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Published in:
Journal of Proteome Research

DOI:
10.1021/acs.jproteome.8b00259

Published: 30/10/2018

Document Version
Peer reviewed version

Development of a parallel reaction monitoring-MS method to quantify IGF proteins in dogs: and a case of non-islet cell tumor hypoglycemia

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Abstract

Non-islet-cell tumor hypoglycemia (NICTH) is a rare paraneoplastic phenomenon well described in dogs and humans. Tumors associated with NICTH secrete incompletely processed forms of insulin-like growth factor-II (IGF-II), commonly named big IGF-II. These forms have increased bioavailability and interact with the insulin and IGF-I receptor causing hypoglycemia and growth-promoting effects. Immunoassays designed for human samples have been used to measure canine IGF-I and -II, but they possess some limitations. In addition, there are no validated methods for measurement of big IGF-II in dogs. In the present study, a targeted PRM MS-based method previously developed for cats has been optimized and applied to simultaneously quantify the serum levels of IGF-I, IGF-II, and IGFBP-3, and for the first time, the levels of big IGF-II in dogs. This method allows the absolute quantification of IGF proteins using a mixture of QPrEST™ proteins previously designed for humans. The method possesses good linearity and repeatability, and has been used to evaluate the IGF-system in a dog with NICTH syndrome. In this dog, the levels of big IGF-II decreased by 80% and the levels of IGF-I and IGFBP-3 increased approximately 20 and 4 times, respectively, after removal of the tumor.

Keywords

Big IGF-II, dog NICTH, IGF system, PRM, targeted proteomics.

INTRODUCTION

Non-islet-cell tumor hypoglycemia (NICTH) is a rare paraneoplastic phenomenon that is well described in both dogs and humans. NICTH denotes the syndrome of hypoglycemia produced by or associated with any neoplasm other than an insulinoma.[1] In most of the human cases, this syndrome is derived from an overproduction of insulin-like growth factor-II (IGF-II) by the tumor, including mature IGF-II or incompletely processed forms of IGF-II, that are referred to as big IGF-II.[2-4] In humans and animals, IGF-I and -II are both important regulators of growth and metabolism, and circulate bound to one of a family of six insulin-like growth factor-binding proteins (IGFBP 1-6), that modulate IGF-bioavailability.[5] Of the IGFBPs, IGFBP-3 is the most abundant in the circulation and transports more than 75% of IGF-I or -II in a ternary complex with an acid-labile subunit (ALS).[6] The large size of this complex (~ 150 kDa) limits the passage of IGFs across the capillaries,[7] thus prolonging their half-life from a few minutes, to hours or days, and determining their circulating concentrations.[8] IGFs and IGFBPs also form binary complexes (40-50 kDa) that are sufficiently small to cross the capillary membrane and gain access to most tissues.[9] In NICTH cases, big IGF-II can contribute up to 60% of the total circulating IGF-II concentration. It has been proposed that impaired proteolytic processing is due to the absence of glycosylation in the big isoforms,[10] as well as the exceeded proteolytic capacity of the tumor cells.[11] Moreover, although big IGF-II isoforms bind to IGFBPs with the same affinity as mature IGF-II, ternary complexes are formed less readily, and the formation of binary complexes is promoted.[12,13] Finally, some big IGF-II isoforms have reduced affinity for the IGF-II receptor, which may decrease its degradation and thereby increase bioavailability.[14] All of these effects increase the amount of bioavailable IGF-II and big IGF-II, that can interact with the insulin and IGF-I receptor causing hypoglycemia and growth-promoting effects.[1,15]

In normal circumstances, growth hormone (GH) induces synthesis of IGF-I and ALS by the liver. In addition, serum IGFBP-3 increases as a result of increased availability of ternary complex formation.[16] In

55 vitro studies demonstrate that IGF-I and IGF-II have inhibitory effects on pituitary GH.[17] Consistent with
56 a negative feedback by big IGF-II forms in NICTH, patients have suppressed IGF-I, ALS and IGFBP-3 which
57 increase in response to growth hormone treatment.[18,19] Total IGF-II may be either within the reference
58 interval, decrased or increased in patients with NICTH.[1] Currently, reduced circulating IGF-I
59 concentrations are routinely used in the investigation of suspected NICTH.[1,20] However, since IGF-I may
60 be suppressed in severe catabolic illness,[21] measurement of big IGF-II is more specific for the disorder.
61 Simultaneous measurements of IGF-I, IGF-II, big IGF-II and IGFBP-3 could aid in diagnosing NICTH as well
62 as improving understanding of the GH-IGF-system in health and disease. To the best of our knowledge,
63 the serum concentrations of big IGF-II protein in dogs have never been measured before.

64 Canine IGF-I, IGF-II and IGFBP-3 concentrations have been measured using a variety of methods.
65 Radioimmunoassays (RIA) have been used to study the levels of IGF-I and -II,[22-25] enzyme-linked
66 immunosorbent assays (ELISA) to measure the levels of IGF-I,[26] and Western Ligand Blotting (WLB) to
67 evaluate the levels of IGFBP-3.[22-23] However, all these methods have limitations. The use of human
68 immunoassays for analysis of samples from other species can result in weak reactivity or unwanted
69 cross-reactivity. Moreover, IGFBPs can interfere with IGFs measurements, causing both false high or low
70 values depending on the assay.[27] In addition to this problem, data available from the College of American
71 Pathologists proficiency testing program for IGF-I demonstrated that interlaboratory variability of human
72 IGF-I immunoassays has an RSD up to 33.5%,[28] and there is no available reference standard for
73 quantification of canine IGF-II.

