suggest that saliva CST shows some promise as a biomarker for psychological stress also in dogs.

The plasma CST concentrations in the psychologically stressed dogs did not significantly differ from the controls and did not significantly correlate with saliva CST concentrations. These findings further indicate an active secretion of CST in saliva in dogs.

The concentrations of plasma CST and VS in the stress group overlapped to a large degree with the reference ranges for healthy low-stressed dogs, suggesting that plasma CST and VS may be unaffected by psychological stress in a clinical setting. Little is known about CgA and its degradation peptides' secretion (onset, peak, and duration) in dogs. All samples included in Paper IV were collected within 60 minutes after arrival at the veterinary hospital. In humans, different studies have reported half-life varying from 18–257 minutes (Stridsberg *et al.*, 2008; O'Connor & Bernstein, 1984). However, no studies on CST and VS half-life have yet been reported. Possibly we missed the peak values when sampling. However, all dogs still showed behavior signs of ongoing psychological stress based on the S-VAS evaluation in conjunction with sampling.

Intensity of stress stimuli may be one factor influencing the CgA secretion. In humans, prolonged low-intensive physical stress has been shown not to

significantly change plasma CgA concentrations, while intensive physical stress i.e. a marathon may lead to increased concentrations (Nickel *et al.*, 2012; Nakane H, 1998; Takiyyuddin *et al.*, 1990). Additionally, plasma CgA increased significantly in dogs, under experimental conditions, experiencing severe stress induced by hypoglycemia after insulin injection (Akiyoshi *et al.*, 2005). The degree of psychological stress in Paper IV may have been insufficient to change plasma CST and VS concentrations in the stress group. One of our aims was, however, to evaluate CST and VS potential as biomarkers for stress in a clinical setting. This degree of stress experienced by the dogs in our studies is probably more indicative of the level of stress that needs to be identified in a clinical setting.

Our findings illustrated that saliva CST may have potential as a psychological stress biomarker whereas plasma CST and VS concentrations have limited potential in dogs. The findings further show the importance and usefulness of the established reference ranges in healthy dogs in a clinical setting.

5.2 Chromogranin A epitopes catestatin and vasostatin in dogs experiencing pain

The potential of CST and VS as biomarkers for pain-induced stress was investigated by comparing concentrations in dogs experiencing different painful situations with healthy control dogs without pain (Paper II and III). In Paper II, CST and VS concentrations were compared in healthy dogs before and after undergoing elective OHE. The OHE procedure was chosen because this is a surgical technique that can be reasonably standardized and has been used in previous studies on pain and surgical stress in dogs (Morgaz *et al.*, 2013; Kim *et al.*, 2012; Kongara *et al.*, 2012; Shih *et al.*, 2008; Devitt *et al.*, 2005; Mastrocinque & Fantoni, 2003). In Paper III, previously healthy dogs with traumatic bone fractures were included and CST and VS concentration compared before and 35–70 minutes after morphine administration. Although difficult to standardize, we chose to perform this study on patients rather than using research animals to reduce the need for inflicting pain experimentally. If the results were to show that CST and VS significantly deviated from the reference range, further studies would be warranted using more standardized experimental protocols. In both Paper II and III, CST and VS concentrations were compared with serum cortisol levels, S-VAS, OP-VAS and CMPS-SF.

Although the dogs in the OHE study received preemptive morphine analgesia, CMPS-SF and OP-VAS indicated some degree of pain at 3 hours after extubation. Plasma CST but not VS significantly decreased at 3 hours after extubation compared with before surgery but the concentrations were within the previously

established normal reference range for low-stressed dogs. At recall for suture removal, plasma CST and serum cortisol concentrations significantly decreased compared with before surgery, but not at 3 hours after extubation. All subjective assessments of stress and pain (decreased S-VAS, increased CMPS-SF and OP-VAS scores) at 3 hours after extubation differed significantly with before surgery and at recall; however, no significant differences were found between before surgery and at recall. Catestatin has an inhibitory role as a negative feedback for the release of catecholamines and CgA (Mahata *et al.*, 2004), which may contribute to the decreased concentrations of plasma CST seen in this study. Many different stimuli can induce stress reactions, including fear and psychological anxiety. The high concentrations of serum cortisol and saliva CST seen before surgery could possibly be due to the white coat effect with less response at recall, perhaps because the dogs were more accustomed to the animal hospital environment. However, the S-VAS scores did not differ significantly between before surgery and at recall. Because both the CMPS-SF and the OP-VAS scores indicated pain at 3 hours after extubation, it is tempting to attribute the significant decrease in circulating CST to be indicative of a pain-induced stress. However, although not in line with CMPS-SF and OP-VAS, there was no significant difference in plasma CST levels between 3 hours after extubation and at recall. The CMPS-SF and OP-VAS differed significantly between 3 hours after extubation but not before surgery and at recall. Further studies with more subjects are needed to fully evaluate plasma CST potential for monitoring pain within an individual.

