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Validity and application of immunoturbidimetric and enzyme-linked immunosorbent assays for the measurement of adiponectin concentration in ponies

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Summary

Background: Circulating adiponectin concentrations were lower in ponies with a history of endocrinopathic laminitis and in non-laminitic ponies that subsequently developed laminitis. The assays used in these studies have been discontinued or are no longer valid.

Objectives: i) To determine the validity of immunoturbidimetric (IT) and enzyme linkedimmunosorbent (ELISA) assays for equine total and high molecular weight (HMW) [adiponectin] measurement and ii) to investigate the association between [adiponectin] measured using these assays and endocrinopathic laminitis.

Study design: Method validation and cohort study.

Methods: Accuracy and precision of IT and ELISA assays for measuring total (TAC) and HMW (HMWAC) [adiponectin] were determined. Using the IT assay, the effects of anti-coagulant and storage temperature were assessed, TAC was measured in previously laminitic (PL) and never laminitic (NL) ponies (n = 6/group). Comparison with a previously validated radioimmunoassay was made in NL ponies (n = 223). Association between TAC and subsequent laminitis development in NL ponies was investigated using univariable logistic regression and ROC curve analysis.

Results: The IT assay was precise and demonstrated good agreement with the previously validated radioimmunoassay. TAC was significantly ($p<0.01$) lower in PL (mean ± s.d. 8.9 ± 2.9 μg/ml) compared to NL (24.2 ± 11.8 μg/ml) ponies and in NL ponies that developed laminitis within 12

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months (median 4.8 μg/ml; IQR 2.65-13.4 μg/ml) compared to those that remained non-laminitic (19.9 μg/ml; 9.95-31.5 μg/ml). TAC was significantly (p = 0.01) associated with laminitis occurrence within 12 months. Use of the area under the ROC curve to distinguish animals that did and did not develop laminitis showed good accuracy (0.76). None of the ELISA methods validated satisfactorily.

Main limitations: Laminitis risk is based on data from ponies in one region.

Conclusions: The IT method is suitable for measurement of equine TAC. TAC is lower in ponies with previous or future laminitis. The ELISA methods are not suitable for measurement of equine HMWAC or TAC.

Introduction

Equine metabolic syndrome (EMS) is a collection of clinical abnormalities including insulin dysregulation (ID), dyslipidaemia and altered circulating adipokine concentrations, with or without obesity, associated with increased laminitis risk [1]. Whilst there are multiple circulating adipokines, leptin and adiponectin have been evaluated in horses in relation to the pathogenesis of, and as diagnostic markers for, EMS. Whilst leptin concentrations were strongly associated with obesity, they were not useful for identifying previously laminitic animals [2]. Contrastingly, concentrations of the anti-inflammatory [3] and insulin-sensitising adipokine adiponectin [4] decreased significantly when horses were fed a carbohydrate-rich diet sufficient to induce obesity and ID, but not when fed a high fat diet that induced obesity alone [5]. Additionally, circulating adiponectin concentrations were lower in previously laminitic compared to never laminitic ponies [2,6]. Finally, in non-laminitic

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ponies, hypoadiponectinaemia was an independent predictor of future laminitis development in a multivariable model of laminitis risk [7].

Investigation of equine adiponectin production has been limited. In other species, adiponectin is synthesised in white adipose tissue as monomers and secreted into the peripheral circulation where it circulates as trimers, low molecular weight hexamers and high molecular weight (HMW) multimers [8]. In human type 2 diabetes, impaired multimerisation may occur before an overall reduction in adiponectin secretion [9]. Thus, concentrations of HMW adiponectin (HMWAC) decrease before reductions in total adiponectin concentration (TAC) and HMWAC may be a more sensitive marker of metabolic disease than TAC; although the evidence is conflicting [10,11]. The HMWA complex may also be more biologically active than lower molecular weight forms due to greater receptor affinity [8]. The relative merits of measuring different adiponectin fractions in equids requires further study. Both TAC and HMWAC have been associated with obesity [12] and are negatively correlated with insulin concentrations [13].