74 To solve these problems, and due to the good capabilities offered by liquid chromatography (LC) coupled
75 to mass spectrometry (MS) in other fields,[29] MS-methods have been proposed to measure IGFs
76 proteins.[30] In comparison to RIA, ELISA and WLB, LC-MS-methods require smaller volumes of samples
77 and allow the unambiguous identification and quantification of multiple proteins in a single experiment.

Published studies have applied this methodology to quantify IGF-I,[28,31-33] or IGF-I, IGF-2, IGFBP-2 and IGFBP-3 simultaneously in humans,[34] as well as in other species.[35,36] In most of these experiments, and due to the wide dynamic range of protein concentrations, a tedious and/or time-consuming step of protein depletion is necessary to remove the most abundant proteins and to avoid the suppression of the signal of the least abundant. To achieve this, different protocols have been developed using acetonitrile,[28] SPE columns,[32] or specific antibodies for protein purification.[31] After sample preparation, the analysis of the proteins is often performed using a triple quadrupole MS spectrometer, where a peptide (precursor) is targeted for its fragmentation, and the quantification is performed at the MS/MS level using 3 to 6 transitions previously selected (commonly known as selected/multiple reaction monitoring (SRM/MRM)).[37] However, the development of more advanced mass spectrometers has enabled the use of other methods for quantification, such as the parallel reaction monitoring (PRM).[38,39] This method is mainly performed using quadrupole-Orbitrap mass spectrometers, where a precursor is selected, all transitions are measured, and the selection of fragments for quantification is done post-acquisition. Since full MS/MS spectra of the targeted peptides are acquired with high resolution and high mass accuracy, a PRM-based targeted method of protein quantification is highly selective and specific,[40,41] making it a very good method for targeted proteomics in complex matrices such as serum or plasma.[42] In addition, the great capabilities of this instrumentation have recently allowed the analysis of different IGFs in feline serum without extensive sample preparation.[43] In targeted MS-methods, heavy isotope-labelled peptides or full-length proteins are used for quantification of the endogenous proteins, which allow the comparison of their signals with the internal standards that are spiked into the samples during processing. The most commonly used peptides contain arginine or lysine with ^{13}C and ^{15}N isotope at the C-terminal end, and the most used synthetic proteins are QconCAT and PSAQ.[44,45] Other synthetic proteins are available, such as the QPrEST™ proteins.[46] QPrEST™ proteins are 50-150 amino acid-long segments of human proteins with heavy isotope-labelled (^{15}N, ^{13}C) lysine and arginine, covering more

than 80% of the human protein-coding genes.[47] However, these proteins are rarely used to quantify human and non-human proteins.

To the best of our knowledge, no MS-based method has been published that quantifies canine IGF-I, IGF-II, big IGF-II and IGFBP-3 simultaneously. Therefore, the aim of this study is to optimize and use a previously targeted PRM MS-based method developed for cats, to absolutely quantify the levels of these IGF proteins and, for the first time, the levels of big IGF-II in dogs. The method was applied to diagnose and monitor a recent case of canine NICTH that was successfully managed surgically.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (ACN), formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (NH_4HCO_3), urea, dithiothreitol (DTT) and iodoacetamide (IAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). For the tryptic digestion, trypsin (sequencing grade modified, Promega, Madison, WI, USA) was used. Ultrapure water was prepared by the Milli-Q water purification system (Millipore, Bedford, MA, USA).

Canine serum samples

Healthy dogs (n=7) were sampled at the University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala (Sweden), as well as in a smaller private clinic. All dogs were considered healthy by their owners and after a clinical examination by a veterinarian. Since weight is strongly related to IGF-I concentrations,[48-49] dogs were selected to represent a wide range of size. The breeds included Labrador Retriever, Australian Shepard, Russkiy Toy, German Shepard, Cane corso, Border Collie and Mixed breed. Age ranged from 1-9 years with median (IQR) of 4 (2-7) and weight between 2.4-46 kg with median (IQR) of 24 (14-36). There were 1 spayed female, 3 intact females and 3 intact males. The study was approved

by the Swedish Animal Ethics Committee (no. C193/14) and all owners provided written informed consent. Blood was drawn into plain tubes and centrifuged within 30-60 minutes. Serum was either frozen in -20°C or sent to the laboratory by post at ambient temperatures. All blood samples arrived within 24 hours after sampling. Blood samples from a dog with suspected NICTH syndrome, before and after tumor removal, were also used for validation.

Protein standards

To be able to match tryptic peptides in QPrEST™ proteins with dog sequences, Clustal Omega (1.2.4)[50] was used for sequence alignment of *Homo sapiens* and *Canis familiaris* amino acid (aa) sequences (**Figure 1**). QPrEST™-containing peptides matching with *Canis familiaris* IGF-II (Cat. Number 22489) and IGFBP-3 (Cat. Number 23429), with ≥99% isotopic purity and ≥80% peptide purity, were provided by Atlas Antibodies (Stockholm, Sweden). For IGF-I, the peptide GPETLCGAELVDALQFVCGDR synthesized with heavy-labelled (^{15}N, ^{13}C) lysine and arginine (≥99% isotopic purity and ≥95% peptide purity) was purchased from New England Peptides (Gardner, MA, USA). The uniqueness of the sequences to the targeted proteins was checked using the “peptide search” tool of the Uniprot database against Canine proteins, and using Skyline.

