

INSULIN-LIKE GROWTH FACTOR-I IN THE DOMESTIC CAT

Studies in Health and Disease

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Insulin-like growth factor-I in the Domestic Cat: Studies in Health and Disease

Abstract

Insulin-like growth factor-I (IGF-I) has growth promoting effects as well as insulin-like actions on metabolism. IGF-I associates with a family of six high affinity IGF-binding proteins (IGFBPs) and these interfere in immunoassays for IGF-I. The role of binding of IGF-I to IGFBP-3, together with a third protein (the acid-labile subunit), to form a high molecular mass ternary complex, is not known in cats. In adult humans the ternary complex is the dominant circulating form. The cat is a strict carnivore with different metabolism to other species, which may include differences in the IGF system. In clinical practice serum IGF-I is used routinely for screening for acromegaly in cats with diabetes mellitus (DM). The overall aim of this thesis was to determine factors regulating IGF-I concentrations in health and disease.

Enzyme-linked immunosorbent assays for measuring feline IGF-I and insulin were validated. It is recommended that laboratories, in validating their assays, should be aware of position effects on assay plates and, for IGF-I assays, interference by circulating IGFBPs. For IGF-I, between animal variation was high (~65%) while within animal variation was considerable lower (~8%). These values for biological variation can now be used in interpreting clinical results after repeated sampling in screening for, and in the management of, acromegaly.

IGF-I concentrations were related to the amount of the ternary complex in healthy and diabetic cats. The ternary complex was the dominating circulating form only in cats with high IGF-I concentrations. There was a wide range of IGF-I concentrations in both healthy and diabetic cats that was in part related to variation in weight.

When using IGF-I as a screening tool for acromegaly in diabetic cats, glycaemic control should also be taken into consideration. IGF-I concentrations increased in response to insulin treatment and concentrations at 2-4 weeks were higher in cats that later went into remission.

In conclusion, in contrast to adult humans, circulating IGF-binding forms vary across the wide range of IGF-I concentrations in the cat. It is recommended that reference intervals for healthy cats are developed, stratifying by weight. IGF-I shows promise as a predictive marker for remission in feline diabetes mellitus.

Keywords: Acid-labile subunit, Acromegaly, Diabetes mellitus, Growth hormone, Insulin, Insulin-like growth factor binding proteins, Ternary complex

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To Peter, Emil and Elsa

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List of Publications

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

- I Strage EM, Ström Holst B, Nilsson G, Jones B, Lilliehöök I (2012). Validation of an enzyme-linked immunosorbent assay for measurement of feline serum insulin. *Veterinary Clinical Pathology*, 41(4), 518-528.
- II Strage EM, Theodorsson E, Ström Holst B, Lilliehöök I, Lewitt MS (2015). Insulin-like growth factor-I in cats: validation of an enzyme-linked immunosorbent assay and determination of biologic variation. *Veterinary Clinical Pathology*, E-pub, September 29th. DOI:10.1111/vcp.12289.
- III Strage EM, Fall T, Lewitt MS. Distribution of circulating IGF-binding protein complexes is similar in healthy and diabetic cats. Submitted manuscript.
- IV Strage EM, Sundberg M, Ström Holst B, Andersson Franko M, Ramström M, Fall T*, Lewitt MS*. Insulin-like growth factor I in cats with diabetes mellitus during insulin treatment: a longitudinal study. Manuscript.
*Denotes equal contribution

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In Paper I Figure 2 and 3 are printed with higher resolution in this thesis than in the original version.

The contribution of Emma Strage to the papers included in this thesis was as follows:

- I Took major part in planning the study, sampled all cats, did all laboratory work except analysis of glucose and fructosamine, interpretation of results, wrote the paper with input from co-authors.
- II Took major part in planning the study, sampled all cats, did all laboratory work except analysis of glucose and fructosamine, interpretation of results, wrote the paper with input from co-authors.
- III Took major part in planning the study, sampled almost all cats, did most of the laboratory work, interpretation of results, wrote the manuscript with input from co-authors.
- IV Took major part in planning the study, sampled most of the cats, did all laboratory work except analysis of glucose, fructosamine and mass spectrometry, interpretation of results, wrote the manuscript with input from co-authors.

Related publications not included in the thesis

- **Strage EM**, Lewitt MS, Hanson JM, Olsson U, Norrvik F, Lilliehöök I, Holst BS, Fall T. (2014). Relationship among insulin resistance, growth hormone, and insulin-like growth factor I concentrations in diestrous Swedish Elkhounds. *Journal of Veterinary Internal Medicine* 28(2), 419-428
- Jitpean S, Holst BS, Hoglund OV, Pettersson A, Olsson U, **Strage E**, Sodersten F, Hagman R. (2014). Serum insulin-like growth factor-I, iron, C-reactive protein, and serum amyloid A for prediction of outcome in dogs with pyometra. *Theriogenology* 82(1):43-48.

Abbreviations

ALS	Acid-labile subunit
ANOVA	Analysis of variance
AUC	Area under the curve
CLSI	Clinical and Laboratory Standards Institute
CPM	Counts per minute
CT	Computed tomography
CV	Coefficient of variation
CV _A	Analytical coefficient of variation
CV _G	Between animal coefficient of variation
CV _I	Within animal coefficient of variation
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
GH	Growth hormone
IGFBPs	Insulin-like growth factor binding proteins
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IND _I	Index of individuality
ISO	the International Organization for Standardization
JCGM	Joint Committee for Guides in Metrology
MRI	Magnetic resonance imaging
MS	Mass spectrometry
OGTT	Oral glucose tolerance test
PRM	Parallel reaction monitoring
RCV	Reference change value
RI	Reference interval
ROC	Receiver operating characteristics
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TE	Total error
TEa	Total error allowable
WLB	Western ligand blot

1 IGF system

1.1 IGF physiology

Insulin-like growth factors (IGFs) are important for growth and metabolism. In mammals there are two IGFs, IGF-I and IGF-II, which are structurally similar to insulin with overlapping effects through their structurally similar receptors (Clemmons, 2012; Bonefeld & Moller, 2011). In contrast to insulin, which is secreted by pancreatic beta cells, IGFs are expressed ubiquitously with production by the liver being the major source to the circulation (Daughaday & Rotwein, 1989). IGF-I in humans is a 7.6 kDa protein (Rinderknecht & Humbel, 1978) and cats have identical amino acid sequence (Sundberg, 2015). Insulin-like growth factors bind to a family of high-affinity insulin-like growth factor binding proteins (IGFBP-1 to IGFBP-6) which modulate the function of IGFs and also have IGF-independent roles (Firth & Baxter, 2002). In healthy adult humans almost all IGFs circulate in a ternary complex form comprising IGFBP-3 or IGFBP-5 and a third protein of ~85kDa, the acid-labile subunit (ALS) (Baxter, 2001b). IGFs and IGFBP-3 or IGFBP-5 form a binary complex before associating with ALS (Baxter *et al.*, 1989). Binary complexes of IGFs and IGFBP-3 bind to ALS with twice the affinity of IGF-IGFBP-5 complexes (Twigg & Baxter, 1998). In healthy adult humans ALS circulates in excess of the ternary complex and is therefore considered not to be rate limiting for ternary complex formation (Baxter, 1988). While binary complexes, (~30-50 kDa) and free IGFs can pass the endothelial barrier, the ternary complex (~150kDa) cannot exit the circulation and this form is considered a storage pool that prolongs the half-life of IGFs (Boisclair *et al.*, 2001). GH is secreted from the anterior pituitary gland in a pulsatile pattern and stimulates hepatic synthesis of ALS and IGF-I and thus is a regulator of the ternary complex (Olivecrona *et al.*, 1999). The GH-dependence of IGFBP-3 and IGF-II is mainly due to binding in the ternary complex, so that their circulating half-lives

are prolonged (Olivecrona *et al.*, 1999). In humans IGF-II circulates in 3:1 molar ratio of IGF-I (Livingstone & Borai, 2014).

The physiology of the IGF system has not been well characterized in cats but one study found that in healthy cats ALS did not circulate in excess of the ternary complex (Lewitt *et al.*, 2000). In contrast to healthy cats, diabetic cats in that study seemed to have excess ALS and higher IGF-I concentrations. In support of this finding another report demonstrated that diabetic cats had higher IGF-I concentrations than healthy cats (Starkey *et al.*, 2004). Other studies however documented low IGF-I concentrations in newly diagnosed diabetic cats which normalized during treatment (Alt *et al.*, 2007; Reusch *et al.*, 2006). The reasons for these discrepancies are not known but it was suggested that differences in methodology or treatment status could be contributing factors (Tschuor *et al.*, 2012; Reusch *et al.*, 2006).

1.2 Predictors of IGF-I concentrations in cats

There are few studies on the regulation of the IGF system in healthy cats. There is a correlation between GH and IGF-I in acromegalic cats (Niessen *et al.*, 2007) and pituitary GH is considered to be a stimulator of IGF-I synthesis. In one study 14 days of caloric restriction was reported to decrease IGF-I concentrations, but IGF-II immunoreactivity and total IGFBP-3 visualized by Western ligand blotting (WLB) did not seem to change (Maxwell *et al.*, 1999). In that study, in contrast to studies in humans (Baxter, 2001a) IGFBP-1 identified by WLB was not increased by short term fasting (18h)(Maxwell *et al.*, 1999).

In two studies of newly diagnosed diabetic cats IGF-I concentrations increased to similar values as the control group during insulin treatment and it was considered likely that this related to improved nutritional status (Alt *et al.*, 2007; Reusch *et al.*, 2006). However, in one study, IGF-I concentrations did not vary between well-controlled and poorly-controlled diabetic cats (Berg *et al.*, 2007).

Weight has been reported to have a positive association with IGF-I concentrations in healthy and diabetic cats (Niessen *et al.*, 2015; Berg *et al.*, 2007; Maxwell *et al.*, 1999) however one study did not find any association (Reusch *et al.*, 2006).

Age and gender are often used for stratifying reference intervals for IGF-I in humans. In one study of healthy cats age and IGF-I concentrations were not associated (Maxwell *et al.*, 1999) whereas another study of healthy cats found a weak negative correlation, and no effect of sex (Reusch *et al.*, 2006). In one study male cats had higher IGF-I concentrations but this was considered

dependent on male cats being heavier (Maxwell *et al.*, 1999). Most studies do not adjust for any effect of castration.

1.3 Measurements of GH, IGFs, and IGFBPs in feline medicine

GH is produced in a pulsatile pattern which may cause difficulties if interpreting one single measurement. Nevertheless, in studies of cats, single measurements have proven useful for diagnosis of acromegaly and may aid in diagnosis (Niessen *et al.*, 2007). However commercial laboratories do not routinely offer feline GH analyses. Instead GH-related diseases are diagnosed by measuring IGF-I, which is provided by a few specialist veterinary laboratories. Circulating IGF-I concentrations are stable during multiple sampling over the course of 12h (Reusch *et al.*, 2006). In feline medicine the most common application for IGF-I analysis is in screening for acromegaly, which is almost exclusively diagnosed in insulin resistant diabetic cats (Hurty & Flatland, 2005). Dwarfism due to lack of pituitary GH has been reported in a cat with proportional dwarfism (Donaldson *et al.*, 2008) but the syndrome is extremely rare or underdiagnosed in cats

There is a notable paucity of literature in relation to non-islet cell tumour hypoglycaemia in cats and the use of IGF-II measurement in this setting has not yet been investigated. IGF-II and IGFBP-assays are at present not available in feline medicine.

2 Feline diabetes mellitus and acromegaly

2.1 Diabetes mellitus

Prevalence of feline diabetes mellitus (DM) in animal hospitals is increasing and part of this increase is thought to be due to lifestyle changes with obesity as a consequence (Rieder *et al.*, 2008; Prah *et al.*, 2007). It is estimated that obesity in cats increases the risk of developing DM by threefold (Scarlett & Donoghue, 1998).