Previous equine studies used an enzyme linked immunosorbent assay (ELISA) to measure HMWAC [12] and a radioimmunoassay (RIA) [7,14] to measure TAC. Production of the validated ELISA has been discontinued and the antibody used in the RIA has been altered adversely affecting the validity of the assay in equids (E Knowles, unpublished data). The objectives of this study were i) to determine whether a latex enhanced immunoturbidimetric (IT) assay used for measurement of human TAC or ELISA methods for measuring TAC or HMWAC provide reliable means of measuring equine adiponectin concentrations and ii) to determine whether adiponectin concentrations measured using reliable assays provide a diagnostic marker for laminitis risk.

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Materials and Methods

Part 1. Assay Validation

The study was performed using residual serum and plasma from samples submitted to a commercial laboratory for the investigation of EMS or routine health screening (IT assay). In addition, serum and plasma samples collected under Home Office project licence (PED1AA054) and approved by the Royal Veterinary College Ethics and Welfare Committee from never laminitic (NL; n = 6; all mares; aged 19.8 ± 4.8 years; native breed ponies) ponies (HMWAC and TAC ELISA) were used. All samples were collected by jugular venepuncture into vacutainer tubes containing clot activator (serum samples), ethylenediaminetetraacetic acid (EDTA) and heparin vacutainers, allowed to clot (serum samples only), separated by centrifugation, aliquoted and stored at -20°C (commercial samples) or -80°C (research samples) until analysed. Two different sets of samples were used due to ease of availability.

The assays to be validated were 1) an IT assay[a] for measurement of TAC; 2) human (Mercodia Adiponectin ELISA [10-1193-01][b]) and equine (Nori® Equine Adiponectin ELISA kit [GR106557][c]) specific ELISA methods for measurement of TAC; and 3) an equine specific ELISA (Horse High molecular weight adiponectin ELISA kit [E10A0530][d]) or measurement of HMWAC.

Assay methods

The IT assay for measurement of TAC was performed using commercial clinical chemistry analyser (Biolis[e]) according to the manufacturers' instructions. The three ELISAs were similarly all performed according to the manufacturer's instructions and the optical density read at 450 nm using a plate reader (Tecan infinite pro 200 plate reader using Magellan 7 software[f]). A commercial control kit

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(Mercodia Obesity Control Kit [10-1241-01][b]) was used in conjunction with the human ELISA for measurement of TAC as an internal quality control.

Assay precision

For the IT assay, serum samples of known adiponectin concentration were pooled into high and low pool samples (n = 3 samples/group), respectively. Each pooled sample was analysed three times within a run on each of three consecutive days. Similarly made pooled heparin plasma samples were assayed three times within each of two assays run on consecutive days for the equine specific ELISA for HMWAC. Aliquots that were analysed on subsequent days were kept stored at -20°C. Coefficients of variation were determined for intra-day/assay and inter-day/assay variability for each test assay. No signal was obtained from the equine or human specific ELISAs for measuring TAC using serum or plasma.

Dilutional parallelism

High pool samples were serially diluted from 1:2 up to 1:32 with low pool samples (IT assay for TAC) or phosphate buffered saline[g] (equine specific ELISA for HMWAC) as per the manufacturers' recommendations to produce samples that spanned the working range of each assay. Samples were then analysed in duplicate within the same assay run/kit and measured concentrations were compared to calculated concentrations.

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Spiked recovery

Low pool samples were spiked with the high calibrator (IT assay for TAC) or highest standard (equine specific ELISA for HMWAC) for the assay to produce five samples within the working range of each assay. These samples were analysed in duplicate within the same assay run/kit and the mean of each pair compared to expected concentrations.

Effect of temperature, time and anti-coagulant

For the IT assay, EDTA plasma, heparin plasma and serum samples (n = 5 for each) were separated into aliquots and individual aliquots were analysed immediately (T0) and then either stored at -20°C in a domestic freezer or at 4°C, 23°C or 30°C in temperature-controlled cabinets. Samples were analysed after 24, 48 and 72 hours of storage. Data were expressed as a percentage of the T0 serum sample.

Part 2. TAC as a diagnostic marker for laminitis

TAC in previously laminitic and never laminitic ponies

Blood samples were collected by jugular venepuncture into vacutainers[h] containing clot activator from previously laminitic (PL; n = 6; 5 mares, one gelding; aged 16.7 ± 2.9 years [mean ± s.d.]; native breed ponies) and never laminitic (NL; n = 6; all mares; aged 19.8 ± 4.8 years; native breed ponies) ponies with no other clinical signs consistent with pituitary pars intermedia dysfunction and plasma ACTH concentrations within the seasonally adjusted reference range. Vacutainers were incubated at 37°C in a water bath for 30 minutes, centrifuged and the serum separated and frozen at -80°C until analysis.

