NOTE *Biochemistry*

Molecular Characterization and Tissue Distribution of Feline Retinol-Binding Protein 4

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ABSTRACT. Retinol-binding protein 4 (RBP4) is a specific transporter of retinol and was recently identified as an adipokine potentially involved in type 2 diabetes in humans and rodents. However, the function and structure of feline RBP4 have not been reported. In this study, we describe the molecular cloning and expression analysis of feline RBP4. The complete feline *RBP4* cDNA encodes a precursor protein comprising an 18 amino acid signal peptide and a 183 amino acid mature protein. Feline *RBP4* was mapped to chromosome D2. Mature feline RBP4 is 83–94% homologous to the RBPs of humans, cows and rodents. RT-PCR analysis revealed feline RBP4 expression in liver and adipose tissues.

KEY WORDS: adipose tissue, diabetes mellitus, feline, liver, retinol-binding protein 4.

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Retinol-binding protein 4 (RBP4), which is mainly produced by hepatocytes and adipocytes, is a serum protein that transports retinol from the liver to peripheral tissues [2]. In 2005, RBP4 was reclassified as an adipokine that was potentially involved in the pathogenesis of insulin resistance. Yang *et al*. reported that RBP4 has a crucial role for onset of type 2 diabetes mellitus (T2D) in the adipocyte specific GLUT4−/− mice [25]. These mice developed insulin resistance in muscle and liver. Furthermore, serum RBP4 concentrations were significantly higher in another mouse model on obesity and insulin resistance. These data suggested that RBP4 is an adipokine associated with obesity and insulin resistance in animal models [25], and the increased serum RBP4 concentrations could be a suitable biomarker for insulin resistance or adiposity. In humans, several groups showed that elevated serum RBP4 concentrations are correlated with the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance and T2D [3, 21, 26]; however, other studies have not confirmed these results [5, 11, 12, 16]. Thus, the role of RBP4 in the pathogenesis of insulin resistance in humans remains controversial.

Feline diabetes mellitus closely resembles human T2D, including similar clinical characteristics and pathological abnormalities [6, 9]. Important risk factors for the development of feline diabetes are age, gender, neuter status and obesity [14, 15, 18]. Obesity is most likely responsible for the development of T2D in overweight cats [7, 20], and obesity in domesticated cats has recently become much more common [1, 4, 13]. However, the role of RBP4 in the development of feline T2D and insulin resistance in obesity remains unclear. In addition, the complete nucleotide and amino acid sequences of feline RBP4 have not yet been reported.

Understanding the pathophysiological role of feline RBP4 requires the identification and characterization of RBP4. In this study, we cloned feline *RBP4* to determine its sequence and structure and subsequently determined the tissue distribution of *RBP4* mRNA.

To clone full-length feline *RBP4* cDNA, the RT-PCR and rapid amplification of cDNA ends (RACE) methods were used. First, one pair of PCR primers, RBP4F (5'-CTT CCG AGT CAA GGA GAA CTT CGA-3') and RBP4R (5'-GGG AAA ACA CGA AGG AGT AGC TGT C-3'), were designed on the basis of a region that was highly conserved among several species, including human RBP4 (X00129), murine (NM_011255) and rat (XM_215285). Partial cDNA fragments encoding RBP4 were amplified using liver cDNA as a template. The resultant PCR products were subcloned and sequenced using BigDye Terminator v3.1 chemistry and an ABI PRISM 310 DNA Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). After identifying and confirming the partial sequence encoding feline RBP4, 5'- and 3'-RACE were performed using GeneRacer Kit (Invitrogen, Carlsbad, CA, U.S.A.) to obtain full-length cDNA sequences. RACE-PCR was performed between GeneRacer primers and the RBP4F or RBP4R primer according to the manufacturer's instructions. Resultant PCR fragments were subcloned and sequenced as described above. The assembled full-length feline RBP4 nucleotide sequence was deposited in the DNA Data Bank of Japan (DDBJ) with the accession number AB771450.