In humans DM can be classified into type 1 or 2 DM according to the patterns of clinical characteristics, endogenous insulin secretion and islet-cell and insulin autoantibodies. Type 2 DM is typically characterized by insulin resistance and beta cell dysfunction and insulin concentrations may be high, normal or low (Saisho, 2015; Vague & Nguyen, 2002). Type 1 DM is typically characterized by insulin deficiency (ADA, 2004). Most cats present with a form of DM which resembles human type 2 (Nelson & Reusch, 2014), however insulin concentrations are usually lower than in healthy cats (Nelson *et al.*, 1999; Lutz & Rand, 1996; Kirk *et al.*, 1993). One hypothesis for the low insulin concentrations in cats with type 2 DM is beta cell impairment due to glucotoxicity (Zini *et al.*, 2009), which can be reversible with adequate treatment (Tschuor *et al.*, 2011; Nelson *et al.*, 1999). The statement that cats mainly present with type 2 DM is based on the presence of amyloid in the pancreas, lack of antibodies against beta cells and lack of lymphocytic infiltration of beta cells (Hoenig, 2002). The classification of type 1 or type 2 is at present not considered clinically relevant because it is recommended that all cats presenting with clinical DM are treated with insulin (Rucinsky *et al.*, 2010). Furthermore insulin concentrations at diagnosis of DM do not predict whether a cat goes into remission (Tschuor *et al.*, 2011; Nelson *et al.*, 1999). A remission rate, defined by insulin independence, of 14-78% has been reported for cats with DM (Hafner *et al.*, 2014; Nack & DeClue, 2014; Hall *et al.*, 2009;

Michiels *et al.*, 2008; Alt *et al.*, 2007) and depends on achieving early glycaemic control (Gottlieb & Rand, 2013).

2.1.1 Diagnosis

Diagnosis of feline DM is based on hyperglycaemia and glycosuria together with clinical signs, such as polyuria/polydipsia, weight loss and polyphagia (Sparkes *et al.*, 2015). The renal threshold for glycosuria in cats is about 15 mmol/L and thus diagnosis of feline DM is usually made when cats present with profound hyperglycaemia. The use of hyperglycaemia alone as a diagnostic tool for DM in cats is complicated by the phenomena of stress hyperglycaemia and elevated serum glucose concentrations cannot solely be used for diagnosing DM in cats (Laluha *et al.*, 2004).

Fructosamine is the irreversible complex between serum proteins and glucose. Fructosamine depend on protein half-life and usually reflects glucose concentrations during the last 1-2 weeks; and so it is not affected by acute stress hyperglycaemia (Link & Rand, 2008; Crenshaw *et al.*, 1996).

There has been an interest in developing prognostic markers for feline DM. Insulin concentrations at DM diagnosis are usually low, and do not predict whether cats go into remission or not (Tschuor *et al.*, 2011; Nelson *et al.*, 1999). In one study of non-diabetic cats, fasting insulin was used to detect reduced insulin sensitivity (Appleton *et al.*, 2005). In that study it was proposed that cats with reduced insulin sensitivity could be at risk for developing type 2 DM and identifying these cats would enable preventive actions.

2.1.2 Treatment

To achieve remission of DM in cats it is recommended that insulin treatment is started promptly to decrease blood glucose and hence reverse glucotoxicity. Since lente insulin has a suboptimal duration of action, the longer acting glargine has been recommended as the first line of therapy in cats with DM (Rucinsky *et al.*, 2010). Even if glargine is prescribed, cats typically require insulin twice a day to achieve glycaemic control.

Cats are strict carnivores, and low carbohydrate and high protein diets, together with insulin have been found useful and are recommended for achieving glycaemic control in diabetic cats (Sparkes *et al.*, 2015; Rucinsky *et al.*, 2010).

Oral hypoglycaemic agents are ineffective in most diabetic cats (Palm & Feldman, 2013). When they are used, a sulfonyleurea, which increase insulin secretion from beta cells, is the most common choice. However, if the beta cells are incapable of producing insulin the drug has little effect. Another

concern has been that increased insulin secretion stimulated by sulfonylureas is accompanied by amyloid deposition in the pancreas which may further reduce beta cell function (Palm & Feldman, 2013).

2.2 Acromegaly

Acromegaly in cats is caused by a GH-secreting pituitary adenoma (Rijnberk *et al.*, 2003). GH is an insulin antagonist and insulin resistant DM is typically found in acromegalic cats (Niessen, 2010). At presentation acromegalic cats may have clubbed paws, broad facial features, prognathism and organomegaly. However, in contrast to dogs, clinical signs may not be prominent, especially not early in the disease. A typical finding in diabetic cats is weight gain in the face of poor glycaemic control, which would usually have been accompanied by weight loss (Niessen, 2013).

2.2.1 Diagnosis

IGF-I is generally considered a screening marker for acromegaly in cats with a cut-off of 1000 ng/mL used for further investigation (Niessen, 2013). Clinical signs may not be prominent and one can only be truly confident of a diagnosis of acromegaly where pituitary tissue is available for immunohistochemistry. In humans with acromegaly, lack of GH suppression in response to an oral glucose load is an aid in diagnosis (Giustina *et al.*, 2010). However, in healthy cats given glucose intravenously there is no effect on GH secretion (Kokka *et al.*, 1971).

Diagnostic imaging (computed tomography, CT, and magnetic resonance imaging, MRI) are often used in the diagnostic workup. However, the incidence of pituitary “incidentalomas” is unknown in cats: in humans the prevalence is estimated to be approximately 9% (Orija *et al.*, 2012). In my opinion there are no studies to date that been designed in a way that identifies the best IGF-I cut-off for acromegaly. Recently a claim has been made that there is a prevalence of acromegaly of 25% in diabetic cats in the United Kingdom (Niessen *et al.*, 2015). This was based on screening 1221 cats and identifying 319 cats with IGF-I >1000 ng/mL. Of these, 63 were further investigated and 60 were diagnosed with acromegaly based on diagnostic imaging or post-mortem examination. In the study by Niessen (2015) the number of cats with pituitary abnormalities on diagnostic imaging appears very high. It would be interesting to know the prevalence of pituitary abnormalities in a wider population of cats with and without DM.

2.2.2 Treatment

Specific treatment for acromegaly in cats consists of hypophysectomy or radiotherapy (Niessen, 2013), options that are not yet available in Sweden. The most common approach for managing acromegalic cats is to tailor insulin dosages. Somatostatin analogs have been tried in cats. Octreotide has had mainly disappointing results (Slingerland *et al.*, 2008; Peterson *et al.*, 1990). Pasireotide has shown more promising results with a decrease of IGF-I in 12 acromegalic cats after treatment (Scudder *et al.*, 2015); more studies are needed before this treatment can be widely recommended.

3 Validation of analytical methods

In veterinary medicine assays intended for humans are often used for other species. It is therefore particularly important to validate each analytical method. The cost of developing species-specific immunoassays for many hormones used in the diagnosis of rare diseases is too high to be affordable for the veterinary laboratory, because the cost is ultimately borne by the animal owners. Nevertheless, the assays must provide reliable results. When analysing IGF-I, interference of IGFBPs can substantially influence test results and it is especially important that validation of the method focuses on this problem.

The uncertainty of a test result can be due to preanalytical-, analytical- or postanalytical errors. Pre- and postanalytical errors can be reduced by standardization of routines and correct sample handling but these are still considered major sources of variation (Wiwanitkit, 2001). The analytical errors are often divided into random and systematic error, and can be expressed separately or combined. There are different approaches to dealing with the concept of error. Total error (TE) is advocated by Westgard and colleagues (2008) and describes the probability distribution around a true concentration. To calculate TE the systematic error and the random error are combined to a total error. The observed TE is often compared to the allowable total error (TEa) which can for example be based on biological variation or clinicians' expertise (Westgard, 2008). In another approach, "Uncertainty", used by the International Organization for Standardization (ISO) expresses the dispersion of values during measurement (JCGM, 2008). With this approach it is recommended that different sources of uncertainties are identified, combined and preferably the systematic error corrected. Often the expanded uncertainty is calculated and this corresponds to a confidence interval for the measured value (JCGM, 2008).

3.1 Random error - precision

Random error can be evaluated from repeated measurements and is often expressed as coefficient of variation (CV). In many clinical laboratories measurements of precision are often expressed as CV within and between analytical runs. It is possible to take this further, for example to evaluate between run CV during longer time periods using different batches or to calculate CV between laboratories. For clinicians it is useful to know total CV because they often compare samples analysed on different days. One recommendation from the Clinical and Laboratory Standards Institute (CLSI) is to run 5 replicates on 5 days and evaluate results based on an ANOVA (CLSI, 2014). Precision may differ depending on concentrations and should be evaluated at different concentrations.

3.2 Systematic error

3.2.1 Bias

Bias is defined as systematic deviation from the true value. Bias can be investigated by analysing reference material, recovery of spiked samples or comparison with a gold standard method (Magnusson & Örnemark, 2014). In veterinary medicine there are very few species-specific reference solutions that can be used as quality controls or for spiking, or gold standard methods for comparison, and true bias is seldom evaluated. Guidelines from the American Society for Veterinary Clinical Pathology recommend that veterinary laboratories should determine the observed TE by combining precision and bias, and these values can be derived from quality control material (ASVCP, 2013). Most quality control material used in veterinary medicine is not species-specific and it is therefore difficult to express bias as deviation from the true value. Clinical laboratories that set their own reference intervals for a given assay already include possible bias into the reference intervals.

Identifying all essential sources of variation is important but may be difficult. There are reports that sample positioning on ELISA plates can contribute to systematic errors (Miller *et al.*, 2009; Shekarchi *et al.*, 1984) but including or identifying this effect in validation studies is seldom undertaken.

3.2.2 Dilution experiments

Dilution of samples is sometimes performed with the objective to investigate whether there is a linear relationship between the dilution factor and concentration. This problem is discussed in a guide from CLSI, where it is recommended that at least five dilutions in 2-4 replicates are analysed and a

first, second- and third order polynomial curve fitted to the data (NCCLS, 2003). If second or third order polynomial curve fits better than a linear one, the assay is considered non-linear in statistical terms. To evaluate the degree of non-linear error the difference between the linear and the non-linear curve is calculated and this value compared to predetermined limits. It has been proposed that TEa can be used as an acceptable limit for non-linear error (Westgard, 2008).

Another approach to express agreement of results from dilution experiments is to investigate consistency after dilution. The undiluted sample is usually considered the true value. The ratio between the expected value (E) and the observed value (O) can be used for evaluating consistency and is often expressed as a percentage. For example, in a dilution experiment with negligible random error and perfect consistency, the O/E ratio will be 100% at all dilution points after adjusting for dilution factors.

3.2.3 Interference

Interfering substances can cause erroneous results during analysis. IGFBPs have been identified in feline serum (Lewitt *et al.*, 2000; Maxwell *et al.*, 1999) and are likely to potentially interfere during analysis as in other species. When analysing IGF-I the IGFBPs may cause bias leading to either false low or false high concentrations depending on which assay is used (Frystyk *et al.*, 2010). When validating IGF-I assays for use with human samples it has been recommended that serum samples from a variety of clinical conditions, in which IGFBP concentrations or profiles may potentially affect IGF-I assay results, are included; information about this interference should be reported to the end user (Clemmons, 2011). The gold standard procedure for removing IGFBPs is size-exclusion chromatography under acidic conditions (Clemmons, 2011). This is a cumbersome procedure and may not be feasible in routine practice. Instead other procedures have been developed to remove IGFBPs, for example acid-ethanol extraction and acid-ethanol followed by cryoprecipitation. In many species these extraction methods do not remove all IGFBPs and these may interfere in the assay (Mohan & Baylink, 1995; Frey *et al.*, 1994; Srivenugopal *et al.*, 1994). In competitive immunoassays truncated IGF-I with reduced affinity for IGFBPs has been effectively used (Bang *et al.*, 1991). Another approach is to add excess amounts of IGF-II (Frystyk *et al.*, 2010). The sample is acidified to dissociate IGFs from their binding proteins and excess amounts of IGF-II, which does not interact with the IGF-I antibody, are added to the sample. When the sample is neutralized again, the excess IGF-II binds to the IGFBPs thus reducing their availability for interference in the assay. To determine interference exogenous IGFBPs can be added to serum

samples and interference calculated. Recommendations for human samples are to add IGFBP-2 and -3, up to twice the reference limit (Clemmons, 2011). No reference interval (RI) for any of the six feline IGFBPs has been reported and there are no purified feline IGFBPs available.