In-solution tryptic digestion

The protein content in dog sera was measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For normalization, a volume of sera that contained 30 µg of total protein was taken from each sample, and submitted to in-solution digestion. Firstly, the volume was adjusted with 0.4 M NH_4HCO_3, 1 M Urea, pH 8 to a total volume of 200 µL, and then the QPrEST™- containing peptides were spiked into the samples at different concentrations depending on the study (see below). The samples were sonicated for 3 minutes and after that, 10 µL of 45 mM DTT was added and the samples were kept at 50°C for 15 minutes to reduce the proteins. To irreversibly carbamidomethylate the cysteines, 10 µL of

100 mM IAA was added, followed by 15 minutes incubation at room temperature in darkness. For the digestion, 5% (w/w) of trypsin was added and the samples were incubated over night at 37°C. The heavy-labelled synthetic peptide was then spiked into the samples, and they were completely dried in a SpeedVac system. The samples were re-suspended in 40 µL 0.5% TFA and desalted using the SPE Pierce® C18 Spin Columns (ThermoFisher Scientific). These columns were activated by 2 x 200 µL of 50% ACN and equilibrated with 2 x 200 µL of 0.5% TFA. The tryptic peptides were adsorbed to the media using 2 repeated cycles of 40 µL sample loading and the column was washed using 2 × 200 µL 0.5% TFA. Finally, the peptides were eluted in 2 × 30 µL of 70% ACN and dried. Before they were analysed on a nanoLC-LTQ-Orbitrap mass spectrometer, the peptides were re-suspended in 160 µL of 0.1% FA in Milli-Q water.

NanoLC-Q Exactive Plus-PRM analysis

The PRM analysis was performed on a Q Exactive Plus Orbitrap mass spectrometer (ThermoFisher Scientific). An EASY-nLC 1000 system (ThermoFisher Scientific) was used for the peptide separation. A volume of 4 µL of sample was loaded onto a pre-column (EASY-Column, 2 cm, inner diameter 100 µm, 5 µm, C18-A1, ThermoFisher Scientific) at a maximum pressure of 280 bar. The peptides were then eluted onto an EASY-column, 10 cm, inner diameter 75 µm, 3 µm, C18-A2 (ThermoFisher Scientific), which was used for the separation. A flow rate of 250 nL/min using mobile phase A (Milli-Q water with 0.1% FA) and B (ACN with 0.1% FA) was set for the separation. A 40 minutes gradient from 5% B to 40% B followed by 10 minutes from 40% B to 75% B, and a washing step with 10% B for 10 min was used. The system was controlled through Q Exactive Plus Tune 2.5 and Xcalibur 3.0. The PRM method combined two scan events starting with a full scan event followed by targeted MS/MS for the doubly and/or triply charged precursor ion scheduled in an inclusion list. The full scan event employed a m/z 300-800 mass selection, an Orbitrap resolution of 140,000 at m/z 200, a target automatic gain control (AGC) value of $3*10^6$, and maximum fill times of 250 ms. The targeted MS/MS was run at an Orbitrap resolution of 35,000 at m/z

200, target AGC value of $1*10^6$, and maximum fill times of 200 ms. The targeted peptides were isolated using a 1.2 m/z unit window. MS/MS fragmentation was performed using the high energy collision dissociation (HCD) mode, with normalized collision energy (NCE) of 27 eV. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[51] partner repository with the data set identifier PXD009277.

Determination of linearity, repeatability and limits of detection (LOD) and quantification (LOQ)of the method

The IGF-II and IGFBP-3 QPrEST™ proteins and the heavy-labelled GPETLCGAELVDALQFVCGDR peptide were used in a spike-in experiment to determine the linearity and the repeatability of the targeted proteins, applying the PRM-method. For the calibration curves, the QPrEST™ were spiked into canine sera to final concentrations of 0.058, 0.29, 1.4, 7.2 and 36 fmol/µL for IGF-II, and 0.019, 0.094, 0.47 and 2.3 fmol/µL for IGFBP-3. The samples were then digested and thereafter, the GPETLCGAELVDALQFVCGDR peptide was spiked into the sample to final concentrations of 0.0024, 0.012, 0.059, 0.29 and 1.5 fmol/µL. These samples were analysed in triplicate. For the intraday repeatability study, three serum samples (technical replicates) from the NICTH dog after surgical removal of the splenic tumor were spiked-in with the QPrEST™ or the heavy GPETLCGAELVDALQFVCGDR peptide the same day. The concentrations were selected based on the ratios obtained in the calibration curves between the native and the synthetic peptides, to obtain an approximate ratio of 1. The final concentrations were 7.2 fmol/µL for IGF-II, and 0.47 fmol/µL for IGFBP-3. These samples were then digested and the GPETLCGAELVDALQFVCGDR peptide was spiked-in to a final concentration of 0.29 fmol/µL. These samples were analysed in triplicate, and all data were processed together (n=9). For the interday repeatability study, the same sample preparation was applied as for the intraday repeatability study, but in this case, serum from the dog before and after the surgery of the splenic tumor was used.

The same sample was prepared in three different and non-consecutive days, and they were analysed in triplicate (n=9). For data analysis, the SkyLine 3.7.0 software[52] was applied to extract area under curve (AUC) of the fragments. For the statistical analysis, the built-in tool Data Analysis in Microsoft Excel Professional 2010 was used. Ordinary least squared regressions along with ANOVA were used to evaluate the fitness of the calibration curves (one predictive variable), the relation between IGF protein concentrations and weight (two variables), and the relation between IGF-I concentrations calculated using the PRM MS-based method and the ELISA method (two variables). To evaluate the effect of the splenic tumor removal in the concentration of IGF proteins, the normal distribution of the data was verified by visual examination and by the Anderson-Darling normality test, and a paired sample T-test was applied. In all these analyses, p-values < 0.05 were considered statistically significant. The LOD and LOQ for IGF-I, IGF-II and IGFBP-3 were calculated using the following formulas: $LOD = 3Sa/b$, $LOQ = 10Sa/b$, where Sa is the standard deviation of the minimum detectable concentration with an RSD ≤ 20%, and b is the slope of the standard dilution curve.[53] LOD for big IGF-II was determined by manual inspection of analysed samples in the low concentration range, as described in the results.