The cloned feline *RBP4* cDNA was 942 base pair (bp), including a 79 bp 5'-untranslated region (UTR) and 257 bp

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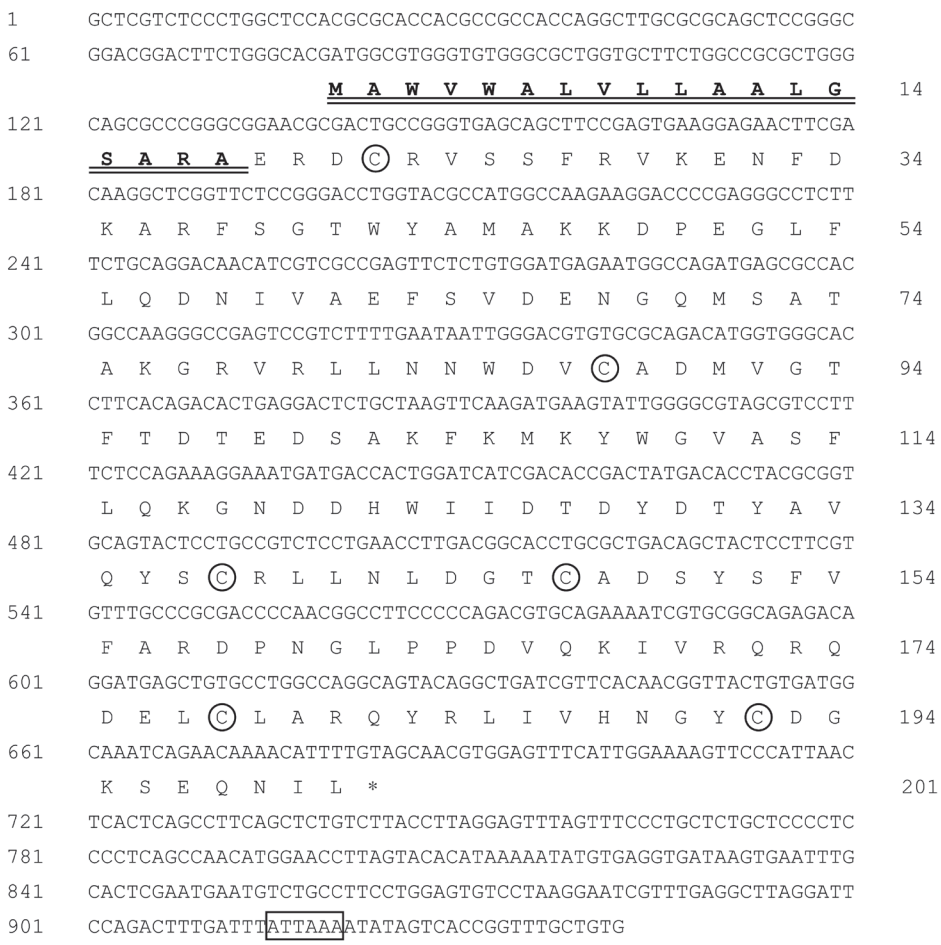

Fig. 1. Nucleotide and encoded amino acid sequences of feline RBP4. The nucleotide sequence of the 942 bp *RBP4* cDNA is shown on the top line, and its predicted amino acid sequence is shown below using one-letter amino acid code. Numbers on the left and right refer to the nucleotide and amino acid residue, respectively. The cDNA encodes a precursor protein of 201 amino acids. The asterisk indicates the translation termination codon. The polyadenylation signal (ATTAAA) is boxed. The signal peptide sequence is double-underlined. Six cysteine residues which are required for the formation of three disulfide bonds are indicated by a circle.