Insulin assays are subjected to interference by insulin antibodies. Diabetic cats treated with insulin may develop insulin-antibodies (Hoenig *et al.*, 2000) and these are a potential source of interference in insulin immunoassays. One group has reported that insulin auto-antibodies exist in healthy cats (Takashima *et al.*, 2013; Nishii *et al.*, 2010).

3.3 Biological variation and assay performance

In addition to preanalytical, analytical and postanalytical variation, repeated test results from an individual animal vary depending biological factors. Most analytes vary similarly in health and disease; however, for some analytes disease status may affect variation (Borai *et al.*, 2013; Ricós *et al.*, 2007). Biological variation can be classified into within-individual variation (CV_I) and between-individual variation (CV_G).

3.3.1 Quality specifications

In 1999 there was a conference in Stockholm addressing quality specifications for analytical performance. A hierarchic model was proposed in which quality specifications based on biological variation was considered high in the hierarchy (Kenny *et al.*, 1999).

To decide quality specifications and the acceptable uncertainty for analytical assays it should be decided what effect different magnitudes of variation have on decision making for a specific test. If we ignore preanalytical and postanalytical variation, total variation for an analyte would consist of analytical variation (CV_A) and biological variation. Based on data from biological variation suggested limits for optimal, desirable and minimal CV_A are calculated as: optimal $CV_A < 0.25CV_I$; desirable $CV_A < 0.5CV_I$; minimum $CV_A < 0.75CV_I$. These limits for CV_A have been calculated to add 3.1, 11.8 and 25.0 %, respectively, to test result variability (Fraser, 2001).

In a similar manner, recommendations for acceptable bias have been proposed. If RI is not determined by the laboratory, the assay bias affects the number of individuals that fall within the RI being used. RI usually include the central 95% of the reference population. If bias is positive, more than 2.5% of individuals would be above the upper reference limit and less than 2.5%, below. It has been proposed that desirable bias should be less than $0.25(CV_I^2 + CV_G^2)^{0.5}$ so that 5.8% are outside the RI instead of the usual 5%

(Fraser, 2001). Westgard and Fraser suggests that TEa can be calculated using limits for CV_A and bias derived from biological variation (Westgard, 2008; Fraser, 2001). The use of TEa is recommended by the American Society for Veterinary Clinical Pathologists (ASVCP, 2013; Harr *et al.*, 2013).

3.3.2 Reference intervals and repeated sampling

For analytes that have a marked individuality (i.e. CV_I is considerably lower than CV_G), population-based RI may be of limited utility for follow-up of patients (Fraser & Harris, 1989). In these cases a significant change of an analyte for that individual may not be detected because the value does not exceed the reference limits. The utility of population-based RI can be evaluated by an index of individuality (IND_I) which is calculated as $(CV_A^2 + CV_I^2)^{0.5} / CV_G$. If $IND_I < 0.6$ the use of populations-based RI may be of limited use and subject-based RI are recommended. If IND_I is between 0.7 and 1.4, population-based RI can be used but with caution, and for $IND_I > 1.4$ observed values can be interpreted with respect to population-based RI (Walton, 2012). If IND_I indicates that population based RI are too wide, stratification for example by age or gender, may improve its utility.

Animals are often sampled multiple times to evaluate change of treatment or progress of disease. Since test result variability in one animal depends on CV_I and CV_A , it is possible to calculate the magnitude of change in test results considered to be significant. This value is usually expressed as a Reference Change Value (RCV) and is based on CV_A , CV_I and the desirable level of significance. For example a bi-directional change between two samples and 95% probability is calculated as $1.96(2(CV_A^2 + CV_I^2))^{0.5}$ (Walton, 2012; Fraser, 2001).

4 Aims of the thesis

The general aim was to determine factors regulating feline IGF-I concentrations in health and disease.

Specific aims were:

- To validate assays for measurement of feline serum IGF-I and feline serum insulin, including determination of biological variation.
- To determine the range of IGF-I concentrations in cats.
- To determine factors contributing to circulating IGF-I concentrations, including IGF-binding forms, in order to improve interpretation of test results for feline medicine.
- To investigate the effect of diabetes mellitus on IGF-I concentrations at diagnosis and during insulin treatment in cats.
- To investigate the role of IGF-I and insulin in predicting remission of feline diabetes mellitus.

5 Materials and methods

5.1 Animals

Healthy and diabetic cats were recruited by advertising at a cat exhibition and the reception area at Bagarmossen Animal Hospital, and by sending e-mails to all staff at Bagarmossen Animal Hospital, the University Animal Hospital and the Department of Clinical Science. Cats were considered healthy based on answers to a health questionnaire by owners, a clinical examination by a veterinarian and a biochemical profile. Cats were excluded if they were pregnant or under one year of age. For cats sampled solely for these studies, animals were excluded if they were stressed as exhibited by hissing and struggling. Diabetes mellitus was diagnosed based on clinical signs, hyperglycemia, glucosuria and elevated fructosamine. Cats were fasted for 12h before sampling, except for Manuscript IV where cats on insulin treatment were fasted for 5-12h. In addition, left-over serum from samples submitted for investigation to the Clinical Pathology Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences, were used for validation experiments (Paper I, II, IV). All serum samples were centrifuged within one hour after sampling.

In total, 60 healthy and 44 diabetic cats were sampled during the study period of Papers I and II and Manuscripts III and IV. Of the 44 diabetic cats 3 were diagnosed with acromegaly based on clinical signs, high IGF-I concentrations and a pituitary tumour detected at post mortem examination or computed tomography (CT). The cat in which CT was used for detecting a tumour was later subjected to post mortem examination and was found to have a pituitary adenoma. Of the 60 healthy cats, five had abnormal biochemical results, clinical findings or missing information and were excluded, leaving 55 healthy cats.

In Paper I, 36 healthy cats and 22 diabetic cats were included. Ten of these healthy cats were subjected to an oral glucose tolerance test (OGTT). Baseline sampling was performed with a permanent catheter placed in the cephalic vein from which blood samples were withdrawn at 30 and 60 minutes.

In Paper II 55 healthy cats, four cats with DM, three cats with DM and acromegaly, one cat with chronic renal failure and two cats with liver disease were included. Chronic renal failure was diagnosed based on clinical signs, ultrasonography and azotemia. Liver disease was diagnosed by elevated liver enzyme activity, increased bile acids and histopathological findings (hepatic lipidosis and biliary liver cirrhosis).

Ten healthy cats and one acromegalic cat were enrolled in the study of biological variation (II and Section 6.3). One healthy cat had a heart murmur and two cats were stressed during sampling. Cats excluded due to stress were not sampled at later occasions. Thus one acromegalic and seven healthy cats completed the study period. Cats were sampled from the cephalic vein, in their home environment in the morning after a 12h fast, once a week for five weeks (sample occasion A-E) by the same veterinarian using the same equipment each time.

For Manuscript III, 41 diabetic cats and 55 healthy cats were included. Samples for size-exclusion chromatography were selected to represent a range of age and IGF-I concentrations in both healthy (n=13) and diabetic (n=10) cats.

For Manuscript IV, 24 diabetic cats were included and were sampled five times over the course of one year from diagnosis (T0=at diagnosis, T1=2-4 weeks, T2=8-10 weeks, T3=5-7 months, T4=10-13 months). Serum for size-exclusion chromatography in this study was selected from cats that went into remission (n=3) and cats that did not (n=4). Serum from the three cats used for chromatography and which went into remission were also subjected to measurement by mass spectrometry.

5.2 ELISA validation

5.2.1 Feline insulin ELISA

Paper I validated the Mercodia Feline Insulin ELISA (Uppsala, Sweden) which is a commercial sandwich ELISA. The wells of the plate are coated with mouse monoclonal anti-human insulin antibodies and a second monoclonal antibody is used for detection. In the beginning of the study this assay used standards based on human insulin but during the validation process the manufacturer changed standards to feline origin. Cross-reactivity for insulin glargine (Lantus) and lente insulin (Caninsulin) reported by the manufacturer was 8.4%

and 57.4%, respectively (Mercodia, 2008). All the samples were pipetted within the 30 minutes recommended by the manufacturer.

Position effects were evaluated by pipetting the same standard in all wells and results evaluated by two-way ANOVA with rows and columns as factors. This was further investigated by evaluating precision and position effects by pipetting samples with low, medium and high insulin concentrations in three fixed duplicate positions on the ELISA plate. The assay was run on 8 different days and results evaluated by two-way ANOVA with microplates and positions as factors. Feline insulin extracted from a feline pancreas, as described in Paper I, was used to develop a solution for spiking studies.

Consistency upon dilution was evaluated by diluting samples up to 1:8 with the zero calibrator as recommended by the manufacturer. The expected concentration, E , was calculated from the original concentration and the dilution factor or the spiking procedure. O denotes the observed concentration for the spiked or diluted sample, and the result was expressed by the ratio O/E . The standard error of O/E was calculated from the estimated values of different components of variation (I, supplement). To test the hypothesis of consistency, the critical limit was obtained by multiplying the standard error by k (coverage factor, Paper I, Appendix). According to recommendations for situations when the standard error is very uncertain, k was set to 3 (JCGM, 2008).

Stability was evaluated by analysing insulin concentrations on samples stored at room temperature (20°C) and in the refrigerator (2-8°C) daily for four days. The critical limit, based on the expanded uncertainty of the assay, was calculated as described in detail in the appendix for Paper I.

To test utility of the insulin assay in measuring a known biological response samples from an OGTT were assayed. Glucose (dextrose 500 mg/mL) 1 g/kg body weight was given as a solution orally by syringe at the hospital. Insulin (I) and glucose (Section 6.1) were measured at baseline and after 30 and 60 minutes. Statistical analyses were performed using Wilcoxon signed-rank test. Insulin concentrations between healthy and diabetic cats were compared by Mann-Whitney test.

5.2.2 IGF-I ELISA

The Mediagnost IGF-I ELISA (Reutlingen, Germany) was validated and presented in Paper II. Although this assay is intended for human use it is stated in the instructions that the assay can be used in various species, including cats. It uses a sandwich technique based on two specific high-affinity antibodies. The sample is first diluted with an acidic buffer containing excess IGF-II. The acidic buffer destroys ALS and dissociates IGFs from their IGF-BPs allowing the excess IGF-II to later associate with IGF-BPs when neutralized. The plates

are coated with monoclonal anti-human IGF-I antibodies to which the second polyclonal biotinylated antibody is added. When the sample is pipetted into the wells it is neutralized by the antibody conjugate and the excess IGF-II binds to the IGFBPs, leaving IGF-I free to interact with the antibodies. Standards were recombinant human IGF-I calibrated against WHO reference material 02/254.

Position effect was evaluated by pipetting a high control sample in all wells. When pipetting samples into this ELISA plate, all samples were positioned in duplicates situated in adjacent wells overlapping two columns. Hence, the mean value of duplicates was evaluated by two-way ANOVA with rows and columns as factors. Precision was determined by analyzing samples with low, medium and high concentrations in 2-20 replicates on 8 days. Statistical calculations were performed taking the uneven number of replicates into account (Aronsson & Groth, 1984). Spiking was done by adding recombinant human IGF-I (CU100, IBT Systems, Binzwangen, Germany).