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TAC as a marker of future laminitis risk and comparison with previously validated radioimmunoassay

TAC were measured using the IT assay in stored serum samples collected from a cohort of NL ponies (n = 223; one sample per animal) ≥7 years old that had been the subject of a previous study [7]. As part of the previous study, follow-up was obtained from owners 12 months after samples were collected to determine which animals had subsequently developed laminitis and TAC was measured using a human radioimmunoassay (RIA)[i] that is no longer valid for equine samples due to an antibody alteration. In this previous study, TAC was associated with future laminitis in a multivariable analysis [7].

Data analysis

Data were analysed using statistical and graphing software[j,k]. The normality of continuous data was assessed using the Shapiro-Wilk normality test. The effect of anti-coagulant was determined using a one-way repeated measures ANOVA with Tukey's multiple comparisons test. The effects of storage temperature and time were determined by visual inspection of the data plotted against time using the pre-determined criteria that ≤10% degradation was clinically acceptable and veterinarians are unlikely to be able to freeze samples immediately, but can usually maintain samples at 4-23°C.

Total adiponectin concentration (TAC) in PL and NL ponies were compared using an unpaired t test and between NL ponies that developed laminitis and those that remained non laminitic using a Mann Whitney U test. The relationship between TAC as measured by the IT method and the previously validated RIA method [6] was examined by performing linear regression, creating ratio

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(IT:RIA) Bland-Altman plots to show the limits of agreement between the methods and by calculating the intraclass correlation coefficient (ICC) whereby an ICC of <0.40 is considered poor, 0.40 - 0.59 fair, 0.60 - 0.74 good and ≥0.75 excellent. Univariable logistic regression was used to assess whether TAC was associated with the outcome (namely laminitic after 12 months or not). A ROC curve was constructed for TAC and the accuracy of the test to separate animals into those which did or did not subsequently develop laminitis was determined by calculating the area under the curve (AUC) whereby an area of 0.90-1 is excellent, 0.80-0.89 good, 0.70-0.79 fair, 0.60-0.69 poor and 0.50-0.59 fail [29]. In addition, the co-ordinates of the ROC curve were then used to determine the cut-off value that maximised specificity and sensitivity and the corresponding positive (PPV) and negative predictive values (NPV) using this cut-off value were calculated. Significance was accepted at $p \leq 0.05$.

Results

Part 1: Assay Validation

Assay precision

Mean (± s.d.) intra-assay (within day) and inter-assay (between day) coefficients of variation for the IT assay for TAC were calculated as 1.02 ± 0.15% and 1.87 ± 0.93%, respectively. The mean (± s.d.) intra-assay and inter-assay coefficients of variation for the equine specific ELISA for HMWAC were 7.35 ± 1.6% and 89.1 ± 56.8%, respectively.

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Dilutional parallelism

The IT assay for TAC demonstrated acceptable dilutional parallelism; mean ± s.d. measured concentration was 107 ± 5.83% of the expected concentration. However, the equine specific ELISA for HMWAC did not; mean ± s.d. measured concentration was 2210.8 ± 1847% of the expected concentration.

Spiked recovery

Percentage recovery from spiked samples measured using the IT assay for TAC was acceptable; mean ± s.d. recovery of 111 ± 2%. However, the recovery from spiked samples using the equine specific ELISA for HMWAC was not acceptable; mean ± s.d. recovery of 21.2 ± 11.9%.

Effect of anti-coagulant and storage temperature and time

TAC at T0 measured using the IT assay in samples collected into EDTA (mean ± s.d. 10.5 ± 3.6 μg/ml) was significantly lower than TAC in serum samples ($p = 0.009$; 11.1 ± 4.0 μg/ml) or in samples collected into heparin ($p = 0.003$; 11.1 ± 3.7 μg/ml). TAC in samples stored at different temperatures for up to 72 hours measured using the IT assay are shown in Table 1. On the basis of visual inspection of graphs, EDTA was unsuitable as an anti-coagulant if the sample was not kept frozen (-20°C) or chilled (4°C); heparinised plasma and serum samples gave similar results when stored between -20 and 23°C; heparinised plasma and serum samples should be analysed within 24 hours if stored at 23°C; and no sample type was suitable if the sample was stored at 30°C.