3'-UTR. A polyadenylation signal, ATTAAA, was located 28 bp upstream of the poly (dA) tail (Fig. 1). The 606 bp putative open reading frame encoded a 201 amino acid polypeptide. A search for the signal peptide sequence was conducted in feline RBP4 using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). As shown in Fig. 1, the feline RBP4 precursor polypeptide has a putative 18 amino acid signal peptide at the NH_2- terminal, suggesting that mature RBP4 contains 183 amino acids beginning with a glutamine residue and has an approximately 21.0 kD molecular mass similar to the size of plasma RBP4 detected by immunoblotting, in humans [10] and cats [17, 23]. Six cysteine residues are known to be required for the formation of three disulfide bonds in human RBP4 [19], and all of these residues are conserved in feline RBP4 (Fig. 1). The secondary protein structure was subsequently predicted by homology modeling using the SWISS-MODEL server (http://swissmodel.expasy.org/) and represented as eight-stranded, antiparallel β-sheet accompanied by a short α-helix formed by a region close to the COOH- terminal. A three-dimensional model of feline RBP4 structure was created on the basis of a known structure of the bovine holo-RBP4 bound to retinol (PDB code: 1HBP), exhibiting a typical beta-barrel structure (data not shown). These results indicate that our cloned *RBP4* cDNA encodes a feline RBP4 protein and its structure similar to RBP4 proteins from other mammals.

To determine the distribution of *RBP4* mRNA in various feline tissues, semi-quantitative RT-PCR was performed. DNase-treated total RNAs (Zyagen, San Diego, CA, U.S.A.) from heart, lung, liver, pancreas, stomach, ileum, colon, kidney, skeletal muscle and adipose tissue were reverse transcribed. The cDNA samples were amplified by PCR using the RBP4-specific primer pair, RBP4F and RBP4R, which generated a 392 bp fragment. The glyceraldehyde-

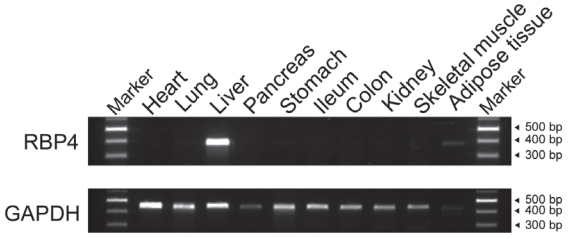

Fig. 2. Tissue distribution of *RBP4* mRNAs in feline tissues. Semi-quantitative RT-PCR was performed for feline *RBP4* mRNA (upper panel) and *GAPDH* mRNA, which was used as an internal control (lower panel). The size of the PCR product was 392 bp for *RBP4* and 453 bp for *GAPDH*. Representative RT-PCR results are shown.

3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control, and a 453 bp fragment was amplified using the primer pair GAPDHU (5'-ACC ACA GTC CAT GCC ATC AC-3') and GAPDHL (5'-TCC ACC ACC CTG TGG CTG TA-3'). PCR was performed using KAPA Taq DNA polymerase (Kapa Biosystems, Woburn, MA, U.S.A.) following the manufacturer's instruction. The amplification products were analyzed on 2.0% (w/v) agarose gels stained with GR Red (Bio Craft, Tokyo, Japan).

As shown in Fig. 2, *RBP4* transcripts were detected in the liver and adipose tissue. Expression in the liver was higher than that in the adipose tissue (Fig. 2). This result is consistent with a previous report that the liver is the major site of *RBP4* mRNA expression, whereas adipose tissue expressed only 32% *RBP4* mRNA compared to liver in mice [8]. In addition, mouse kidney is reported to express *RBP4* mRNA at approximately 2% of the amount expressed in liver [8]; however, we could not detect any *RBP4* mRNA expression in the feline kidney. Thus, liver and adipose tissue could play a crucial role in feline retinol metabolism.