Linearity after dilution was evaluated by diluting five feline serum samples, as well as a serum sample that had been subjected to size-exclusion chromatography under acidic conditions in order to remove IGFBPs. Results were evaluated according to CLSI and Westgard (Westgard, 2008; NCCLS, 2003). In addition to this, sera from 16 cats were serially diluted 4 times. In 2 dilution experiments the samples were analyzed both with and without added recombinant human IGFBPs. In the first experiment a total concentration of 30 mg/L was added: 10 mg/L of BP-1 (871-B1-025; R&D Systems, Oxfordshire, UK), 10 mg/L of BP-2 (674-B2-025; R&D Systems) and 10 mg/L of BP-3 (10-663-45149; Genway, San Diego, CA, USA). The samples were incubated at room temperature for 3h before analysis. In the second experiment 8 mg/L was added (1 mg/L of BP-1, 2 mg/L of BP-2 and 5 mg/L of BP-3).

Stability of IGF-I (IV) was determined by analysing 2 samples stored at room temperature and in refrigerator at day 0, 3, 6 and 14. In one cat there was insufficient serum stored at 20°C and 6 days was the longest storage time.

5.2.3 Biological variation

Biological variation of IGF-I is presented in Paper II. The biological variation of insulin has not been included in any paper or manuscript. Outliers between replicates and within cats were tested by Cochran's test and between cats by Reed's criterion (Fraser & Harris, 1989). Data was analysed using nested ANOVA with both balanced and unbalanced designs. Variances for between cats, within cats and between replicates were obtained by using nested ANOVA by the command "proc nested" in SAS (SAS 9.3 SAS Institute Inc., Cary, NC, US). The variances and overall mean were used to calculate the corresponding CVs (CV_G , CV_L , and CV_A).

For insulin there was not enough serum for duplicate analysis from one occasion in one cat (sample occasion A). Results of the remaining samples indicated one sample occasion as an outlier in another cat and hence that sample occasion was also deleted (sample occasion A). Hence the balanced design was based on excluding all A samples, leaving seven cats and four sampling occasions. The results are presented in Section 6.3.

For IGF-I, one duplicate in one cat was considered an outlier. To achieve a correct design for the balanced ANOVA two approaches were used: 1) this sample number was excluded in all cats, or 2) the cat was excluded from analysis. The first approach is presented in Paper II and the second approach in Section 6.3.

By using CV_G , CV_I and CV_A from the method validations at relevant concentrations, the reference change value was calculated as $1.96(2(CV_A^2+CV_I^2))^{0.5}$ and index of individuality as $(CV_A^2+CV_I^2)^{0.5}/CV_G$. Desirable limit for TEa was calculated as $1.65(0.5CV_I)+0.250(CV_I^2+CV_G^2)^{0.5}$ (Harr *et al.*, 2013; Friedrichs *et al.*, 2012; Walton, 2012; Fraser, 2001).

5.3 Size-exclusion chromatography for IGFs and IGFBPs

Size-exclusion chromatography is a useful technique to separate proteins. Under acidic conditions, ALS is destroyed and IGFBPs dissociate from IGFs. Size-exclusion chromatography under acidic conditions, with a column that can separate proteins with molecular sizes of IGFBPs (about 24-50 kDa) and IGFs (about 7.6 kDa), is considered gold standard method for separating IGFBPs from IGFs (Clemmons, 2011).

Size-exclusion chromatography under neutral conditions will separate the ternary (approximately 150kDa) and binary (approximately 30-50kDa) complexes, as well as free IGF-I. To detect these complexes, concentrations of IGFs or IGFBPs can be measured directly in the eluted fractions. Alternatively, tracer amounts of iodinated IGF-I or IGF-II can be used, by addition to serum and then incubating to equilibrium so that incorporation of label reflects the pattern of endogenous IGF-binding proteins. After size-exclusion chromatography radioactivity (counts per minute, C.P.M.) in the fractions is then detected by gamma-counting (Renes *et al.*, 2014; van Duyvenvoorde *et al.*, 2008; Lewitt *et al.*, 2001; Lewitt *et al.*, 2000).

5.3.1 Acidic conditions

Size-exclusion chromatography under acidic conditions on a Superdex column (17-5174-01; GE Healthcare, Little Chalfont, UK) was used for separating IGFBPs from IGFs in Paper II. Serum from a healthy cat as well as from cats

with DM and acromegaly, chronic renal failure and liver cirrhosis was used for this experiment. Firstly serum (100 μ L) was incubated with 400 μ L of 1.25 M acetic acid and chromatographed with 1 M acetic acid as running buffer as recommended (Mohan & Baylink, 1995). However, under these conditions and using feline serum there was a precipitate and lower IGF-I immunoreactivity than when 1 M acetic acid was used. Thus 100 μ L feline serum or recombinant human IGF-I was instead mixed with 400 μ L 1 M acetic acid and incubated for 30 min. Although no particles were macroscopically visible the sample was filtered (Whatman resist, 0.2 μ m) to remove any remaining particulate matter before loaded onto the column. Recombinant human IGF-I was chromatographed and used for comparison.

The samples were eluted at 0.5 mL/min using 1 M acetic acid as running buffer. To determine when IGF-I eluted, fractions were collected every two minutes and lyophilized (SpeedVac) before being analysed in the IGF-I ELISA. In addition, size-exclusion chromatography was performed on feline serum and fractions known to be positive for IGF-I immunoreactivity were pooled and dried. The immunoreactivity of the pooled fractions was compared to that in the loading material. Insoluble material was visible after lyophilisation and reconstitution of the loading material, therefore the volume was reduced to concentrate the samples and the volumes adjusted to equivalence.

The immunoreactivity of IGF-I in the sample before and after size-exclusion chromatography was determined by the IGF-I ELISA. The sample after size-exclusion chromatography was used as expected (*E*) and was compared to the non-chromatographed sample (*O*).

5.3.2 Neutral conditions

In Manuscript III and IV size-exclusion chromatography under neutral conditions was used to separate IGF-binding forms in serum. A Superose column (17-5173-01; GE Healthcare) was used to separate feline serum incubated with human h-¹²⁵I-IGF-II, as well as size-separation of non-labelled serum. Feline serum (50 μ L) was incubated with 50 μ L PBS-buffer (50 mM, pH 7.4) and 100 000 C.P.M. h-¹²⁵I-IGF-II (1096 Ci/mmol, T-033-23, Phoenix Pharmaceuticals, CA, US) until equilibrium (>17h at 4°C). The tubes were centrifuged at 10 000 G and the supernatant loaded onto the column. In total, 25 μ L serum in a final volume of 100 μ L of PBS were loaded on the column and eluted at 0.5 mL/min. Running buffer was PBS (50 mM, pH 7.4) run at 0.5 mL/min and fractions collected every 0.5 minutes. The C.P.M. for each fraction was counted in the gamma counter (Wallac Wizard-2 2470, Perkin Elmer, Massachusetts, USA).

Since the affinities of feline IGFs and IGFbps are unknown and may differ from human IGFs, the 150kDa and 30-50kDa peaks were expressed as a 150/30-50kDa ratio, estimated as a ratio of the sums of the five top-fractions for the 150kDa peak and 30-50kDa peak, respectively.

In Manuscript III size-exclusion chromatography of non-labelled serum was performed and IGF-I immunoreactivity determined by ELISA. Serum (100 μ L) was diluted with 100 μ L of ammonium acetate (0.2 M, pH 7.4). The column was run at 0.5 mL/min and fractions collected every two minutes. Fractions were lyophilized, reconstituted with assay buffer containing IGF-II and analysed in the IGF-I ELISA. Molecular weight markers (HWM 24-4038-42, and LMW 28-4038-41, GE Healthcare, Uppsala, Sweden) showed the same elution patterns in PBS and ammonium acetate. In Manuscript IV changes in the 150/30-50kDa complex between T0 and T1 was evaluated by paired t-test after transformation to the natural logarithmic scale.

5.4 SDS-PAGE and Western ligand blots of IGFbps

In Paper II, in order to visualize IGFbps in fractions after size-exclusion chromatography under acidic conditions, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reduced conditions, followed by WLB. In this thesis SDS-PAGE under non-reduced conditions is also reported. Fractions from size-exclusion chromatography under acidic conditions were lyophilized and reconstituted with 100 μ L distilled water. The reconstituted fractions were diluted 50% with Laemmli buffer, with or without mercaptoethanol, and 15 μ L loaded on to a TGX gel (Any kD, BioRad, Hercules, CA, USA). On every gel a molecular weight marker was loaded (Precision Plus Protein Western C Standards, 1610376 or Precision Plus Protein Dual Xtra Standards, 1610377, BioRad). SDS-PAGE was run at 100 V (Mini-Protean Tetra Cell, BioRad). The proteins were transferred to nitrocellulose membrane (0.2 μ m) at 100 V for 1h in the cold room (Mini-Trans-blot, BioRad). Membranes were incubated with biotinylated IGF-II (1:2500, FU100, IBT-systems) overnight in the cold room. The membrane was washed and neutravidin-HRP (1:5000, high sensitivity neutravidin-HRP, Pierce, ThermoFischer Scientific, MA, US) was added and incubated for 1h. If the Western C standard was run as weight marker StrepTactin-HRP (1:30 000, Precision Protein™ StrepTactin-HRP Conjugate, 1610381, BioRad) was added together with the neutravidin-HRP. After washing protein bands were detected by using Amersham ECL Prime (GE Healthcare).

5.5 Mass spectrometry

A targeted mass spectrometry based method (MS) with parallel reaction monitoring (PRM) analysis, previously validated for cats (Sundberg, 2015) was used to measure IGF-I, IGF-II, IGFBP-3, and IGFBP-5 in Manuscript IV. These analyses were performed in serum at baseline (T0) and after 2-4 weeks (T1) from three cats that went into remission. The method is described in detail elsewhere (Sundberg, 2015). Briefly, protein content of serum was measured with the Bradford protein assay (BioRad Laboratories, Hercules, CA, US) and an aliquot equal to 35 µg was used for digestion. To quantify the proteins, reference peptides (QPrEST, Atlas antibodies, Stockholm, Sweden) were used. QPrESTs are recombinantly produced human protein fragments, with heavy labelled arginine and lysine, also containing the peptides of interest, in this case sequences of feline IGF-II, IGFBP-3 and IGFBP-5. For IGF-I one stable isotope label peptide containing the feline IGF-I amino acid sequence (New England Peptide, Gardner, MA, US) was used as standard instead of QPrEST peptides. The serum samples were spiked with QPrEST and peptide to a concentration of 1 fmol/µL for IGF-I, IGFBP-3 and IGFBP-5, and 3 fmol/µl for IGF-II and digested with trypsin (5% w/w). Samples were incubated overnight at 37°C, desalted on a ZipTip C18 column (Merck Millipore) and dried in SpeedVac system. After reconstitution, peptides in the samples were separated on a reverse-phase column using an EASY-nLC 1000 system (ThermoFischer Scientific) followed by a PRM analysis on a Q Exactive Orbitrap Plus mass spectrometer (ThermoFischer Scientific). For data analysis and quantification the Skyline software was used. In Manuscript IV changes in IGFBP-3, IGFBP-5 and IGF-II between T0 and T1 were evaluated by paired t-test after transformation to the natural logarithmic scale.

5.6 Multivariable and logistic regressions

In Paper II and Manuscript III predictors of IGF-I concentrations were assessed by multivariable linear regression models using Minitab (II, Minitab 16, State College, PA, USA) or the “proc GLM” command in SAS 9.3 (III, SAS Institute Inc., Cary, NC, US). Explanatory variables in Paper II were weight, age and sex. In Manuscript III explanatory variables were weight, insulin and age. Assumptions of normality, homoscedasticity and linear relationship between variables were evaluated by QQ-plots, histograms and plotting standardized residuals against the predicted values. If preliminary models showed violation of assumptions, predictors and/or dependent variables were transformed to the natural logarithmic scale. Significance was set to $P < 0.05$.