Plasma CST, serum cortisol concentrations, CMPS-SF, and OP-VAS scores differed significantly in dogs with traumatic bone fractures compared with healthy dogs without fractures. Plasma CST concentrations overlapped largely with previously established normal ranges in dogs. Morphine treatment partially relieved pain and stress according to the subjective pain assessments. Circulating levels of CST and cortisol did not differ significantly before and 35–70 minutes after morphine treatment. The absolute delta values for plasma CST, however, correlated significantly with the delta values for CMPS-SF. Dogs with lower delta values for CMPS-SF scores, indicating good pain relief, had higher absolute delta values for plasma CST. Although our results indicated a possible future use of plasma CST for monitoring pain progression, further studies are needed.

On the other hand, plasma VS did not significantly differ between any of the dog groups and time points. This is in line with previous studies in dogs undergoing OHE and in dogs with pyometra where plasma VS concentrations were also found not to change significantly (Höglund *et al.*, 2015; Jitpean *et al.*, 2015). Further, plasma VS concentrations in all of our studies were largely within the reference range established in Paper I. Our findings indicated that plasma VS has no potential as a biomarker for pain-induced stress in dogs.

Obtaining saliva samples was difficult in dogs with CMPS-SF and OP-VAS indicating pain. Too few samples were obtained to be able to fully evaluate saliva CST and VS concentrations and therefore we could not completely assess their potential as biomarkers for pain-induced stress. However, if methods for analyzing CST and VS that required smaller volume of saliva become available, it would be interesting to pursue further studies because saliva CST has shown promise as a biomarker for psychological stress in dogs.

5.3 Other assessments for psychological and pain-induced stress in dogs

Multimodal assessments and compilation of currently available assessment methods may be beneficial for improving psychological and pain-induced stress evaluation in dogs. In this thesis, behavioral, physiological, and neuroendocrine parameters including CMPS-SF, OP-VAS, S-VAS, temperature, respiratory rate, heart rate, serum cortisol, together with CST and VS concentrations have been used for assessing psychological and pain-induced stress in dogs.

5.3.1 Subjective assessment

Subjective assessments are sensitive and accessible for assessing stress and pain in dogs. The subjective assessments, used in this thesis, for evaluating stress behavioral changes were S-VAS scores and for pain, CMPS-SF and OP-VAS scores.

In this thesis, the S-VAS criteria were based on observed avoidance behavior. Because the S-VAS scores significantly correlated with saliva CST and serum cortisol concentrations in stressed and low-stressed healthy dogs, our results indicated that S-VAS may be useful for evaluating psychological stress (Paper IV). However, subjective assessment is technique sensitive which requires a trained and preferably blinded single observer (Holton *et al.*, 2001).

In dogs undergoing elective OHE (Paper II), at 3 hours after extubation, CMPS-SF and OP-VAS scores were significantly increased compared with the baselines, indicating some degree of pain. However, the S-VAS scores were significantly decreased at this time point. Dogs with traumatic bone fractures (Paper III) also had significantly decreased S-VAS scores compared with the healthy control group and, further, S-VAS scores significantly decreased after morphine treatment. CMPS-SF and OP-VAS scores clearly indicated pain prior to morphine treatment. After morphine treatment, CMPS-SF and OP-VAS scores decreased significantly but still indicated some degree of pain. Moreover in these dogs, S-VAS scores were significantly decreased after analgesia. Sedatives, analgesia, and anesthetic drugs can reduce anxiety and stress in dogs. However, the

sedative effect may inhibit the dogs' ability to show avoidance behaviors. S-VAS should therefore be interpreted with caution in dogs receiving sedative or analgesic drugs. Although subjective assessments are sensitive and useful, their interpretation may have limitation as a sole assessment for monitoring psychological and pain-induced stress in dogs.