A DNA database search revealed that a predicted feline *RBP4* gene sequence has already been deposited in GenBank (XM_003994245), which was predicted by computational analysis of the feline genome sequence (assembly name: Felis_catus-6.2). This predicted *RBP4* gene contains 963 bp and lacks a 5'-UTR and the ATG start codon. The cloned and predicted RBP4 nucleotide sequences differ only at the 5'-region (Fig. 3A); the first 280 bp of our cloned sequence has only 52% homology with the first 312 bp of predicted RBP4, whereas the distal 662 bp is highly homologous (99%) between both sequences. Although the amino acid sequence of RBP4 has been reported to be highly conserved among mammals [2, 8], the NH_2-terminal region of this predicted RBP4 sequence is longer and different from RBP4 sequences of other mammals (data not shown). The homology of the NH_2- terminal region is low (16.4%) between cloned RBP4 (67 amino acids) and the predicted RBP4 (104 amino acids) proteins. However, the 134 amino acid residues at the distal COOH-terminal of both sequences are completely identical. Because they include a putative signal peptide, it is possible to be expressed as functional secretory proteins from both the cloned cDNA and computationally predicted genes. It is not yet clear whether this difference is reflected by true coding sequence and/or different splice variants and by a feline-specific difference.

To determine which transcript was expressed in feline tissues, we conducted RT-PCR analysis using primers specific for each transcript. The reverse primer, RBP4R2 (5'-GCA CAC GTC CCA ATT ATT CA-3'), was designed to target a sequence common to both predicted and cloned *RBP4* cDNA. Using the primers designed on the basis of the cloned *RBP4* cDNA, RBP4F2 (5'-CCG AGT AAG GAG AAC TTC G-3') and the common primer, RBP4R2, we obtained a 184-bp PCR product in both liver and adipose tissue (Fig. 3B). However, no PCR product was found in RT (−) samples. When we used primers designed to target the predicted *RBP4* gene, RBP4F3 (5'-CTC ACA AGA TCC CCC AAA GA-3') and the common primer, RBP4R2, we obtained no PCR product in both liver and adipose tissue. We concluded that it is likely that only the *RBP4* mRNA corresponding to our cDNA clone is expressed in feline liver and adipose tissue.

We could amplify the 5'-region of our cloned cDNA by RT-PCR of the liver and adipose tissue, and this region was highly homologous (89%) to the 5'-region of bovine *RBP4* gene (NM_001040475). Furthermore, Thatcher *et al*. reported that the NH_2-terminal of feline RBP had an ERDCRVSSFRVKENFDKARFSGTXYAMA sequence as determined by Edman degradation analysis [22]. Our cloned cDNA encodes the same polypeptide immediately following the putative signal peptide; however, the computationally predicted gene has no such sequence. In conclusion, our sequenced clone can be a true cDNA for feline RBP4 gene.

According to genomic data, the predicted feline *RBP4* gene is located on chromosome D2. To determine the gene loci of our cloned *RBP4* sequence, we mapped the cDNA against the feline genome (Felis_catus-6.2) using the BLAT program (http://genome.ucsc.edu/cgi-bin/hgBlat). We were successful in mapping most of our cloned sequence to feline chromosome D2 (NW_004065082). However, the 5'-end of our cloned sequence (280 bp) could not be mapped to chromosome D2, because the corresponding genomic region maps to an undefined gap (875 bp) in the genome sequence (data not shown). In the future, if a more precise feline genomic sequence is obtained, we can accurately map the 5'-region of our clone to the feline genome.

Subsequently, we compared the amino acid sequences of mature feline RBP4 with those of several mammalian species, including human (NP_006735), murine (NP_035385), rat (NP_037294) and bovine (NP_001035565). In this study, protein sequence alignment revealed that mature RBP4 is highly conserved among these species and that feline RBP4 shares sequence homology with human (94%), bovine (93%), rat (83%) and mouse (83%). These results suggested that feline RBP4 is also highly conserved and could serve functions similar to RBP4 in other mammals.