In Manuscript IV, a linear mixed-effect model was built to evaluate factors associated with IGF-I concentrations using the statistical software R (The statistical software R, 2014) . Explanatory variables were age, days from initiation of insulin treatment, insulin, fructosamine, body condition score 1-5 (1=underweight and 5=overweight), and weight. The individual cat was considered a random effect and the other variables as fixed effects. Akaike Information Criteria and p-values were evaluated during modelling and predictors not significantly contributing ($p>0.10$) were excluded in the final model. Residuals were evaluated for normal distribution, homoscedasticity and linearity with respect to the predictors. If preliminary models showed non-normality of residuals the predictors were transformed to the natural logarithmic scale.

Predictors of remission were evaluated by logistic regression and receiver operating characteristic (ROC) curves calculating area under the curve (AUC) using the statistical software R. Samples after 2-4 weeks (T1) were considered to give the highest statistical power based on the number of animals and differences between groups. Since glycaemic control has been associated with remission rates (Gottlieb & Rand, 2013), glucose, fructosamine and insulin were considered potential predictors for remission in addition to IGF-I.

6 Results

6.1 Validation of a feline insulin ELISA

In paper I there was a significant effect of sample positioning on the ELISA plate that was dependent on columns. During experiments where standard was pipetted into all wells this column effect was still present after pipetting in reverse order, the plate being washed in reverse order and when columns were randomized but analyses performed on the original column order. Position effect was also present when analysing serum samples in duplicate in three different positions on 8 different days. Total-, intra-, and inter-assay CV as well as position effect is presented in Table 1.

Table 1. Assigned values for CVs depending on insulin concentrations. CV_M indicates the CV between microplates; CV_L , systematic and random position effect; CV_e , the CV between duplicates; $CV_{Tot(2)}$, total CV for the mean values of duplicates.

	Insulin concentrations (ng/L)			
	<50	50-100	100-200	>200
CV_M	15.0	13.0	8.0	8.0
CV_L	10.0	10.0	6.0	8.0
CV_e	10.0	6.0	2.0	2.0
$CV_{Tot(2)}$	19.4	16.9	10.1	11.4

When using human insulin standard, the absorbance in the blank well often overlapped the first standard point. This was not seen with feline standard however the concentration of the lowest standard also increased.

Serum spiked with purified feline insulin yielded an O/E ratio of 86-126% and consistency after dilution of plain serum was 78-105%. This was within the defined critical limits (Paper I). Fasting insulin concentrations in healthy cats demonstrated a wide range of values. Insulin concentrations in 35 out of 36 cats were 31.0-652 ng/L (median 221 ng/L). Diabetic cats had median

insulin concentrations of 60 ng/L (range <5-321 ng/L), significantly lower than in healthy cats ($p<0.0001$). One healthy cat had an insulin concentration of 2139 ng/L. To investigate the presence of natural anti-insulin antibodies in healthy cats others have added purified insulin to serum. In presence of natural insulin-antibodies the recovery after addition has been poor (Nishii *et al.*, 2010). The serum sample with high insulin concentration was spiked with feline insulin. The results demonstrated acceptable recovery (80-90%).

The insulin concentrations measured in feline serum increased after an oral glucose tolerance test consistent with the biological response. There was a significant increase of insulin after 30 and 60 minutes compared to baseline but no significant differences between 30 and 60 minutes (I) despite a further increase in glucose. Glucose concentrations after OGTT have not been presented in any paper or manuscript (Figure 1).

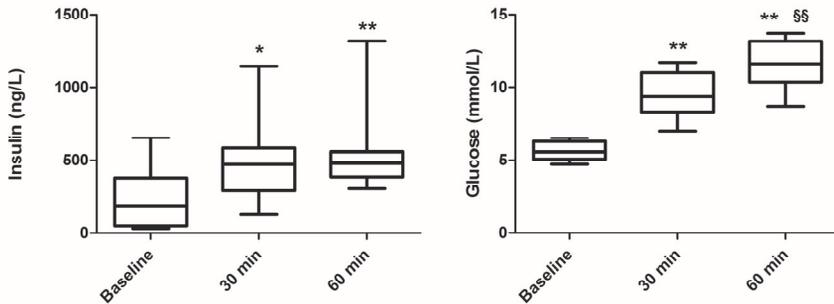


Figure 1. Serum insulin and glucose concentrations in 10 healthy cats after oral glucose tolerance test. Whiskers indicate max and min concentrations. *, $p<0.05$; **, $p<0.01$, compared to baseline. §§, $p<0.01$ compared to 30 minutes.

For samples stored at 20°C, insulin concentrations were within the calculated critical limit for up to 3 days. However, there was a significant difference at day 2 between samples stored at 20°C and 2-8°C, and hence samples stored at 20°C were considered stable for 1 day.

6.2 Validation of an IGF-I ELISA for use in cats

No significant row or column effect ($p=0.11$) was detected in the IGF-I ELISA (II). Total-, intra- and inter-CV are presented in Table 2.

Table 2. Coefficients of variation (CV) expressed in percent (%). Results were derived by analysing samples in 2-20 replicates on 8 different days. Results were obtained by use of ANOVA.

Concentration (ng/mL)	Total CV (%)	Intra-assay CV (%)	Inter-assay CV (%)
86	6.4	4.8	4.3
442	4.3	2.4	3.6
843	5.8	3.1	5.0

Results after dilution of serum samples were considered non-linear according to CLSI. Non-linearity was considered dependent on IGF-BPs since samples diluted with more IGF-II containing assay buffer were linear and a sample previously considered non-linear was linear after size-exclusion chromatography. If using TEa derived from biological variation as limit for the non-linear error (Table 4), samples were considered to have an acceptable non-linear error up to 28 ng/mL on the standard curve. Hence, it is recommended that concentrations of feline IGF-I are determined below 28 ng/mL on the standard curve when using this assay.

As presented in Paper II, there was a wide range of IGF-I concentrations in healthy cats (range 86–1216 ng/mL). Three acromegalic cats had higher IGF-I concentrations (2029, 2869, and 3849 ng/mL) than healthy cats. Unfortunately no serum from cats with pituitary dwarfism was available.

Over six days of storage at 20°C, IGF-I varied between -3.6 and 10.4% and at 2-4°C between -1.7 and 6% (IV). If kept at 2-4°C, the change of results after 14 days was between -8.9 and 6.2%.

IGF-I immunoreactivity and recombinant human IGF-I eluted in the same fractions. IGF-I was detected in fractions 14-22 with a peak in fractions 18-20. When immunoreactivity was compared in serum before and after size-exclusion chromatography the *O/E* ratio was between 98.2 and 115.2%. On WLB IGF-BPs in fractions from size-exclusion chromatography were detected before IGF-I eluted from the column (Figure 2).

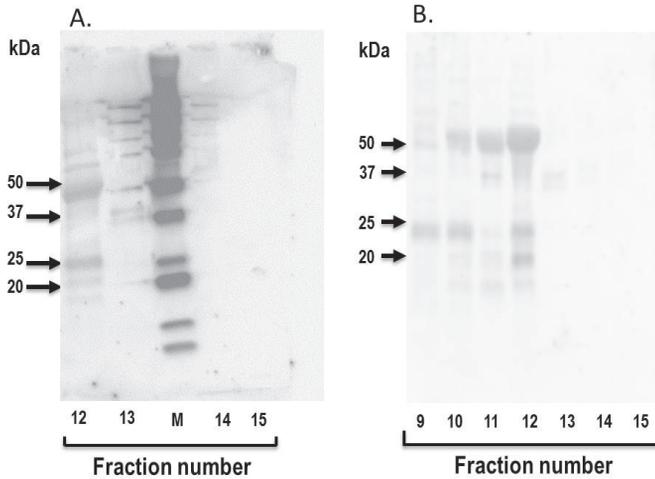


Figure 2. Western ligand blot of IGFFBPs in fractions from size-exclusion chromatography of serum separated under acidic conditions. Samples were lyophilized and run on SDS-PAGE under non-reducing (A) and reducing (B) conditions. Immunoreactive IGF-I eluted from fraction 14 with peak at fraction 18-20. Arrows indicate the molecular weight markers (M).

6.3 Biological variation of insulin and IGF-I

Biological variation of insulin and IGF-I in healthy cats is presented in Table 3. In addition, a cat with acromegaly was sampled and CV_I was 8.1% for IGF-I (II).

Table 3. Biological variation of insulin and IGF-I in healthy cats. Between cat coefficient of variation (CV_G), within cat coefficient of variation (CV_I), analytical coefficient of variation (CV_A), sample occasion (S_{occ}).

	Balanced ANOVA			Unbalanced ANOVA		
	CV_G	CV_I	CV_A	CV_G	CV_I	CV_A
Insulin						
1				46.3	39.7	5.3
2	44.3	42.9	5.5			
IGF-I						
3				65.7	8.2	1.8
4	64.1	7.4	1.5			
5	63.9	7.8	1.7			

1= S_{occ} A deleted in two cats, cats n=7, S_{occ} n=4-5

2= all S_{occ} A deleted, cats n=7, S_{occ} n=4

3= S_{occ} C excluded in one cat, cats n=7, S_{occ} n=4-5

4= All S_{occ} C excluded, cats n=7, S_{occ} n=4

5= One cat excluded, cats n=6, S_{occ} n=5

Based on recommendations (Fraser, 2001) desirable CV_A should be less than $0.5CV_I$. For insulin CV_I varied between 39.7 and 42.9% which makes desirable CV_A for insulin assays in cats to be below 19.9-21.5%. Total CV_A for the feline insulin ELISA presented in Paper I was 10.1-19.4% and thus the assay met the desirable performance. For IGF-I CV_I varied between 7.4 and 8.2% and hence desirable limits for CV_A was 3.7-4.1%. Total CV_A for the IGF-I ELISA exceeded the limit of 4.1% by 0.2-2.3%.

As presented in Table 4, CV_A at low and high concentrations had little effect on IND_I and RCV. Index of individuality for IGF-I indicated marked individuality (defined as <0.6) and hence use of population based RI may be of limited use. For insulin IND_I was in a grey zone (0.6-1.4) indicating that population based RI may be used but with caution (Walton, 2012). Total error allowable for IGF-I and insulin was 23.4 and 47.8%, respectively.

Table 4. *Index of individuality (IND_I), Total error allowable (TEa) and reference change value (RCV) based on data from biological variation derived from unbalanced ANOVA. Total analytical CV (CV_A) from assay validations were used: CV_A IGF-I low and high concentration (6.4 and 5.8%), CV_A insulin low and high concentrations (16.9 and 11.4%).*

	IGF-I	Insulin
$IND_{I\text{low}}$	0.16	0.93
$IND_{I\text{high}}$	0.15	0.89
TEa	23.4	47.8
RCV low	28.8	119.6
RCV high	27.8	114.5

6.4 IGF-I concentrations in health and diabetes

The wide range of IGF-I concentrations in healthy cats (II) was investigated by determining factors associated with IGF-I concentrations. Weight and IGF-I were strongly positively associated in healthy cats (II, $p<0.000001$) and in cats with newly diagnosed DM (III, $p=0.002$). In cats with DM insulin was also positively associated with IGF-I concentrations (III, $p=0.002$). Age and gender were not significant (II, III). There were no significant interactions between any of the significant variables in the prediction of IGF-I. In Manuscript IV the effect of insulin treatment was determined in a longitudinal study design. In these cats weight and fructosamine were negatively correlated, which resulted in collinearity and, based on Akaike Information Criteria and p-values, it was decided to exclude weight from the final statistical model. In the final model

insulin was positively associated with IGF-I and fructosamine negatively associated with IGF-I ($p=0.005$ and $p<0.0001$, respectively). The positive relationship between insulin and IGF-I (Manuscript IV) was seen up to insulin concentrations of 60 ng/L, but not at higher concentrations. This concentration-dependent association was also seen in Manuscript III where the association between IGF-I and insulin did not reach significance in healthy animals with higher insulin concentrations ($p=0.09$). In the final model presented in Manuscript IV age did not reach statistical significance but improved the model fit and was kept in the final model. Results from Paper II and Manuscript III indicate that in healthy cats 1 kg increase in weight is associated with an increase of IGF-I of approximately 40% (II, III). In diabetic cats, sampled before initiation of insulin treatment, doubling of insulin concentration is associated by an increase of IGF-I by 30% (Manuscript III) and in diabetic cats under treatment, at concentrations up to 60 ng/L doubling of insulin concentration was associated with an increase of IGF-I by 95 ng/mL (Manuscript IV).