Method comparison

Measurements of IGF-I is used routinely for diagnosis of GH-related diseases in dogs. Seven samples were analysed both with MS and an IGF-I ELISA (Mediagnost, Reutlingen, Germany) previously validated for use in dogs.[54]

RESULTS

Internal standard selection

The first step for the optimization of the targeted MS-based method used for quantification of IGF-I, IGF-II and IGFBP-3, and the incompletely processed form of IGF-II (big IGF-II), was the selection of the internal standard. IGF-I protein from dog is well defined in the UniProt database, but IGF-II and IGFBP-3 are classified as “uncharacterized proteins”, with three different identifiers for IGF-II (J9NYS6, F1PBX5 and 9P961) and one identifier for IGFBP-3 (F1PQ91). To study if the human-derived QPrEST™ could be used as internal standard, the canine sequences of those identifiers were aligned with the human sequences (**Figure 1**). The chosen sequences were verified as unique to the targeted proteins.

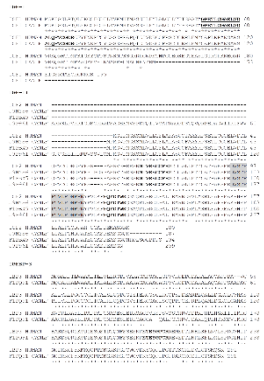

Figure 1. Homology study of IGF-I, IGF-II and IGFBP-3 dog proteins and their human analogues (* indicates the same amino acid). The shorter mature protein is underscored. The tryptic peptides in QPrEST™ or the heavy-labelled synthetic peptide matching peptides found in the canine sequence and used for quantification are market in bold. In grey, is marked a QPrEST™ tryptic peptide that could be theoretically used for big IGF-II quantification.

For IGF-I, while the sequences of the pre-processed form differ between both species (195 aa in humans vs 153 aa in dogs), the mature form of (70 aa) is identical. However, human QPrEST™ proteins that match this sequence are not available. Therefore, as was done in a previous study with cats,[38] a synthetic heavy isotope-labelled peptide (GPETLCGAELVDALQFVCGDR) was used for the quantification. For IGF-II, the mature form (67 aa in humans) differs in comparison with the three UniProt canine identifiers, as well as the E-peptide (or pre-processed) sequence (89 aa in humans). Nevertheless, some regions are conserved, and the same QPrEST™ used in our previous study was selected. This QPrEST™ protein contains five tryptic peptides that match well with the three canine IGF-II identifiers. Two of these peptides,

GIVEECCFR and SCDLALLETYCATPAK, match perfectly with a region of the mature IGF-II. However, when a shotgun analysis of the QPrEST™ standard was performed, a miss-cleavage in SCDLALLETYCATPAK peptide but not in GIVEECCFR peptide was observed; therefore the latter was selected to increase the reliability of the quantification. The other three peptides of the QPrEST™ protein match with unprocessed forms of IGF-II. Among them, GLPALLR peptide matches with the pro-IGF-II obtained after the cleavage of a 24 amino acid signal peptide (known as IGF-2[1-156]). However, this peptide was not found in the shotgun analysis of the QPrEST™ standard. Another peptide is DVSTPPTVLPDNFPR, which matches with one of the big IGF-II forms (known as big IGF-2[1-87]). In this case, a miss-cleavage of the peptide was observed in the shotgun analysis, and it was discarded for the quantification. The last peptide is FFQYDTWK, which matches with the big IGF-II form known as [1-104]. This peptide was found in the shotgun analysis without any miss-cleavage, therefore it was chosen to quantify the levels of big IGF-II. For IGFBP-3, the human and the reported canine sequences have the same length (291 aa) but they differ in 20% of the aa. In spite of these differences, the QPrEST™ contains a tryptic peptide that matches well with the F1PQ91 identifier, and it was selected for IGFBP-3 quantification.

Method evaluation

The parameters of the Q Exactive Plus Orbitrap MS were similar to those used in our previous study,[43] but we also included the analysis of a peptide that matches with the big IGF-II form [1-104]. The final settings for the method can be found in the material and methods section. The retention times of the selected peptides were confirmed using a shotgun analysis, and an inclusion list with the native and the heavy isotope-labelled peptides was included in the PRM-method (see **Table 1**). For IGF-I and IGF-II peptides, six MS/MS-fragments were used for quantification, and for big IGF-II and IGFBP-3, three MS/MS-fragments were used. After the selection of the PRM-method parameters, evaluation of the linearity, the repeatability and the LOD and LOQ was performed. Linear regression of the normalized

signals to the internal standard peptides over the entire investigated ranges resulted in determination coefficients of 0.9997 ($p<0.001$), 0.9978 ($p<0.001$) and 0.9983 ($p<0.001$) for IGF-I, IGFBP-3 and IGF-II (**Figure 2**, Raw data and calculations are given in **Supporting Table S1**). In the case of big IGF-II, linearity was observed in the range 0.058 - 1.4 fmol/µL, resulting in a determination coefficient of 0.9939 ($p<0.001$).

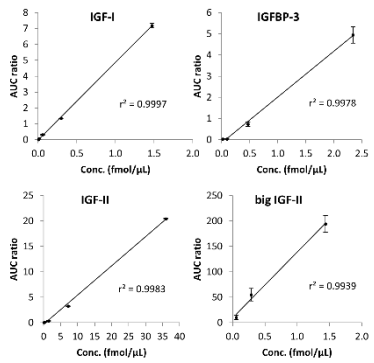

Figure 2. Calibration curves for IGF-I, IGFBP-3, IGF-II and big IGF-II dog proteins. The ratio synthetic/native peptide is plotted against the spiked concentration.