In this study, we have reported for the first time the identification and complete sequence of feline RBP4, as well as its tissue distribution. The results from this study could provide important information regarding a new molecule that can be

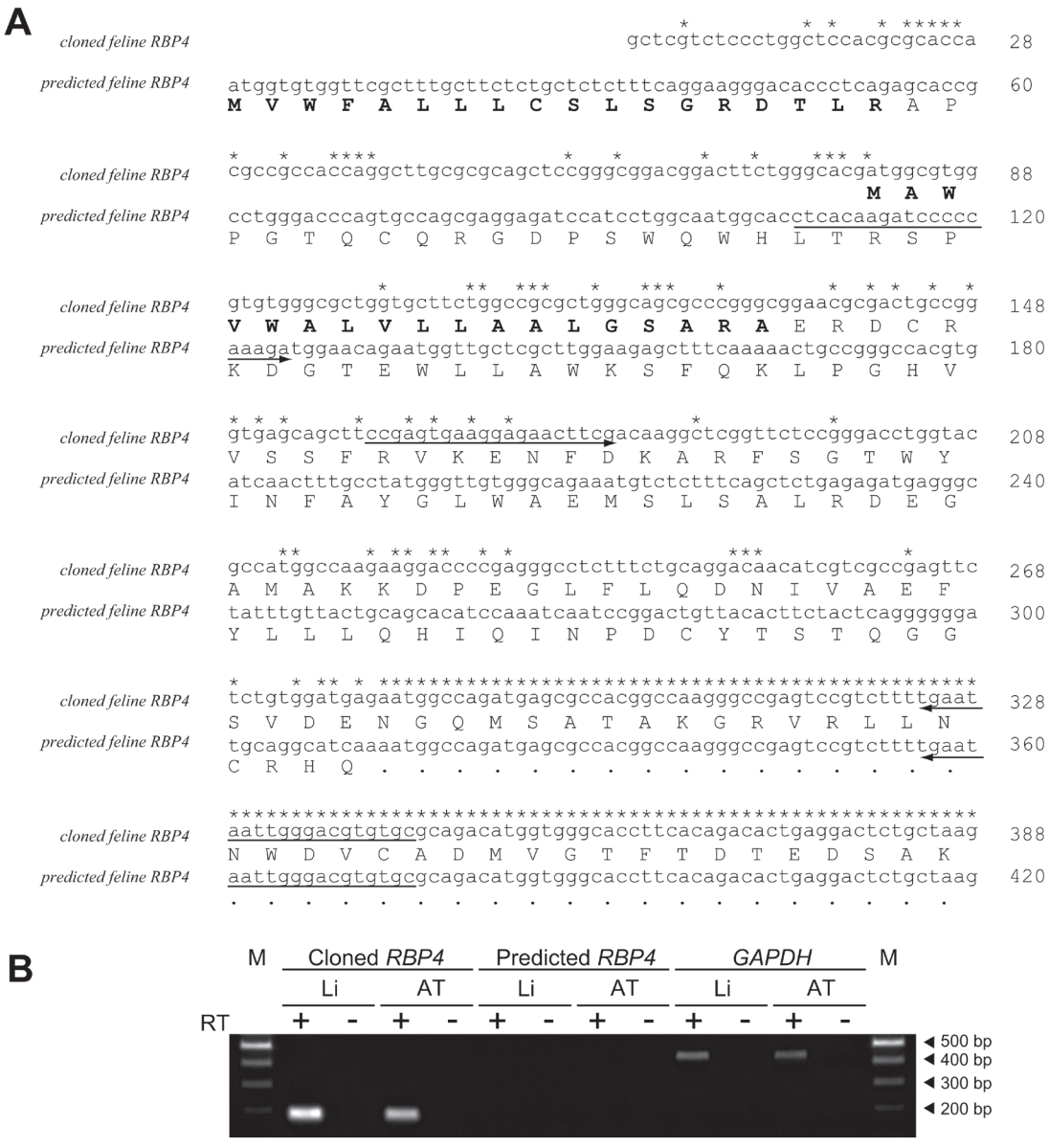

Fig. 3. (A): Alignment of the nucleotide and amino acid sequences of cloned feline RBP4 and the computationally predicted RBP4 (XM_003994245). An asterisk indicates an identical nucleotide. Identical amino acid residues across both sequences are indicated as dots (•). The predicted signal peptide sequences are in bold. The primer sequences and directions are indicated as arrows. (B): RT-PCR analysis of the *RBP4* gene transcript in liver and adipose tissue. RT-PCR was performed using transcript-specific primers. The expected size of PCR products was 184 bp for cloned RBP4 and 270 bp for predicted RBP4. *GAPDH* mRNA was used as an internal control. RT, reverse transcription; Li, liver; AT, adipose tissue; M, DNA size marker.