6.5 Circulating IGF-binding forms

To further investigate the wide IGF-I concentrations in cats (Paper II and Manuscript III) the distribution of endogenous IGF-binding forms was studied by size-exclusion chromatography under neutral conditions (Manuscript III, IV). Two major peaks representing the ternary complex (150kDa) and binary complexes (30-50kDa) were present. Peaks of similar molecular masses have been identified previously in cats, humans and rodents using the same methodology (Renes *et al.*, 2014; van Duyvenvoorde *et al.*, 2008; Lewitt *et al.*, 2001; Lewitt *et al.*, 2000). In Manuscript III and IV IGF-binding profiles were visualized by incubating serum with tracer amounts of h-¹²⁵I-IGF-II to equilibrium. The data is presented by generating three equal IGF-I intervals derived from the RI presented in Paper II (IGF-I ≤ 462 ng/mL, 463-833 ng/mL and ≥ 834 ng/mL). Figure 3 presents one healthy and one diabetic cat representative of each interval. Over the range of IGF-I concentrations, more relative binding of h-¹²⁵I-IGF-II into the 150kDa complex was associated with increasing IGF-I concentrations. When performing univariate linear regression analyses on diabetic and healthy cats altogether (III), results indicated a significant association between ln-150kDa/30-50kDa-ratio and ln-IGF-I concentrations ($p<0.0001$) and ln-150kDa/30-50kDa-ratio and ln-insulin ($p=0.006$).

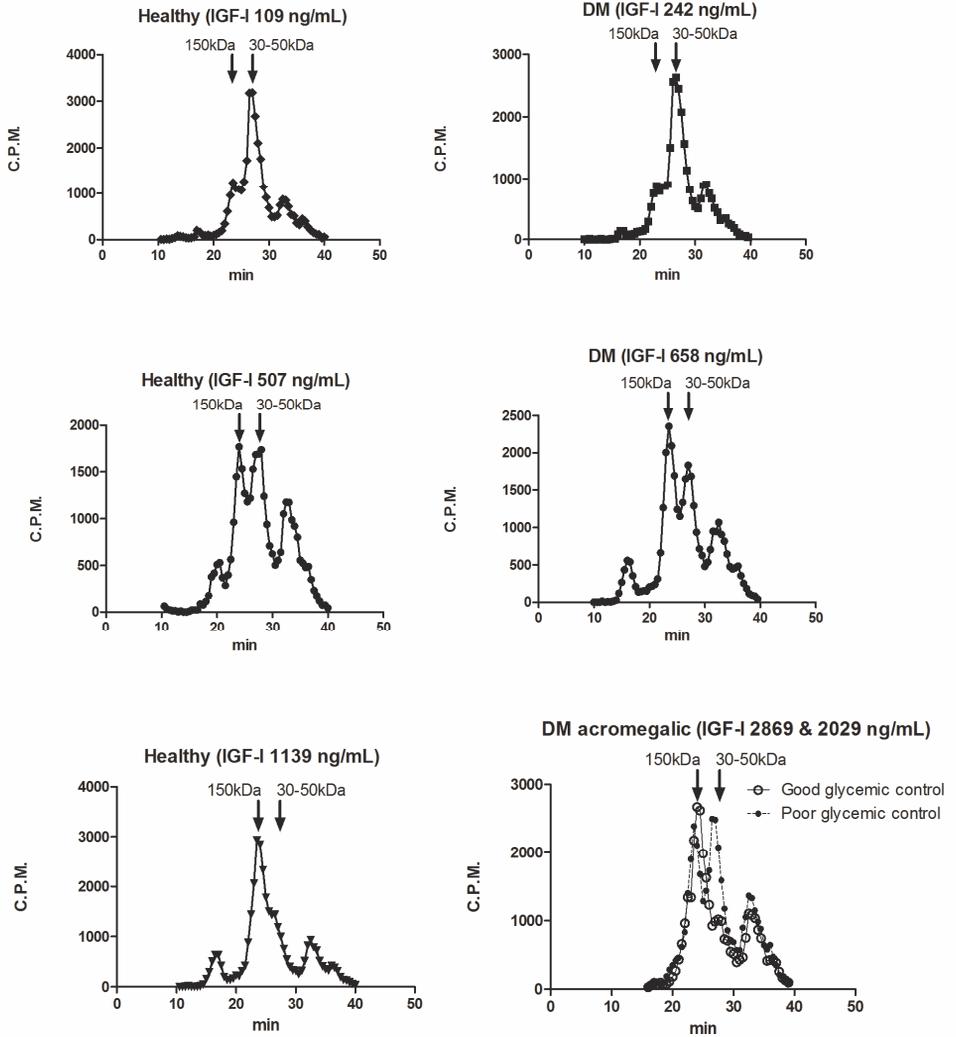


Figure 3. IGF-binding profiles after size separation in healthy and diabetic (DM) cats before insulin treatment after incubation with human ^{125}I -IGF-II and radioactivity (C.P.M., counts per minute) detected by gammacounting. Each graph shows a representative cat. IGF-I concentrations were analyzed with an IGF-I ELISA. The acromegalic cats were on insulin treatment.

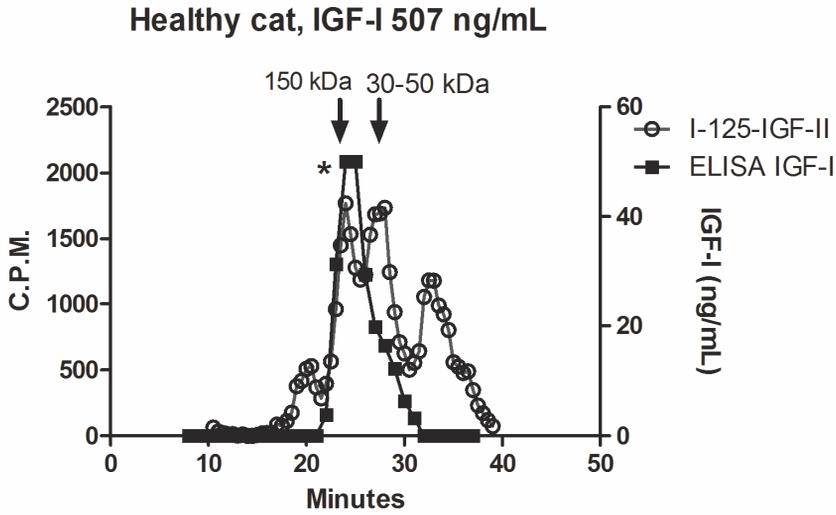


Figure 4. Size separation under neutral conditions using a Superose 12 column. Serum was either incubated with human ^{125}I -IGF-II before separation and counts per minute (C.P.M) counted in each fraction, or serum was run over the column and IGF-I immunoreactivity in the fractions measured by an IGF-I ELISA. The asterisk (*) indicates that the IGF-I concentration was above the highest standard point (50 ng/mL).

In Manuscript III, three samples were size-separated without tracer and fractions analysed with the IGF-I ELISA. IGF-I immunoreactivity was mainly seen in the 150kDa complex regardless of the size of 30-50kDa complex. A representative cat is presented in Figure 4.

In Manuscript IV, IGF-binding forms were studied using tracer methodology in seven cats at diagnosis (T0) of DM and 2-4 weeks after initiating insulin treatment (T1). As shown in Figure 5, in three cats that went into remission the 150/30-50kDa ratio increased ($p=0.03$) along with IGF-I, whereas no change in the 150kDa/30-50kDa ratio was seen in four cats that had poor glycaemic control and did not go into remission ($p=0.7$, $n=4$). IGFBP-3 also increased between T0 and T1 in cats that went into remission ($p=0.03$) but IGFBP-5 and IGF-II did not show any obvious patterns. IGF-II concentrations were higher than IGF-I concentrations. Samples in which IGF-I concentrations were measured by both MS and ELISA ($n=6$) yielded higher concentrations when analysed with MS.

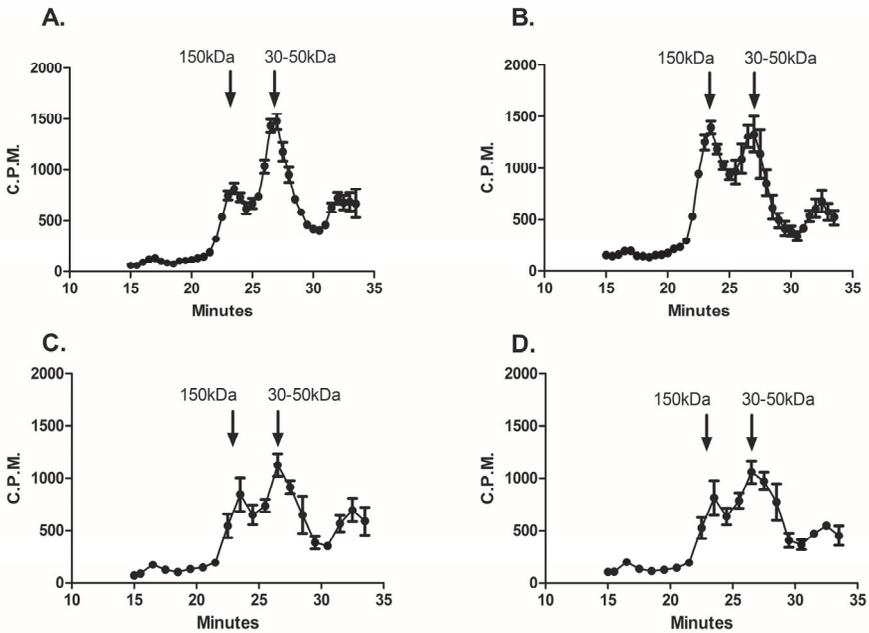


Figure 5. Distribution of IGF-binding forms after incubation with ^{125}I -IGF-II in diabetic cats achieving remission (n=3; A,B) or not (n=4; C,D). Cats were sampled at diagnosis (A, C) and 2-4 weeks after insulin treatment (B,D). Results are expressed as counts per minute (C.P.M., mean \pm SEM).

6.6 Predictors of remission of diabetes mellitus

Using a longitudinal study design (IV) diabetic cats were sampled up to one year after the commencement of insulin treatment. There was a significant increase in IGF-I concentrations between T0 (baseline) and T1 (2-4 weeks, $p=0.0001$) but no significant changes at later time-points. Seven cats went into remission during the study period, all within 5 months from T0. At T1, there were 22 diabetic cats still in the study and of these six went into remission. The cats achieving remission were all treated by the same veterinary practitioner and all of these owners used home glucose monitoring. The effect of “veterinary practitioner” was not significant (data not included). In predicting remission, IGF-I was significant at $p=0.046$ and glucose, fructosamine and insulin were not ($p=0.11$, $p=0.24$ and 0.31 , respectively). The estimated AUC for IGF-I in the ROC analysis was 0.80 (CI 0.62-0.99), for glucose 0.76 (CI 0.54-0.97), fructosamine 0.65 (CI 0.39-0.90) and for insulin 0.61 (CI 0.33-0.90).