For the repeatability study, the retention times of the selected peptides, and the quantification based on the normalized AUC signals were used (**Tables 2 and 3**, Raw data and calculations are given in **Supporting Table S2**). The data indicate that the retention times of IGFBP-3, IGF-II, big IGF-II and IGF-I, were 14, 23, 29 and 40 minutes respectively, and the combination of all data shows a RSD lower than 5%. These results demonstrate the good repeatability of the method. In terms of quantification, the RSD values were good for IGF-I and acceptable for the other proteins. LOD and LOQ were calculated based on a standard statistical approach **(Supplementary Table S1)**. Due to few calibration points in the linear range in combination with very low concentrations of big IGF-II in samples from healthy dogs and in the dog with NICTH after surgery, the LOD of big-IGF-II was estimated based on the observation of well defined MS/MS-fragments and the standard deviation in the repeatability study (**Table 3**). In this sample there were at least two well defined MS/MS-fragments in all intraday measurements and at least one well

defined fragment in all interday measurements. In addition, we considered RSD on the interday measurements to be acceptable. The LOD was estimated to be 6.59 fmol/µL. The LOQ was not estimated for this protein.

Quantification of IGF-I, IGF-II, big IGF-II and IGFBP-3 in dogs

The concentrations of IGF-I, IGF-II, big IGF-II and IGFBP-3 were determined in serum from 7 healthy dogs (**Table 4**, Raw data and calculations are given in **Supporting Table S3**). Due to the low concentrations of big IGF-II in healthy dogs two healthy dogs were below LOD. All samples were analysed in two replicates using the developed PRM-method. The measurements gave RSD values similar as those observed in the repeatability study., As expected based on previous studies,[43,44] there was a significant correlation between weight and IGF-I ($r = 0.8526$, $p<0.05$) (**Figure 3**). There was no correlation between weight and IGF-II ($r = 0.1767$, $p=0.705$) and the trend to a correlation between weight and IGFBP-3 did not reach statistical significance ($r = 0.6617$, $p=0.105$). The IGF-II:IGF-I ratio was inversely correlated to weight ($r = -0.8313$, $p<0.05$).

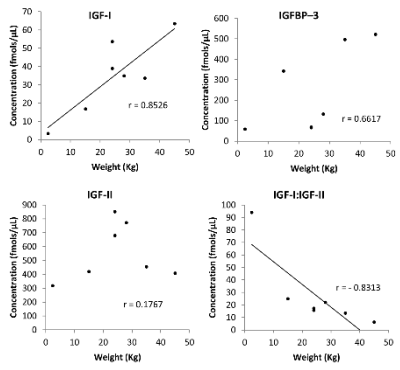

Figure 3. Regression curves obtained after plotting the quantified concentrations of IGF-I, IGF-II and IGFBP-3 proteins, and the IGF-II: IGF-I ratio against the weight of seven dog samples serum.

Method comparison

There was good agreement between IGF-I concentrations calculated using the PRM MS-based method and the ELISA method ($r = 0.9254$, $p<0.05$) (Raw data and calculations are given in **Supporting Table S4)**.

Canine case study of NICTH syndrome

A 9-year old male Labrador retriever, weighing 40 kg, was admitted to a large Veterinary Hospital in Stockholm, Sweden, with sudden ataxia and weakness. At presentation, the dog was unable to stand up and was treated as an in-house patient. Body temperature, heart and pulmonary auscultation were normal. No abnormal findings were detected on neurologic exam but postural reactions were difficult to assess due to difficulties of standing.

Serum and EDTA-blood were submitted to the laboratory for a basic panel. The results revealed profound hypoglycemia, low insulin concentrations and very mild increase in sodium (**Supporting Table S5**). The dog was treated with i.v. fluids containing glucose and despite this there was persistent hypoglycemia (2.1, 1.2, 1.4 and 3.4 mmol/L, reference interval 3.7-6.6) although the signs of weakness and ataxia disappeared with glucose infusion. The dog received one injection of prednisolone (1 mg/kg sc) which was followed by oral administration (1 mg/kg once daily) the next day. Ultrasound revealed an 11 x 15 cm mass in the spleen. There were no abnormal findings in the pancreas. No signs of metastasis were found on radiographs of lungs. The spleen was removed and sent for histopathology and the dog was sent home on prednisolone and frequent meals. Histopathology demonstrated a leiomyoma. At re-check one week after the tumor was removed, the dog was euglycemic (5.9 mmol/L) and had no abnormal clinical signs. Prednisolone was tapered and withdrawn. At recheck 3 months post-surgery, the dog was still euglycemic and insulin had increased to 200 ng/L which was within reference interval (40-380 ng/L). Based on hypoglycemia and low insulin concentration together with a splenic tumor, NICTH was suspected. In humans with NICTH, feedback inhibition by increased concentrations of big IGF-II will usually induce a pattern with low IGF-I and IGFBP-3. The concentrations of IGF proteins were determined in this dog. As shown in **Figure 4**, there was a significant increase of IGF-I (from 3.73 to 71.2 fmol/µL,

p<0.001) and IGFBP-3 (from 73.6 to 315 fmol/µL, p<0.001) between samples taken before and 3 months after tumor removal off any medications. Concentrations of big IGF-II decreased by 80% (from 35.4 to 6.59 fmol/µL, p<0.001) whereas concentrations of total IGF-II did not change significantly (p=0.85).

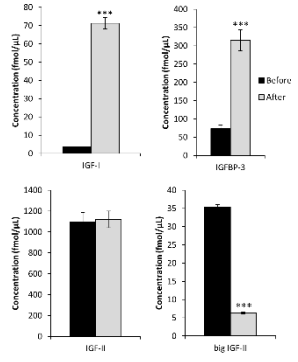

Figure 4. Mean concentration and standard error of the mean of IGF-I, IGFBP-3, IGF-II, big IGF-II proteins in a dog with NICTH syndrome, before and after the removal of the splenic tumor (*** indicates significant differences after a two-sample T-test, p-value < 0.001).