used for the diagnosis of feline insulin resistance and T2D. In addition, a recent study revealed that urine RBP, which reflects renal damage, is also considered as a renal marker in cats [23]. To the best of our knowledge, feline RBP4 specific antibody or detection kit is not commercially available. Raila *et al*. [17] and Hoek *et al*. [23, 24] reported that commercial human RBP4 immunodetection kit and antibody can be used for detecting feline RBP4 in serum. However, Kahn's group pointed out that there is the limitation of dynamic range in several commercial ELISA kits in insulin-resistant human cases [26]. The reliability of the human ELISA kit to apply feline T2D samples is uncertain. Therefore, we are planning to develop feline RBP4 specific immunodetection assay; a recombinant feline RBP4 will be expressed in a bacterial cell and used as an antigen to generate antiserum against feline RBP4.Further studies will be needed to assess the serum

feline RBP4 concentrations in health and when insulin resistance and related conditions occur.

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REFERENCES

1. Baral, R. M., Rand, J. S., Catt, M. J. and Farrow, H. 2003. Prevalence of feline diabetes mellitus in a feline private practice. *J. Vet. Intern. Med.* **17**: 433.
2. Blaner, W. S. 1989. Retinol-binding protein: the serum transport protein for vitamin A. *Endocr. Rev.* **10**: 308–316. [Medline] [CrossRef]
3. Cho, Y. M., Youn, B. S., Lee, H., Lee, N., Min, S. S., Kwak, S. H., Lee, H. K. and Park, K. S. 2006. Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and Type 2 diabetes. *Diabetes Care* **29**: 2457–2461. [Medline] [CrossRef]
4. German, A. J. 2006. The growing problem of obesity in dogs and cats. *J. Nutr.* **136**: 1940S–1946S. [Medline]
5. Henze, A., Frey, S. K., Raila, J., Tepel, M., Scholze, A., Pfeiffer, A. F., Weickert, M. O., Spranger, J. and Schweigert, F. J. 2008. Evidence that kidney function but not type 2 diabetes determines retinol-binding protein 4 serum levels. *Diabetes* **57**: 3323–3326. [Medline] [CrossRef]
6. Hoenig, M. 2006. The cat as a model for human nutrition and disease. *Curr. Opin. Clin. Nutr. Metab. Care* **9**: 584–588. [Medline] [CrossRef]
7. Hoenig, M., Thomaseth, K., Waldron, M. and Ferguson, D. C. 2007. Insulin sensitivity, fat distribution and adipocytokine response to different diets in lean, and obese cats before and after weight loss. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**: R227–R234. [Medline] [CrossRef]
8. Jessen, K. A. and Satre, M. A. 2000. Mouse retinol binding protein gene: cloning, expression and regulation by retinoic acid. *Mol. Cell. Biochem.* **211**: 85–94. [Medline] [CrossRef]
9. Johnson, K. H., Hayden, D. W., O'Brien, T. D. and Westermark, P. 1986. Spontaneous diabetes mellitus-islet amyloid complex in adult cats. *Am. J. Pathol.* **125**: 416–419. [Medline]
10. Kanai, M., Raz, A. and Goodman, D. S. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47**: 2025–2044. [Medline] [CrossRef]
11. Khovidhunkit, W., Pruksakorn, P., Plengpanich, W. and Tharavanij, T. 2012. Retinol-binding protein 4 is not associated with insulin resistance in pregnancy. *Metabolism* **61**: 65–69. [Medline] [CrossRef]
12. Lewis, J. G., Shand, B. I., Frampton, C. M., Elder, P. A. and Scott, R. S. 2008. Plasma retinol-binding protein is not a marker of insulin resistance in overweight subjects: a three year longitudinal study. *Clin. Biochem* **41**: 1034–1038. [Medline] [CrossRef]