7 Discussion

This thesis highlights the importance of validating IGF-I assays with special focus on potential interference by IGFBPs. Commercial assays for IGF-I and insulin were validated for feline samples. To set objective limits for assay performance, and aid in interpretation of repeated sampling in clinical settings, biological variation for IGF-I and insulin were determined, to my knowledge, for the first time in cats. In the feline insulin ELISA there was a statistically significant effect of sample position on the plate, however in the light of biological variation, assay variation was within the desirable limits. In the IGF-I assay the interference of IGFBPs was overcome by the addition of IGF-II in greater quantities than recommended for human serum.

In the studies presented in this thesis a number of factors were evaluated for their relationship to serum IGF-I concentrations. This is important for interpretation and understanding of the clinical value of IGF-I measurements. Weight, for example, was strongly associated with IGF-I concentrations in healthy and diabetic cats.

Since IGF-I is almost exclusively used in diabetic cats when screening for acromegaly it was considered important to study the IGF system in diabetic cats. It has been suggested that pancreatic beta cell function is important for IGF-I concentrations in diabetic cats and for the first time an association between serum insulin and IGF-I is demonstrated in newly-diagnosed diabetic cats. Because many insulin treated diabetic cats can achieve remission there is interest in developing prognostic markers. In this thesis IGF-I emerges as a promising prognostic marker of remission of feline DM.

There follows a discussion for each of the most important findings in this thesis. Please refer to Paper and Manuscript I-IV for detailed discussion of each paper.

7.1 Position effects in ELISA validation

Paper I reported significant effect of sample positioning on ELISA plates and recommended that this should be investigated when performing validation studies. Finding of position effects on ELISA plates have been reported previously (Miller *et al.*, 2009; Shekarchi *et al.*, 1984), however to my knowledge this is not often taken into account when validating assays.

For the feline insulin ELISA, the mechanism behind this effect was not elucidated. The position effect was of the same magnitude as between plate variation and hence should be taken into account when identifying sources of assay variation.

ISO recommends all sources of variation be taken into account, however the application of these guidelines may be found tedious and laborious by laboratories (Rozet *et al.*, 2011). One feasible way to evaluate position effect is to incorporate it into the study design for precision. It has been recommended that precision studies should be performed by analysing samples with different concentrations in 3-5 replicates on 5 days (CLSI, 2014; CLSI, 2005). Instead I would recommend analysing samples with different concentrations in 6 replicates, with 3 duplicates pipetted to fixed positions on the ELISA plate on several days.

The best allocation of samples on an ELISA plate would be to randomly distribute all replicates, including standards. However, this would be cumbersome with 96 wells and a time limit. Manufacturers of ELISA often recommend to analyse 1-3 controls with different concentrations but no recommendations are usually made on how the controls should be situated on the plate. Depending on whether there is a significant effect of rows or columns it could be a valuable procedure to pipette the same controls in both the first and last columns or rows.

7.2 Measurements of feline insulin and biological variation

Feline insulin differs four amino acids from human insulin (Hallden *et al.*, 1986) and previous studies have shown that not all insulin assays intended for human use are suitable for feline samples (Lutz & Rand, 1993). The precision of the feline insulin ELISA validated in Paper I met the desirable specifications based on biological variation at all concentrations, it detected a biological response and had acceptable *O/E* ratio after dilution. Hence it is concluded that this assay can be used for measuring feline insulin.

Evaluation of stability, consistency upon dilution and spiking studies for insulin in Paper I were performed by calculating critical limits. During analysis of insulin stability in Paper I it was apparent that the concentrations declined

systematically even before the critical limit was exceeded. The critical limit is purely based on statistical calculations and does not imply whether the assay is clinically useful or not. In clinical practice insulin is not used as a diagnostic tool and the clinical importance of a decline of this magnitude has not been evaluated.

The critical limits were also used in Paper I to evaluate consistency upon dilution. The critical limits for *O/E* ratio given in percent were in the magnitude of $\pm 46-66\%$ which is considered a clinically unacceptable consistency for insulin assays in humans where $\pm 15\%$ has been recommended (Robbins *et al.*, 1996). However, the *O/E* ratios were between 80 and 105% which was close to the recommended limits for human insulin assays.

Previous studies have found lower insulin concentrations at diagnosis in the majority of cats with DM compared to healthy cats (Tschuor *et al.*, 2011; Kirk *et al.*, 1993) and the results of study I support those findings. It has been suggested that fasting insulin can be used for evaluating insulin sensitivity in healthy cats (Appleton *et al.*, 2005), and by detecting abnormal results cats with impaired insulin sensitivity and risk of developing diabetes may be identified, and preventive actions can be taken. The wide range of insulin concentrations in healthy cats can make interpretation difficult and more healthy cats need to be sampled to determine reference intervals. The results of biological variation of insulin indicated that care should be taken when evaluating results with respect to population based RI and RCV may be a better approach.

Depending on statistical analysis CV_I for insulin varied between 40 and 43%. In one study CV_I of fasting serum insulin in normal people was 24.2% (Borai *et al.*, 2013), however, as glucose tolerance declined CV_I increased to 28.1-33.3%. The cats in study I were not evaluated for impaired insulin sensitivity and it is possible this could have affected the results. In one study of cats sampled twice, CV_I of insulin was 23% (Appleton *et al.*, 2001). However, in contrast to that study where cats were sampled by central venous catheters, the cats in this study were sampled from the cephalic vein. This may affect results since sampling techniques can influence stress at sampling. The stress induced hyperglycemia seen in cats is accompanied by increased insulin concentrations (Rand *et al.*, 2002) and may be a contributing reason for the high CV_I and CV_G reported in Section 6.3. Even though the cats sampled for biological variation did not appear stressed at sampling, some cats are immobilized by fright and the magnitude of stress may be difficult to assess.

7.3 Measurements of feline IGF-I and biological variation

For the IGF-I ELISA the same sample pipetted in all wells did not show any significant position effect and hence this was not further evaluated. However the sample used for the position effect was one high control sample supplied with the ELISA-kit, based on human serum, and it is possible there would have been another result if feline serum had been used.

Feline serum samples measured by the IGF-I ELISA were non-linear according to CLSI. It is difficult to establish clinically acceptable limits for non-linear errors of IGF-I in cats. However, by determining the biological variation and hence TE_a of IGF-I, the guidelines from Westgard were used to calculate the acceptable non-linear error. The non-linear error did not exceed TE_a up to 28 ng/mL on the standard curve and hence values determined below this point were considered acceptable.

The finding of non-linearity above 28 ng/mL in the IGF-I ELISA was likely due to interference of IGFBPs which was seen at high IGF-I concentrations. When results were determined at the upper end of the standard curve, the amount of IGF-II added to prevent interference was not enough. In most species GH stimulates synthesis of IGF-I and ALS and, by ternary complex formation, IGFBP-3, the most abundant IGFBP. Thus high IGF-I concentrations may be accompanied by high IGFBP-3 concentrations. The affinity of feline IGFBPs for human IGF-II has not been studied and it is for example possible that an increase of any feline IGFBP with low affinity for human IGF-II could influence the results. In humans there is a consensus on how to perform method validation for analysis of IGF-I (Clemmons, 2011) and it seems reasonable that this consensus is followed in cats until evidence suggest otherwise. For example we have no studies on which diseases that may increase IGFBPs in cats and thus no indications of what sera to preferably include in method validations. One study compared different IGF-I assays in cats and IGF-I concentrations varied in both healthy and diseased cats depending on which assay was used (Tschuor *et al.*, 2012). In that study no comparison was made with serum subjected to size-exclusion chromatography for removal of IGFBPs and it is not known if discrepancies were due to IGFBPs. Nevertheless, since serum concentrations of the six IGFBPs may differ between healthy and diseased cats it is important to not only include sera from healthy animals in validation studies.

Even if there was interference of IGFBPs in the assay it seemed that the assay could correctly identify acromegaly. However, few acromegalic cats were used in this validation study and the diagnostic performance of the assay needs to be evaluated in a larger population. Unfortunately no cats with pituitary GH-deficiency were available, however it is clear that one should be

cautious if using the IGF-I ELISA for diagnosing this disease since interfering IGF-BPs will cause false low concentrations.

Biological variation of IGF-I in cats was investigated in Paper II and has not been reported before. In humans CV_1 of IGF-I is reported to be 9-21% when results were obtained using ANOVA (Ankrah-Tetteh *et al.*, 2008; Nguyen *et al.*, 2008). In cats CV_1 of IGF-I was calculated to 7.4-8.2% in healthy cats and in one cat with acromegaly, which was similar to, or slightly less than in healthy people.

When measuring IGF-I in healthy adult cats there was a wide RI and IND_1 indicated that RI should be stratified. To identify possible factors for stratification of RI, study II, III and IV aimed at identifying factors associated with IGF-I concentrations.

7.4 Predictors of IGF-I concentrations

7.4.1 General characteristics and markers of glycaemic control

In study II and III a strong association between weight and IGF-I concentrations was seen in both healthy and diabetic cats and it is proposed that weight should be taken into account when interpreting IGF-I concentrations in cats. This finding contrasts to IGF-I in humans where the reference interval for IGF-I often is stratified on gender and age (Brabant & Wallaschofski, 2007). The association between weight and IGF-I has been presented previously (Berg *et al.*, 2007; Maxwell *et al.*, 1999) whereas another study did not see this relationship (Reusch *et al.*, 2006). It is not clear whether the association between IGF-I and weight is due to size or body composition. In dogs the same association between weight and IGF-I has been demonstrated. A genetic variation in the *IGF1* gene has been found between breeds of different body sizes (Sutter *et al.*, 2007). However, IGF-I is not only dependent on size but also on body composition in dogs. IGF-I increased in Beagle dogs that were overfed (Gayet *et al.*, 2004). Similar studies have not been done in the cat and the effect of body size versus body composition on IGF-I concentrations is currently not known. In study IV body composition measured as body condition score was not significantly associated with IGF-I and in study II and III this differentiation was not made.

In healthy cats neither age nor sex was significantly associated with IGF-I. Most cats in study I-IV were castrated which could be one explanation for the lack of association with sex; and another study presented similar results (Reusch *et al.*, 2006). Age was not a significant predictor of IGF-I (II, III) however in the longitudinal study (IV) age improved the model fit and was near the set α -threshold ($p=0.052$). In this study (IV) the association between

age and IGF-I was positive whereas another study presented a negative relationship (Reusch *et al.*, 2006). Any reason for the contradictory results is not known but may be due to confounders not included in the model.

In newly diagnosed diabetic cats sampled before insulin treatment (III) insulin, in addition to weight, was associated with IGF-I. There were no significant interactions between weight and insulin and this indicates that insulin concentrations did not modify the effect of weight on IGF-I concentrations. In diabetic cats (IV) the independent association between IGF-I and insulin was present up to 60 ng/L but not at higher concentrations. An association between beta cell capacity and IGF-I has been demonstrated in type 1 DM in people (Sorensen *et al.*, 2015; Hedman *et al.*, 2004) and it has been proposed that increased beta cell function in cats during insulin treatment is a cause of increased IGF-I concentrations (Alt *et al.*, 2007; Reusch *et al.*, 2006). However, no one has previously presented evidence that demonstrates this relationship in cats. One contributing factor suggested for this association is that expression of hepatic GH-receptors are stimulated by insulin (Leung *et al.*, 2000; Baxter *et al.*, 1980).