5.3.2 Objective assessment

Cortisol is secreted when the HPA axis is stimulated and has traditionally been used for stress and pain evaluation in both humans and animals (Höglund et al., 2015; Hekman et al., 2014; Mastrocinque et al., 2012; Michelsen et al., 2012; Tennant & Hermann, 2002). Acute stress induces hypersecretion of cortisol leading to increased circulating concentrations. However, circadian variation, age, gender, and episodic secretion may affect serum cortisol concentrations in dogs (Giannetto et al., 2014; Kemppainen & Sartin, 1984). Therefore, serum cortisol as a sole biomarker for stress should be interpreted cautiously. In this thesis, cortisol was used for evaluating psychological and pain-induced stress together with subjective assessments to evaluate the potential of CST and VS as psychological and pain-induced stress biomarkers. In Paper IV, both serum cortisol concentrations and S-VAS scores were significantly higher in dogs unaccustomed to the animal hospital environment indicating that the dogs were in fact experiencing psychological stress. Further, dogs accustomed to the animal hospital environment had significantly lower serum concentrations and S-VAS scores. Our findings suggest that the combination of S-VAS scores and serum cortisol concentrations may be useful for monitoring stress in dogs. In Paper II, serum cortisol concentrations did not differ significantly between before surgery and at 3 hours after extubation in dogs undergoing elective OHE. The sustained high serum cortisol concentrations seen in this study may reflect HPA axis stimulation both due to psychological stress and surgical stress induced by tissue damage (Desborough, 2000). At recall, unlike S-VAS scores, serum cortisol concentrations were significantly decreased compared with before surgery. Although sampling may have occurred at a peak or trough in plasma cortisol secretion (Giannetto et al., 2014; Rijnberk & Kooistra, 2010; Kooistra et al., 1997; Kemppainen & Sartin, 1984), it cannot be excluded that the dogs might have experienced slightly decreased psychological stress due to "the white coat effect" at recall.

Long-standing pain can lead to a downregulation of the HPA axis resulting in decreased circulating cortisol concentrations (Muhtz et al., 2013; Tennant, 2013; Rijnberk & Kooistra, 2010; Fries et al., 2005). In Paper III, serum cortisol concentration were significantly lower in dogs with traumatic bone fractures

compared to healthy dogs. Furthermore, serum cortisol concentrations in dogs that had not received analgesia before inclusion were significantly lower than those that had received analgesia. CMPS-SF and OP-VAS scores indicated pain in dogs with traumatic bone fractures and differed significantly from healthy dogs. However, unlike CMPS-SF, and OP-VAS scores, circulating cortisol did not differ significantly after morphine administration. Although CMPS-SF and OP-VAS scores indicated a decreased level of pain after morphine administration, trauma-induced tissue damage may still have influenced the HPA axis. However, as stated previously, because of the episodic and pulsatile secretion, single measurements of cortisol must be evaluated cautiously.

Physiological parameters, including heart rate, respiratory rate, and temperature, may, in addition to SAM and HPA stimulation, be affected by several factors limiting their used as a sole assessment for stress and pain. These parameters are, however, included in some multimodal assessments such as Melbourne Pain Scale (Firth & Haldane, 1999). In dogs undergoing elective OHE, temperature and respiratory rate, but not heart rate, significantly decreased at 3 hours after extubation. Premedication, anesthesia, and analgesia can influence several physiological parameters. Morphine may induce cardiovascular and respiratory depression, hypothermia, hypotension, sedation, anxiety, and bradycardia (Martin *et al.*, 1976). In dogs with traumatic bone fractures, temperature and heart rate significantly decreased after morphine treatment. However, temperature, respiratory rate, and heart rate did not differ significantly between dogs with traumatic bone fractures prior to morphine treatment and healthy dogs without fractures further illustrating the limitation of these physiological parameters for monitoring stress and pain.

6 Conclusions

- Reference ranges of the CgA epitopes CST and VS in plasma and CST in saliva were first established in healthy dogs accustomed to the sampling procedures. CST and VS were unaffected by age, gender, breed, and time of day. Plasma CST and VS concentrations differed significantly, did not correlate, and should therefore be evaluated separately.
- The CgA epitopes CST and VS were studied in association with serum cortisol concentrations, CMPS-SF, OP-VAS, S-VAS, and different physiological parameters in dogs undergoing a standardized elective OHE receiving analgesia. At 3 hours after extubation the subjective measurements suggested pain requiring additional analgesia. At this time point, only plasma CST had changed significantly suggesting that it might be useful for monitoring pain progression in a canine patient undergoing surgery. However, because CST concentrations to a large degree overlapped with the reference range, a single measurement of plasma CST has limited potential as a pain-induced stress biomarker for monitoring pain in dogs receiving analgesia.
- In dogs with acute traumatic bones fractures, the CgA epitopes CST and VS were studied in association with serum cortisol concentrations, CMPS-SF, OP-VAS, S-VAS, and different physiological parameters before and after morphine administration, and the results were further compared with healthy control dogs. In addition to the subjective measurements, plasma CST and serum cortisol differed significantly between dogs with bone fractures and the healthy control dogs. However, unlike the subjective parameters, neither plasma CST nor serum cortisol changed significantly between before and after morphine administration. CST concentrations again overlapped to a large degree with the reference range. Therefore, the use of plasma CST as a sole biomarker for pain-induced stress was of limited potential.
- The CgA epitopes CST and VS were studied in association with serum cortisol concentrations and S-VAS in healthy dogs experiencing psychological stress. Dogs experiencing psychological stress, saliva CST, serum cortisol, and