13. Lund, E. M., Armstrong, P. J., Kirk, C. A. and Klausner, J. S. 2005. Prevalence and risk factors for obesity in adult cats from private US veterinary practices. *Intern. J. Appl. Res. Vet. Med.* **3**: 88–96.
14. McCann, T. M., Simpson, K. E., Shaw, D. J., Butt, J. A. and Gunn-Moore, D. A. 2007. Feline diabetes mellitus in the UK: the prevalence within an insured cat population and a questionnaire-based putative risk factor analysis. *J. Feline Med. Surg.* **9**: 289–299. [Medline] [CrossRef]
15. Nelson, R. W., Himsel, C. A., Feldman, E. C. and Bottoms, G. D. 1990. Glucose tolerance and insulin response in normal weight and obese cats. *Am. J. Vet. Res.* **51**: 1357–1362. [Medline]
16. Promintzer, M., Krebs, M., Todoric, J., Luger, A., Bischof, M. G., Nowotny, P., Wagner, O., Esterbauer, H. and Anderwald, C. 2007. Insulin resistance is unrelated to circulating retinol binding protein and protein C inhibitor. *J. Clin. Endocrinol. Metab.* **92**: 4306–4312. [Medline] [CrossRef]
17. Raila, J., Mathews, U. and Schweigert, F. J. 2001. Plasma transport and tissue distribution of β-carotene, vitamin A and retinol-binding protein in domestic cats. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* **130**: 849–856. [Medline] [CrossRef]
18. Rand, J. S. and Marshall, R. D. 2005. Diabetes mellitus in cats. *Vet. Clin. North. Am. Small. Anim. Pract.* **35**: 211–224. [Medline] [CrossRef]
19. Rask, L., Anundi, H., Fohlman, J. and Peterson, P. A. 1987. The complete amino acid sequence of human serum retinol-binding protein. *Ups. J. Med. Sci.* **92**: 115–146. [Medline] [CrossRef]
20. Scarlett, J. M. and Donoghue, S. 1998. Associations between body condition and disease in cats. *J. Am. Vet. Med. Assoc.* **212**: 1725–1731. [Medline]
21. Stefan, N., Hennige, A. M., Staiger, H., Machann, J., Schick, F., Schleicher, E., Fritsche, A. and Haring, H. U. 2007. High circulating retinol-binding protein 4 is associated with elevated liver fat but not with total, subcutaneous, visceral, or intramyocellular fat in humans. *Diabetes Care* **30**: 1173–1178. [Medline] [CrossRef]
22. Thatcher, M. D., Shille, V. M., Fliss, M. F., Bazer, F. W., Sisum, W. and Randal, S. 1991. Characterization of feline conceptus proteins during pregnancy. *Biol. Reprod.* **44**: 108–120. [Medline] [CrossRef]
23. van Hoek, I., Daminet, S., Notebaert, S., Janssens, I. and Meyer, E. 2008. Immunoassay of urinary retinol binding protein as a putative renal marker in cats. *J. Immunol. Methods* **329**: 208–213. [Medline] [CrossRef]
24. van Hoek, I., Meyer, E., Duchateau, L., Peremans, K., Smets, P. and Daminet, S. 2009. Retinol-binding protein in serum and urine of hyperthyroid cats before and after treatment with radioiodine. *J. Vet. Intern. Med.* **23**: 1031–1037. [Medline] [CrossRef]
25. Yang, Q., Graham, T. E., Mody, N., Preitner, F., Odile, D., Peroni, O. D., Zabolotny, J. M., Kotani, K., Quadro, L. and Kahn, B. B. 2005. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* **436**: 356–362. [Medline] [CrossRef]
26. Yang, Q., Eskurza, I., Kiernan, U. A., Phillips, D. A., Blüher, M., Graham, T. E. and Kahn, B. B. 2012. Quantitative measurement of full-length and C-terminal proteolyzed RBP4 in serum of normal and insulin-resistant humans using a novel mass spectrometry immunoassay. *Endocrinology* **153**: 1519–1527. [Medline] [CrossRef]