Results of the longitudinal study of diabetic cats (IV) indicated that fructosamine and weight were negatively associated which resulted in collinearity. It is not surprising that high fructosamine concentrations, associated with poor glycaemic control, are associated with a malnourished state in diabetic animals. Based on Aikake Information Criteria and p-values it was decided to exclude weight from the final model. Based on the findings in study II, III and IV it can be concluded that weight is an important predictor of IGF-I concentrations in healthy and diabetic cats but glycaemic control must also be taken into account when interpreting IGF-I concentrations in diabetic animals. In these studies the use of IGF-I as a screening marker for acromegaly was not investigated. However, as a result of the present studies we now have a better understanding of feline IGF physiology and further studies addressing the clinical value of IGF-I measurements in a range of clinical conditions including acromegaly, will be possible.

7.4.2 Molecular distribution of IGF-binding forms in serum

Since the 150kDa ternary complex stabilises IGF-I, prolongs its half-life and therefore affect serum concentrations, Manuscript III and IV investigated the molecular distribution of IGFs in the circulation of healthy and diabetic cats. Results of study III indicate that less relative binding of h-¹²⁵I-IGF-II in the 150kDa complex, is associated with lower IGF-I concentrations and this pattern is seen in both healthy and diabetic cats. In a previous study less binding in the 150kDa complex was only seen in healthy cats (Lewitt *et al.*,

2000). This discrepancy could be explained by the fact that cats sampled for study III represented a range of IGF-I concentrations whereas this was not the case in the study by Lewitt *et al.* (2000).

The results of Manuscript III, together with the previous study published by Lewitt *et al.* (2000), indicate that in both healthy and diabetic cats ALS is not in excess across the range of IGF-I concentrations, but may be at high concentrations. Low IGF-I concentrations are associated with low ternary complex formation and relatively increased 30-50kDa complexes. It is most likely that ALS is the limiting factor for 150kDa complex formation in cats since it was possible to shift the 30-50kDa peak by adding human serum which is known to contain excess ALS, as well as from cats which were hypothesized to have high ALS (Lewitt *et al.*, 2000). Previous results found no differences in IGFBP-3 visualized on WLB between cats with low and high amount of 150kDa complex (Lewitt *et al.*, 2000), which makes the alternative hypothesis that IGFBP-3 is the limiting factor for ternary complex formation less likely.

GH is a major stimulant of hepatic ALS synthesis and one can speculate on the role of GH in determining the patterns observed. GH also stimulates production of IGF-I and IGFBP-3 concentrations, since circulating IGFBP-3 levels depend on ternary complex formation (Olivecrona *et al.*, 1999). In human GH deficiency all three components of the ternary complex are reduced (Domene *et al.*, 2011). GH and IGF-I have been shown to stimulate adipocyte differentiation (Smith *et al.*, 1988; Kuri-Harcuch & Green, 1978) and, since cat serum has been observed to be relatively deficient in a factor that stimulates adipocyte differentiation (Kuri-Harcuch & Green, 1978) it is possible that some cat serum is IGF- or GH-deficient. In the absence of an available validated feline GH assay and no reference material, it is difficult to compare GH concentrations in serum between species.

In Manuscript IV there was a noticeable increase in IGF-I concentrations between T0 and T1. In cats which later went into remission the relative amount of h-¹²⁵I-IGF-II binding into the 150Da complex increased but this was not seen in cats which did not achieve remission. In addition, in three cats that went into remission IGFBP-3 concentrations were measured by MS and were found to be increased. The increase of IGF-I and IGFBP-3 concentrations in cats which went into remission is likely to reflect an increased amount of ternary complex.

In Manuscript IV, IGF-I was measured both by MS and ELISA in six samples. This was done in order to compare concentrations of IGF-I with IGF-II, IGFBP-3 and IGFBP-5 when using the same methodology. As in humans it seemed that IGF-II is higher than IGF-I (Rajaram *et al.*, 1997). In all six samples IGF-I concentrations were higher when analysed with MS compared

to ELISA. Higher concentrations of IGF-I measured by MS compared to immunoassay has been demonstrated when analysing human serum (Kay *et al.*, 2013).

It has been proposed that insulin may have an effect on ALS concentrations and this contributes to ALS regulation under nutritional deprivation (Dai & Baxter, 1994; Dai *et al.*, 1994). Decreased ALS concentrations that increase during insulin treatment have been seen in both diabetic humans and rats (Bereket *et al.*, 1996; Dai & Baxter, 1994). In Manuscript III a positive association was found between insulin concentration and relative amount of 150kDa complex, and in Manuscript IV the relative amount of 150kDa complex increased after treatment with insulin, supporting an association between nutrition and the amount of ternary complex formed.

IGF-I immunoreactivity was mainly found in the 150kDa complex regardless of the size of the 30-50kDa peak and this finding has also been observed in children (Renes *et al.*, 2014). It is therefore likely that the 30-50kDa complex represents binary complexes with IGF-II. However, the affinities of IGFs and IGF-BPs in the cat are unknown and it cannot be excluded that results would have been different if feline IGFs were available and used as tracer. Further studies of affinities and physiology are needed to understand the IGF system in cats.

7.5 Predictors of remission of feline diabetes mellitus

It is considered that early treatment and near euglycaemic control are key factors in achieving remission of DM in cats (Gottlieb & Rand, 2013). In study III and IV IGF-I concentrations were associated with markers of glycaemic control and preliminary evidence indicates that IGF-I concentrations at 2-4 weeks after initiating insulin treatment (T1) predict remission (IV). The finding of increased serum IGF-I concentrations after initiation of insulin treatment has been reported previously (Alt *et al.*, 2007; Reusch *et al.*, 2006). Interestingly, in cats, one study proposed a protective effect of IGF-I on feline beta cells during hyperglycaemia (Link *et al.*, 2013). IGF-I may also itself, by enhancing insulin action (Clemmons, 2012), contribute directly to glycaemic control and therefore remission.

Since glycaemic control is associated with remission, endogenous insulin, fructosamine and glucose concentrations were investigated as predictive markers, in addition to IGF-I. These had lower AUCs than IGF-I and were not statistically significant (IV). Previous studies which investigated serum insulin concentrations at diagnosis as a possible predictor for remission, found that insulin concentrations in diabetic cats were lower than in healthy cats and did

not predict remission (Tschuor *et al.*, 2011; Nelson *et al.*, 1999). It has been proposed that reduction of beta cell function is transient and due to glucotoxicity. When the animal is treated with insulin and glucotoxicity is reversed, the insulin-secreting capacity is restored (Zini *et al.*, 2009; Nelson *et al.*, 1999). Interestingly, hyperlipidemia does not seem to cause decreased beta cell function in cats (Zini *et al.*, 2009). In study IV fasting insulin was measured 2-4 weeks after initiating insulin treatment and there was no difference between cats going into remission or not.

Glucose was not a predictor of remission. Cats are prone to stress-induced hyperglycaemia which may contribute to larger variation and thus affect results. Fructosamine is a glycated protein and is considered to reflect glycaemic control during the last 1-2 weeks (Crenshaw *et al.*, 1996). It is possible that IGF-I is reflecting the glycaemic and nutritional status in a shorter time frame and results would have been different if data from later sampling occasions were used. However, at later sampling occasions there were fewer animals left in the study which resulted in less power. For the clinician and owners a marker for remission early in the course of the disease is preferable.

8 Conclusions

- In validating two ELISAs (IGF-I and insulin) for use in feline medicine it was concluded that laboratories, in validating their assays, should be aware of position effects on assay plates and, for IGF-I assays, interference by circulating IGF-binding proteins.
- Biological variation should be taken into account when recommending limits for assay performance and in interpreting clinical results after repeated sampling. For IGF-I, between animal variation was high (~65%) while within animal variation was considerable lower (~8%).
- There is a wide range of IGF-I concentrations in healthy and diabetic cats that is in part determined by weight. Weight should be used to stratify reference intervals for feline IGF-I.
- IGF-I concentrations are related to the amount of the IGFBP-3 150kDa ternary complex formation in healthy and diabetic cats.
- Glycaemic control should be taken into consideration when interpreting IGF-I concentrations in diabetic cats.
- IGF-I response to insulin treatment shows promise as a predictive marker for remission in feline diabetes mellitus.

9 Populärvetenskaplig sammanfattning

Inom veterinärmedicinen används analys av insulin-liknande tillväxtfaktor-I (IGF-I) framförallt vid diagnos av sjukdomar relaterade till tillväxthormon. Den vanligaste indikationen för mätning av IGF-I hos katt är vid diagnos av akromegali vilket orsakas av en tumör i hypofysen som producerar tillväxthormon. Tillväxthormon ger insulinresistens och akromegali ses nästan uteslutande hos katter med svårbehandlad diabetes mellitus (DM). Katten är en strikt köttätare och det finns indikationer på att regleringen av IGF-systemet skiljer sig från andra djurslag. För att korrekt tolka provsvar för IGF-I hos katt är det viktigt att förstå fysiologin samt identifiera faktorer som påverkar koncentrationerna och som bör tas i beaktande vid tolkning. De flesta katter med DM har en typ som liknar människans typ 2, men till skillnad från människor har katter ofta låga nivåer av insulin vid diagnos. Trots detta kan de flesta katter tillfriskna från sin diabetes (remission) om rätt behandling sätts in. Snabbt insatt insulinbehandling och kontroll över blodsockernivån anses vara den viktigaste faktorn för tillfrisknande av DM hos katt men hittills har ingen blodmarkör identifierats som kan användas för att förutsäga chansen att katten går i remission.

Det övergripande målet med denna avhandling var att studera IGF-I hos friska katter och katter med DM för att identifiera faktorer som bör tas i beaktande vid tolkning av provsvar. Ytterligare ett mål var att undersöka om IGF-I och insulin kan användas för att förutsäga vilka katter som kan tillfriskna från sin diabetes.

En kommersiell analysmetod (ELISA) för IGF-I respektive insulin utvärderades för analys av blodprov från katt och bägge metoderna fungerade bra. För insulin sågs en signifikant effekt på resultaten beroende var proven analyserades på ELISA-plattan men den totala variationen överskred inte uppsatta gränser. En slutsats i denna avhandling är att positionseffekter bör undersökas vid validering av ELISA. För IGF-I ELISA störde IGF-bindande

proteiner analysen vid höga koncentrationer av IGF-I men detta kunde hanteras genom att späda prover mer än vad som rekommenderats för prover från människa. Det var en stor spännvidd av IGF-I koncentrationer hos både friska katter och katter med DM. Vikt hade ett starkt positivt samband med IGF-I hos friska katter men ingen koppling sågs till ålder och kön. Hos katter med DM provtagna före insulinbehandling hade både vikt och insulinkoncentrationer ett positivt samband med IGF-I. Hos katter med DM provtagna under behandling hade markörer för blodsockerkontroll (insulin och fruktosamin) ett samband med IGF-I koncentrationer.

Huvuddelen av IGF-I cirkulerar bundet till sex olika bindarproteiner och distributionen av dessa komplex skiljde sig åt från vad som rapporterats hos friska vuxna människor. Detta tyder på att katten kan ha en annan reglering av IGF-systemet än människa och man skall vara försiktig med att extrapolera vad som är känt från människa till katt.

Hos katter under insulinbehandling sågs en tydlig ökning av IGF-I från diagnos till efter 2-4 veckors insulinbehandling. Katter som senare tillfrisknade från sin diabetes hade högre koncentrationer 2-4 veckor efter insatt insulinbehandling än de som inte tillfrisknade.

Sammanfattningsvis har jag i denna avhandling visat att man vid tolkning av IGF-I hos katt bör ta hänsyn till vikt. Stora katter har normalt högre koncentrationer än små katter. Referensvärden bör tas fram med hänsyn till vikt och inte kön och ålder som för människor. Hos katter med diabetes bör blodsockerkontrollen tas i beaktande vid tolkning av provsvar då dålig kontroll av blodsockret kan ge lägre värden av IGF-I. Koncentrationen av IGF-I efter insatt insulinbehandling kan vara en möjlig markör för chansen att tillfriskna från DM.

10 References

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