Part 2. TAC as a diagnostic marker for laminitis and comparison with a previously validated radioimmunoassay

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TAC in previously laminitic and never laminitic ponies

Mean ± s.d. TAC in NL ponies (24.17 ± 11.83 μg/ml) was significantly (p = 0.01) greater than in PL ponies (8.95 ± 2.94 μg/ml).

TAC as a marker of future laminitis risk and comparison with previously validated radioimmunoassay (RIA)

Linear regression indicated that TAC measured using the IT assay (μg/ml) = 0.1249 x RIA + 1.78 (r^2 = 0.48; Fig 1). The ICC was 0.54 (0.51, 0.59) consistent with fair agreement between the methods and Bland-Altman analysis revealed a mean bias (limits of agreement) methods of 4.4 (1.1, 7.9) in the ratio of IT:RIA (Fig 1).

As previously reported [7], nine of the 223 ponies developed laminitis within 12 months of sample collection. Median (IQR) TAC measured using IT assay in ponies that remained non-laminitic (n = 214; 19.9 μg/ml; 9.95, 31.53) was significantly (p<0.001) greater than in ponies that developed laminitis (n = 9; 4.8 μg/ml; 2.65, 13.4). TAC was significantly (p = 0.04) associated with the outcome (namely laminitic after 12 months or not). The area (95% confidence intervals) under the ROC curve was 0.762 (0.64, 0.88; p = 0.001), which is considered good. From the ROC curve analysis, an optimal cut-off of 24.1 μg/ml was determined, which generated sensitivity and specificity values of 89% and 60% respectively. The odds ratio (OR) (95% confidence interval) was 0.91 (0.87, 0.97) i.e. a pony with a value above this cut-off is 9% less likely to develop laminitis. This is reflected in the high negative predictive value (NPV) for development of laminitis of 98.7%. However, the positive predictive value (PPV) of 11.0% was extremely low using the optimal cut-off of 24.1 μg/ml and did

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not increase even if the cut-off was reduced as low as 11 μg/ml reflecting the overall low prevalence of laminitis in this cohort.

Discussion

This study has demonstrated that a human IT assay for TAC is suitable for the measurement of equine TAC in serum and heparinised plasma stored between -20 and 23°C for up to 72 hours and in EDTA plasma for up to 48 hours provided it is stored at 4°C or below. This assay can be performed quickly and reliably using commercial biochemistry analysers that are available in many equine hospitals and by operators with limited experience. In contrast, the accuracy and precision of ELISA methods evaluated herein for the measurement of equine HMWAC or TAC was unacceptable and their use cannot be recommended. It should be acknowledged, however, that there may be other ELISAs available for measurement of either TAC or HMWAC in equine samples that were not tested in this study.

In stored samples, TAC decreased after 24 hours at higher temperatures; however, in frozen or chilled samples concentrations remained stable. Therefore, whilst the analysis of chilled or frozen serum is preferred, the use of chilled or frozen plasma is acceptable. Whilst TAC was significantly lower in samples collected into EDTA compared to serum samples and samples collected into heparin, the magnitude of this difference is unlikely to be clinically relevant. Investigations of the stability of adiponectin in whole blood were not performed, so samples should be separated before shipping to a postal laboratory.

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TAC measured using the IT assay correlated with, but were higher than those determined using the RIA method that has been validated and used in previous studies [6,7]. Results generated in the current study are, however, consistent with TAC in other species such as 5 to 30 μg/ml in humans, 9 to 17.4 μg/ml in mice, and 0.052 to 33.8 μg/ml in dogs [15,16]. It has been suggested previously that the RIA method may have low affinity for equine adiponectin [12] and if this is true then the IT assay may provide a more accurate estimate of circulating TAC. Further investigations would be required but are unlikely to be possible, as the antibodies used in the RIA have been changed since it was validated such that it no longer produces acceptable results.