subjective assessments were in agreement and significantly differed from the low-stressed healthy dogs. Neither plasma CST nor plasma VS differed significantly between stressed and low-stressed healthy dogs. Saliva CST concentrations were above the reference range established in healthy low-stressed dogs; therefore, saliva CST may have potential as a biomarker for psychological stress in dogs.

- Based on the results of studies in the thesis, plasma VS is not useful for monitoring stress and pain progression in dogs.

7 Future perspectives

Stress and pain are complex responses and the limitation of communication in animals make evaluations challenging. Both subjective and objective assessment methods have been used for monitoring stress and pain in humans and animals. All today's available methods, including the parameters evaluated in this thesis, have both advantages and disadvantages. Currently, there is still no gold standard for assessing stress and pain in humans and animals, therefore the search for new methods continues.

Ideally, it would be preferable to have a single reliable biomarker that can be collected without inducing a stress reaction in patients that is both sensitive, specific, and inexpensive. There is a growing interest in establishing novel objective biomarkers for stress and pain. To evaluate the potential of clinical biomarkers, the analyzing methods first need to be validated and then further studies in specific standardized situations and finally in a clinical setting be done.

Although CgA is a sensitive stress biomarker in humans, the studies in this thesis found that VS has no potential as a stress biomarker in dogs. Saliva CST shows some promise as a potential stress biomarker, but due to difficulties in saliva sampling in dogs, its clinical use today seems limited. Few studies on CgA have been performed in dogs and more future studies are needed to improve the understanding of CgA and its degradation peptides.

In humans, CgA has shown promise as a reliable biomarker for diagnosing neuroendocrine tumors. CgA concentration can discriminate healthy and non-adrenal diseased patients from adrenal tumor patients. In dogs, CgA can be found in neuroendocrine tumor tissues (Myers *et al.*, 1997). Whether or not CST and VS can be used as biomarkers for neuroendocrine tumor in dogs is still unknown. CgA, in humans, has also shown promise as a prognostic biomarker in critically ill patients and patients suffering of cardiovascular disease (D'Amico M *et al.*, 2014; Helle, 2010; O'Connor *et al.*, 1989). It would be interesting to investigate whether CST and VS have similar potentials also in dogs. The reference ranges

for CST and VS in low-stressed healthy dogs established here will be useful in future studies in dogs.

Evaluation of different biomarkers for pain would be interesting to investigate. For instance, tissue damage may induce pain and therefore studies on different acute phase proteins in association with other subjective and objective pain assessments methods may be a way forward in the future. Further studies on psychological stress in different situations may also be interesting to pursue in the future.

Although CST and VS have been found in this thesis to have limited value as a stress biomarker in dogs, there is a clear need for better methods to identify pain and stress in animals to improve animal welfare and it is imperative that the search continues.

Popular science summary

Animals can experience and perceive the unpleasant feeling of stress and pain in a similar manner to humans. Stress and pain can therefore affect animal welfare and need to be addressed and managed properly, illustrating the need for early detection. Humans can report their own feelings and perception of stress using different scoring systems whereas animals' ability to convey their feelings is limited.

Stress and pain evoke similar body responses and can be evaluated using behavioral observation, and physiological and biological testing. Each assessment method has its own advantages and limitations and as yet there is no gold standard for assessing stress and pain either humans or animals. Therefore, new biomarkers for evaluating stress and pain are still needed. Chromogranin A (CgA) is a protein containing different sections called catestatin (CST) and vasostatin (VS) that can be analyzed in dogs. The aim of this thesis was to investigate CST and VS potential as biomarkers for stress and pain assessments in dogs.