DISCUSSION

Measurement of IGF proteins in dogs using RIA, ELISA or WLB possess limitations which may be overcome using MS-based methodologies.[55] In our study, a previously developed PRM-method for cats has been optimized and applied to quantify four canine members of the IGF system (IGF-I, IGF-II, big IGF-II [1-104] and IGFBP-3) in healthy dogs, and in a dog with NICTH syndrome, a rare paraneoplastic phenomenon. To the best of our knowledge, this is the first time that simultaneous MS measurements of the IGF system proteins have been performed in serum from a dog with NICTH, and the first time that big IGF-II [1-104] has been measured by mass spectrometry in any species. The targeted amino acid sequences were identical in humans and dogs, and it is therefore possible that the method can also be applied in humans with NICTH syndrome.

The developed method allows the measurement of all proteins at the same time, decreasing the variation due to the methodology (IGFBPs are not interfering in the assay), and reducing the amount of serum needed for the analysis (less than 1 µL). In addition, the high sensitivity obtained by using a nanoLC coupled to a high-resolution Q Exactive Plus Orbitrap MS method reduced the tedious and time-consuming sample preparation steps needed to increase the sensitivity of other methods,[28,31,32] and more proteins can easily be added to the list of proteins to be quantified if suitable peptides for quantification are identified. Moreover, QPrEST™ synthetic proteins were used for the quantification of IGF-II, IGFBP-3 and big IGF-II, and they were added to the samples prior to the digestion step. As far as we know, we are the first group using QPrEST™ human proteins to quantify non-human proteins, previously in cats,[43] and now in dogs. QPrEST™ synthetic proteins are well characterized, they are cheap to produce and purchase, and they offer extensive human proteome coverage. However, the use of QPrEST™ has the limitation that, in general, fewer tryptic peptides can be selected for quantification. In the present study, several tryptic fragments were available for IGFBP-3, IGF-II, and big IGF-II quantification, but only one peptide for each protein met all the requirements needed for a reliable quantification. In addition, no QPrEST™ proteins were available to target the active sequence of IGF-I, therefore an isotope-labelled tryptic peptide was used. The peptide chosen for IGF-I quantification (GPETLCGAELVDALQFVCGDR) has been used in previous works to quantify IGF-I protein in human,[28,33,56] and cat samples[43] with successful results. This peptide contains a proline in its sequence, which might induce the “proline effect”, characterized by favouring the cleavage of the N-terminal to proline during MS/MS fragmentation. However, this effect is mainly observed when the collision-induced dissociation (CID) fragmentation mode is used and we used the high energy collision dissociation (HCD) fragmentation mode. HCD has no low-mass cutoff, higher resolution and, because it employs higher energy dissociations than CID, it enables a wider range of fragmentation pathways. In addition, the selected peptide contains glutamine, which is prone to deamidation. This modification was observed in

359 the preliminary shotgun analysis, but its abundance was very low compared to the unmodified form. In
360 the case of IGFBP-3, the selected peptide has been used to quantify the levels of IGFBP-3 protein in
361 cats,[43] and our data also demonstrate good linearity and acceptable repeatability based on the RSD
362 value. The high RSD value could be due to the small amount of this protein in the serum, but also
363 because of the chemical properties of the peptide used. This peptide is hydrophilic and it can be lost
364 differentially during the desalting process, or because of the instability of the electrospray at early time
365 points (it elutes early in the gradient, minute 14, and it has the highest RSD of all peptides used). Some
366 difficulties have previously been seen when analysing IGFBP-3 protein by mass spectrometry.[34] The
367 authors of that work suggested that the problems could be due to the formation of complexes between
368 large molecular weight proteins from plasma and IGFBP-3, and they developed a labour intensive sample
369 preparation protocol including precipitation and delipidation steps to concentrate the targeted proteins.
370 However, the authors still had accuracy problems for the quantification of IGFBP-3. In the present work,
371 we aimed to develop a simple and rapid method that require less than 1 µL of serum. The peptide
372 selected for the quantification of IGF-II has been previously used to quantify the levels of IGF-II protein in
373 humans,[34] and cats,[43] and our data also demonstrates good linearity and acceptable repeatability. Finally,
374 the peptide FFQYDTWK was selected to quantify the levels of big IGF-II form, and this peptide has never
375 been used either in humans or dogs.

376 In humans, increased concentration of bioavailable IGF-II can cause negative feedback on pituitary GH
377 secretion, leading to low IGF-I and IGFBP-3 concentrations.[1] Based on that, the recommendation is to
378 consider the diagnosis of NICTH in a patient if hypoglycemia, suppressed insulin, and low IGF-I
379 concentrations are found.[57] In dogs, at profound low glucose concentrations, insulin should be low or
380 undetectable[20] and this is an important difference when separating NICTH from insulinoma, where
381 hypoglycemia is accompanied by normal to high insulin concentrations. However, there are to date no
382 previous case reports of canine NICTH where both insulin and IGF-I have been measured. Therefore, the

383 application of the present method to measure the levels of IGFs proteins in a dog with NICTH syndrome
384 has revealed that IGF-I was decreased, which together with the low insulin levels, suggest the
385 recommendations made for human NICTH are also applicable in dogs.