In agreement with previous studies, low TAC was associated with previous laminitis or future laminitis [2,6,7]. However, these conclusions are drawn on a population basis and its value as a means of predicting laminitis in an individual animal should be considered carefully. Whilst a cut-off value of 24 μg/ml maximised diagnostic accuracy for predicting the future development of laminitis, due to the low prevalence of laminitis within the population, the positive predictive value of the test was only 11% i.e. for every 100 horses that were identified as having a low adiponectin concentration only 11 would go on to develop laminitis. By contrast, the negative predictive value was extremely high (99%). Therefore, the result in an individual animal should be interpreted accordingly with the emphasis on a high value being consistent with an extremely low risk of developing laminitis without over-interpretation of a low value. The positive predictive value of the test for laminitis would be expected to improve if it were used only in ponies selected based on clinical signs and phenotype rather than being used to screen a population; further investigations are required.

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Whilst measurement of TAC is likely to be helpful in individual animals suspected of having metabolic disease, additional testing of insulin dysregulation should be considered to determine a more complete picture of metabolic dysfunction and laminitis risk. Many horses with some of the metabolic alterations consistent with EMS do not develop laminitis and further research is required to determine whether this relates to the severity of the underlying metabolic disease or the interaction between metabolic dysfunction and other factors. There is a far greater body of evidence to support the use of assessments of insulin dysregulation in equids with EMS or pituitary pars intermedia dysfunction to determine laminitis risk and measurement of TAC should be considered an adjunct rather than an alternative. In addition, it should be acknowledged that in the previous study evaluating multiple risk factors, combining the individual significant risk factors such as basal insulin concentration and TAC did not increase the predictive power [7].

The reason for the apparent association between lower adiponectin concentrations and laminitis is unknown and further studies are required. Adiponectin has been shown in other species to open smooth muscle potassium channels and to increase vascular expression of both endothelial nitric oxide synthase and prostacyclin synthase all of which promote vasodilation [17,18] and this may provide a means by which adiponectin is protective against laminitis. In man, adiponectin in association with insulin stimulates insulin receptors and IGF-1 receptors [19] so reductions in circulating adiponectin concentrations may increase the risk of laminitis that occurs in association with hyperinsulinaemia. Alternatively, adiponectin may simply be a marker of the metabolic derangements that lead to laminitis and may have no direct role in the pathogenesis of laminitis.

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In conclusion, the IT assay for the measurement of TAC was reliable and repeatable, but the ELISAs evaluated for the measurement of HMWAC and TAC were not. TAC was significantly lower in previously laminitic ponies and in non-laminitic ponies that subsequently developed laminitis in the next 12 months. The accuracy of TAC in distinguishing between animals that did and did not develop laminitis after 12 months was good and it generated a cut-off value with acceptable sensitivity and specificity. However, it should be acknowledged that this cut-off value was generated using samples obtained at a single time of year (summer) from ponies kept in a single geographic area and that, as no radiography was performed, it is possible that animals with pre-existing subclinical laminitis were included. Further prospective cohort studies that examine animals more frequently so that TAC can be measured within a shorter period in relation to the onset of laminitis are ongoing to assess this risk factor further.

Authors' declaration of interests

D. Rendle, I. Rogers and E. Knowles all have affiliations with commercial veterinary laboratories.

Ethical animal research

Some samples used in Part 1 were surplus from material collected for clinical purposes. Additional samples and protocols for Part 2 approved under Home Office project licence (PED1AA054) and/or by the Royal Veterinary College Ethics and Welfare Committee (URN 2014 1309). Explicit owner informed consent was not obtained, but for samples collected for clinical purposes, owners were given the opportunity to opt out of research studies.

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Authorship

All authors contributed to the study design and interpretation of results. I. Rogers, N. Menzies-Gow and E. Knowles performed the laboratory analyses. N. Menzies-Gow performed the data analysis. D. Rendle and N. Menzies-Gow produced the manuscript with contributions from E. Knowles and I. Rogers.

Manufacturers' addresses

[a]Randox Reagents, Crumlin, Co Antrim, Northern Ireland, UK.

[b]Mercodia AB, Uppsala, Sweden.

[c]Genorise Scientific Inc., Glen Mills, Philadelphia, USA.

[d]Shanghai Bluegene Biotech, Shanghai, China.

[e]Abaxis, York, Yorkshire, UK.

[f]Tecan Group Ltd, Mannedorf, Switzerland.

[g]InvitrogenTM, Gibco, Paisley, Scotland, UK.

[h]BD UK Ltd, Berkshire, UK.

[i]Merck, Massachusetts, USA.

[j]GraphPad Software Inc., La Jolla, California, USA.

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[k]IBM Corp., Armonk, New York, USA.