Normal ranges of CST and VS in blood and saliva need to be established before assessing their usefulness for evaluating stress. We established reference levels for healthy dogs that were familiar with the sampling and hospital environment. While saliva sampling is less stressful for humans, we found that for dogs the degree of stress seems to be similar independent of the sampling method used. We have compared CST and VS levels in dogs with low stress profiles and dogs experiencing stress and found that CST in blood to a large degree overlapped with the levels in low-stressed dogs whereas CST in saliva was significantly higher. However, saliva sampling was difficult because the dogs produced very little saliva when stressed leading to often inadequate volumes for analysis. VS was not useful.

To evaluate whether CST and VS can be used to identify pain in dogs, we examined levels in blood and saliva in dogs before and after spaying and in dogs with bone fractures before and after receiving pain relief. Although the levels

were still within the normal range, blood CST showed some potential for monitoring pain progression in individual dogs whereas VS in blood had no potential as a biomarker for pain assessment in dogs.

Populärvetenskaplig sammanfattning

Precis som människor upplever djur obehag i samband med stress och smärta, och för att säkerställa god djuromvårdnad krävs tidig upptäckt och förebyggande åtgärder. Vi människor kan oftast själva förmedla våra känslor, medan djur i stället är beroende av vår förmåga at upptäcka deras obehag.

Stress och smärta kan ge upphov till liknande fysiologiska förändringar, som i sin tur kan ge förändringar i beteende, hjärt- och andningsfrekvens och olika blodparametrar. Tyvärr har alla idag tillgängliga metoder för att utvärdera smärta och stress både för- och nackdelar och det finns ännu ingen enskild perfekt metod att tillgå varken för människor eller djur. Därför bedrivs forskning för att hitta nya objektiva markörer. Chromogranin A är ett protein som är lovande som markör för stress hos människor. Catestatin (CST) och vasostatin (VS) är två områden på proteinet chromogranin A som kan mätas i blod och saliv hos hund. Syftet med denna avhandling var att utröna om CST och VS har potential att användas som markörer för stress och smärta hos hundar.

I ett första steg har normala nivåer av CST och VS i blod och saliv kartlagts hos hundar med låg stressprofil. Salivinsamling är en ofta använd metod inom humanmedicinen för att undersöka olika stressmarkörer utan att orsaka oro hos patienten eftersom patienten frivilligt spottar saliven i ett uppsamlingskärl. Vi upptäckte att hundar inte reagerar på samma sätt utan att de upplevde salivinsamling lika stressande som blodprovstagning. Vi har jämfört CST och VS nivåer hos hundar med låg stressprofil och stressade hundar och fann att blodnivåerna av CST var i stort sätt inom referensområdet till skillnad från nivåerna i saliv som var signifikant högre vid stress. Salivinsamling är dock oberäkneligt på hund då salivutsöndringen minskar i samband med stress och detta leder till svårigheter att samla tillräckliga volymer av saliv för undersökningar. VS var inte användbar som markör för stress.

Vi ville också undersöka om CST och VS kan användas för att upptäcka smärta hos hundar. Vi undersökte nivåerna i blod och saliv innan och efter kastration av friska hundar och hos hundar, som innan ankomst till kliniken, hade drabbats av

frakturer. Hos hundarna med benbrott undersöktes nivåerna direkt vid ankomst till kliniken i samband med att djuren stabiliserades och direkt efter smärtlindring med morfin. CST i blod tycks ha en viss potential att användas för att utvärdera smärtlindringseffekt hos en enskild individ men även här var koncentrationerna till viss del inom referensspannet för friska hundar. VS i blod saknar potential som biomarkör för smärta hos hund.

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ขอขอบคุณทุกคนมากค่ะ ที่เป็นส่วนร่วมในการทำให้การเรียนปริญญาเอกนี้สำเร็จ ทุกคนทั้งที่มีส่วนร่วมของงานและส่วนร่วมของกำลังใจล้วนมีผลต่อการเรียนและงานในครั้งนี้ การอยู่ในต่างแดนและการเรียนที่ต้องอาศัยความตั้งใจ อดทน และพยายาม ที่มีทั้งความสนุก ความตื่นเต้น การได้เรียนรู้สิ่งแปลกใหม่ตื่นตาตื่นใจ ในขณะเดียวกันก็มีความคิดถึง ความเหงา ความเศร้า ความท้อเกิดขึ้นด้วย หากแต่สิ่งเหล่านี้ได้เกิดมาเพื่อให้เรียนรู้ถึงความเป็นจริงที่มีการเกิดขึ้น เสื่อม และดับไปทั้งสิ้น ขอขอบคุณทุกคนที่มาทำให้การเรียนรู้ ผจญภัย และการเดินทางครั้งนี้ดำเนินไปอย่างน่าจดจำ ขอบคุณค่ะ