386 Moreover, the ratio of IGF-II:IGF-I >10 has been suggested as an additional marker for diagnosis of NICTH
387 in humans.[57] In our study, the IGF-II:IGF-I ratio was inversely correlated to weight. This may be explained
388 by differences of the IGF-system. In dogs, we and others[22,48,49] have observed that IGF-I concentrations,
389 but not IGF-II, are strongly associated with body size. This finding will impact the IGF-II:IGF-I ratio. In our
390 study, the smallest dog (2.4 kg) had an IGF-II:IGF-I ratio of 94, whereas the largest dog (46 kg) had a ratio
391 of 6. The dog with NICTH syndrome weighed approximately 40 kg and the IGF-II:IGF-I ratio decreased
392 from 294 to 16 after removal of the tumor. Dogs were of different breeds, ages and gender but
393 unfortunately there were too few dogs to investigate possible confounding factors. Measurements from
394 a larger population of healthy dogs of different sizes are needed before any ratio between IGF-II and IGF-
395 I can be recommended as an additional marker for canine NICTH.

396 Furthermore, in human patients with NICTH, total IGF-II concentrations measured by immunoassays are
397 reported to be low, within RI or high.[1] NICTH in humans is often caused by precursor forms of IGF-II,
398 usually big IGF-II [1-87] or IGF-II [1-104], and for diagnosis with immunoassays, antibodies targeting
399 these big forms are needed. Big IGF-II represents at least 10% of total IGF-II in the healthy population,[58]
400 and this proportion is increased with NICTH.[59] As well as for IGF-II, there are no standard methods to
401 quantify big IGF-II [1-104] in dogs. Here we present a novel MS-based approach. In serum from the dog
402 with NICTH, the concentration of the protein before surgery was determined with high confidence and
403 an RSD value of ≈ 5%) (**Table 3**). Values decreased considerably after tumor removal and was similar to
404 healthy dogs. LOD was calculated based on the sample from the dog with NICTH after surgery and was
405 6.59 fmol/µl. This assumption restricts the calculations of the absolute concentrations in some healthy

406 dogs. The low concentrations in healthy dogs and the relatively high RSD seen at low concentrations of
407 big IGF-II and IGFBP-3 indicates that one should be cautious about interpreting small canges of these
408 proteins with this method. Based on biological variation of IGF-I in dog, RSD up to 10.5% is considered
409 desirable and up to 15.7% is considered acceptable.[60,61] All the meausurements of IGF-I in the present
410 study demonstrated an RSD of <12.7%. To the best of our knowledge, there are no expert opinions or
411 data on biological variation to help determine a clinically acceptable RSD for big IGF-II, IGFBP-3 and total
412 IGF-II in dogs. Further studies where these proteins are measured in healthy dogs as well as in diseased
413 dogs are needed to evaluate the clinical usefulness of this method. Before surgery, big IGF-II [1-104] was
414 only 3.23% of total IGF-II, which is lower than previous reports in human serum. It is possible that other
415 precursor forms of IGF-II (e.g. big IGF-II [1-87]) are present, and this could explain why big IGF-II [1-104]
416 was such a small percentage of the total IGF-II. Another interesting aspect is that the sequence used for
417 quantifying big IGF-II [1-104] is located in the E-peptide region and the cleaved E-peptide can circulate in
418 serum as well as being a part of big IGF-II.[59] In the study by Daughaday and Trivedi, immunoreactivity
419 against the cleaved IGF-II E-peptide was similar in serum from healthy subjects compared to a NICTH
420 patient.[59] The cleaved E-peptide has been named preptin and found to be co-secreted with insulin by
421 pancreatic beta-cells.[62] There is scarce information about preptin but, as reviewed by Aydin in humans
422 and rats, it seems to be mainly secreted from pancreas and is positively associated with insulin
423 concentrations.[63] In the dog with NICTH syndrome, undetectable insulin concentrations, and high big
424 IGF-II [1-104] concentrations were found before surgery, while insulin increased and big IGF-II [1-104]
425 decreased after surgery. If dogs show the same patterns as humans and rats, most of the measured
426 sequence is likely to represent big IGF-II. However, further studies are needed to evaluate preptin and its
427 role in the IGF-system.

428

CONCLUSION

NICTH is a rare paraneoplastic syndrome and should be suspected in dogs with persistent hypoglycemia, in the presence of low insulin and low IGF-I. High precursor forms of IGF-II would further support diagnosis. We have developed a PRM MS-based method that allows the simultaneous quantification of IGF-I, total IGF-II, IGFBP-3 and, for the first time, the quantification of big IGF-II protein in dogs. This method overcomes the need for antibody availability or potential antigen cross-reactions that are problems in enzymatic or radioimmunoassays, as well as the tedious and time consuming sample preparation. It is also adaptable and scalable to detect other biomarkers of interest if suitable peptides for quantification are identified, and it requires less than 1 µL of serum, making this method potentially useful for clinical laboratories. Moreover, we have used the heavy-labelled synthetic QPrEST™ proteins designed for humans, which enable the correction for possible incomplete proteolysis that could affect the selected peptide and cause variability in the protein quantification. The method was demonstrated to possess good linearity and repeatability, and has been successfully applied to a dog with NICTH syndrome, with results in accordance with previous human studies found in the literature. These measurements may aid in diagnosing NICTH, however further studies with more dogs are needed to evaluate reference ranges and diagnostic performance of this method.

Acknowledgments

The Agria and Swedish Kennel Club Research Foundation, Linnea and Axel Ericsons fund and the Michael Forsgren Foundation are acknowledged for financial support.

Conflict of interest

The authors declare no competing financial interest.

Figure legends

Figure 1. Homology study of IGF-I, IGF-II and IGFBP-3 dog proteins and their human analogues (* indicates the same amino acid). The shorter mature protein is underscored. The tryptic peptides in QPrEST™ or the heavy-labelled synthetic peptide matching peptides found in the canine sequence and used for quantification are market in bold. In grey, is marked a QPrEST™ tryptic peptide that could be theoretically used for big IGF-II quantification.

Figure 2. Calibration curves for IGF-I, IGFBP-3, IGF-II and big IGF-II dog proteins. The ratio synthetic/native peptide is plotted against the spiked concentration.