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Table 1: Mean ± s.d. total adiponectin concentrations in serum, heparinised plasma and EDTA plasma samples collected from 5 horses, stored between -20°C and 30°C and analysed after 0, 24, 48 and 72 hours storage. Results are expressed as a percentage of the T0 sample that was considered the gold standard. Values highlighted in bold were considered unacceptable.

Storage Time (Hours)	-20°C			4°C			23°C			30°C		
	Serum	Hep plasma	EDTA plasma	Serum	Hep plasma	EDTA plasma	Serum	Hep plasma	EDTA plasma	Serum	Hep plasma	EDTA plasma
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
24	97.4 ± 1.4	98. ± 1.5	99.0 ± 1.5	98.7 ± 2.2	97.3 ± 1.9	95.6 ± 1.6	95.2 ± 0.7	92.3 ± 0.9	**86.1 ± 2.2**	**83.8 ± 3.0**	**83.8 ± 3.9**	**77.3 ±3.8**
48	96.6 ± 1.6	95.4 ± 1.4	99.1 ± 2.2	99.4 ± 0.8	98.1 ± 1.1	92.9 ± 3.0	**91.7 ± 0.7**	**90.7 ± 1.0**	**83.2 ± 1.3**	**82.1 ± 3.2**	**81.7 ± 4.5**	**70.7 ± 4.4**
72	98.9 ± 1.5	98.4 ± 1.8	101.8 ± 2.2	100.2 ± 1.7	99.2 ± 1.7	**90.1 ± 3.1**	**91.6 ±0.5**	**89.5 ± 1.6**	**79.7 ± 2.7**	**81.4 ± 4.0**	**80.8 ± 5.5**	**67.9 ± 4.1**

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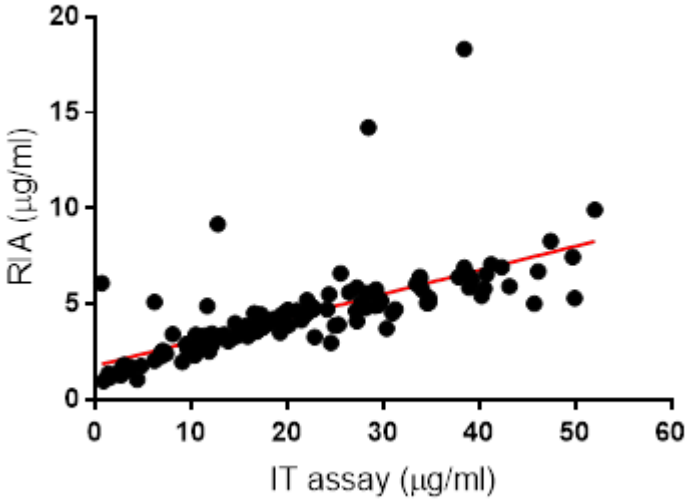
Figure Legends

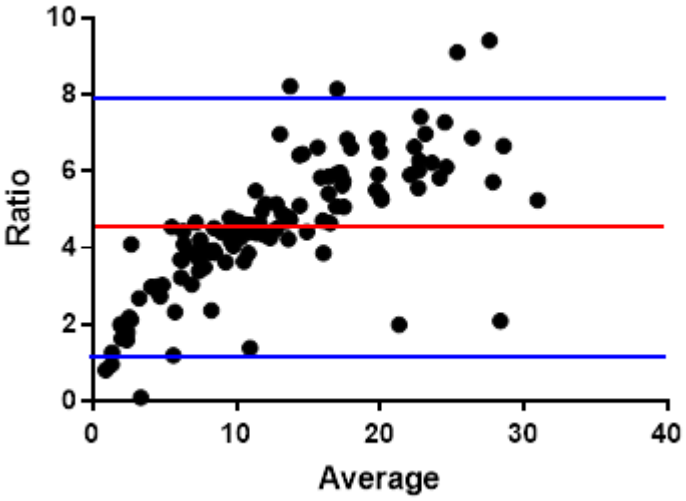
Fig 1: A) Scatter plot of total adiponectin concentration (TAC) measured using immunoturbidimetric assay (IT) and radioimmunoassay (RIA) showing linear regression line (red) and B) Bland-Altman plot of ratio of TAC measured using IT compared to RIA with mean bias (red line) and limits of agreement (blue lines).

Figure 1

A)



B)



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