Figure 3. Regression curves obtained after plotting the quantified concentrations of IGF-I, IGF-II and IGFBP-3 proteins, and the IGF-II:IGF-I ratio against the weight of seven dog serum samples.

Figure 4. Mean concentration and standard error of the mean of IGF-I, IGFBP-3, IGF-II, big IGF-II proteins in a dog with NICTH syndrome, before and after the removal of the splenic tumor (*** indicates significant differences after a two-sample T-test, p-value < 0.001).

Tables

Table 1. Targeted peptides included in the PRM method.

Protein	[m/z] (Th)	Charge	Sequence	Position	RT (min)	Fragments
IGF-I	769.6963	3+	GPETLCGAELVDALQFVCGDR (light)	1-21	40.3	y_4, y_5, y_6, y_7, y_{10},b_9
	773.0324	3+	GPETLCGAELVDALQFVCGDR (heavy)	1-21	40.3	
IGF-II	585.2575	2+	GIVEECCFR (light)	41-49	22.5	y_3, y_4, y_5,y_6, y_7, b_3
	590.2617	2+	GIVEECCFR (heavy)	41-49	22.5	
big IGF-II	567.7664	2+	FFQYDTWK (light)	90-98	29.4	y_5,y_6, y_7,
	571.7735	2+	FFQYDTWK (heavy)	90-98	29.4	
IGFBP-3	506.2136	2+	ETEYGPCR (light)	207-214	14.2	y_2, y_4, y_5
	511.2178	2+	ETEYGPCR (heavy)	207-214	14.2	

C-terminal arginine (R) and Lysine (K) of heavy peptides were labelled with (^{15}N , ^{13}C). All cysteines are carbamidomethylated (underscored).

Table 2. Intraday repeatability data of the peptides in dog's serum after surgery. Three serum samples (technical replicates) were prepared independently and each sample was analysed in triplicate (n=9).

	Retention time		Quantification		
	Mean (min)	RSD (%)	Mean (fmol/µL)	SEM (fmol/µL)	RSD (%)
IGF-I	41.0	0.33	79.1	1.08	4.11
IGF-II	23.1	1.7	977	68.3	19.8
big IGF-II	30.1	1.1	5.49	0.555	26.7
IGFBP-3	14.6	2.8	312	34.9	33.6

Table 3. Interday repeatability data of IGF-peptides in a case of canine NICTH before and after surgery. The same sample was prepared in three different and non-consecutive days, and they were analysed in triplicate (n=9).

	Before surgery, n=9					After surgery, n=9				
	Retention time		Quantification			Retention time		Quantification		
	Mean (min)	RSD (%)	Mean (fmol/µL)	SEM (fmol/µL)	RSD (%)	Mean (min)	RSD (%)	Mean (fmol/µL)	SEM (fmol/µL)	RSD (%)
IGF-I	40.2	1.6	3.73	0.106	8.08	39.8	2.4	71.2	3.01	12.7
IGF-II	22.4	3.5	1100	88.7	24.3	22.0	4.2	1120	79.8	21.4
big IGF-II	29.3	3.0	35.4	0.651	5.20	28.7	4.2	6.59	0.313	14.3
IGFBP-3	14.2	4.2	73.6	8.69	33.4	13.7	4.9	316	28.6	25.7

Table 4. Weight and quantified levels of IGF proteins in 7 different dogs.

	Weight	IGF–I		IGF–II		IGFBP–3		big IGF–II		IGF–II:IGF–I ratio	
	(Kg)	Mean (fmol/µL)	RSD (%)	Mean (fmol/µL)	RSD (%)	Mean (fmol/µL)	RSD (%)	Mean (fmol/µL)	RSD (%)	Mean	RSD (%)
Dog 1	24	39.0	3,70	681	4,92	66,2	28.0	9,68	29,3	17,5	8,61
Dog 2	24	53.7	0,603	852	8,94	69,5	12,4	13,3	3,95	15,9	8,34
Dog 3	2.4	3.39	6,19	319	12.0	59,3	51,2	<6.59[a]	-	94,6	18,2
Dog 4	28	35.0	0,456	773	11,7	133	27.0	11,4	27,2	22,1	11,3
Dog 5	45	63,6	4,85	410	4,11	522	9,75	15	3,99	6,44	0,738
Dog 6	35	33,7	1,80	455	1,21	498	10,9	9,48	1,24	13,5	0,587
Dog 7	15	16,8	0,343	420	3,81	343	1,22	<6.59[a]	-	25.0	3,47

[a] Concentration below the estimated LOD.

Supporting Information

Supporting Table S1. Raw data and calculations for the construction of the linear regression.

Supporting Table S2. Raw data and calculations for intraday and interday repeatability study.

Supporting Table S3. Raw data and calculations for the quantification of IGF proteins in 7 different dogs.

Supporting Table S3. Raw data and calculations for the correlation between IGF-I concentration calculated using the PRM MS-based method and the ELISA method.

Supporting Table S5. Biochemistry and hematological results at presentation.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009277.

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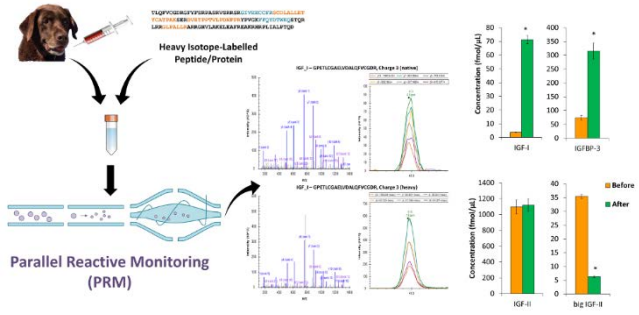

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