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Retinol to Retinol-Binding Protein (RBP) is Low in Obese Adults due to Elevated *apo*-RBP

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Abstract

Elevated serum retinol-binding protein (RBP) concentration has been associated with obesity and insulin resistance, but accompanying retinol values have not been reported. Assessment of retinol is required to discriminate between *apo*-RBP, which may act as an adipokine, and *holo*-RBP, which transports vitamin A. The relations between serum RBP, retinol, retinyl esters, BMI, and measures of insulin resistance were determined in obese adults. Fasting blood (≥8 h) was collected from obese men and women ($n = 76$) and blood chemistries were obtained. Retinol and retinyl esters were quantified by HPLC and RBP by ELISA. RBP and retinol were determined in age and sex-matched, nonobese individuals ($n = 41$) for comparison. Serum *apo*-RBP was two-fold higher in obese (0.90 ± 0.62 μM) than nonobese subjects (0.44 ± 0.56 μM) ($P < 0.001$). The retinol to RBP ratio (retinol:RBP) was significantly lower in obese (0.73 ± 0.13) than nonobese subjects (0.90 ± 0.22) ($P < 0.001$) and RBP was strongly associated with retinol in both groups ($r = 0.71$ and 0.90, respectively, $P < 0.0001$). In obese subjects, RBP was associated with insulin ($r = 0.26$, $P < 0.05$), homeostatic model assessment of insulin resistance ($r = 0.29$, $P < 0.05$), and quantitative insulin sensitivity check index ($r = -0.27$, $P < 0.05$). RBP was associated with BMI only when obese and nonobese subjects were combined ($r = 0.25$, $P < 0.01$). Elevated serum RBP, derived in part from *apo*-RBP, was more strongly associated with retinol than with BMI or measures of insulin resistance in obese adults. Investigations into the role of RBP in obesity and insulin resistance should include retinol to facilitate the measurement of *apo*-RBP and retinol:RBP. When evaluating the therapeutic potential of lowering serum RBP, consideration of the consequences on vitamin A metabolism is paramount.

Keywords

obesity; RBP; RBP4; retinol; vitamin A

Introduction

In addition to its well established role as the major blood carrier of retinol, serum retinol-binding protein (RBP) has recently been referred to as “RBP4,” a new adipokine, by members of the diabetic research community (1). This development was instigated by a series of studies in mice and humans revealing a strong relationship between serum RBP and obesity-induced insulin resistance (2,3). These novel findings spawned widespread fervor to understand the role of RBP in obesity and insulin resistance, generating a considerable pool of publications

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in a relatively short amount of time. While some studies have validated the original observations of elevated RBP in obesity and insulin resistance in humans (3–8), others have not (9–15). Often lacking in these publications are data for serum retinol, arguably RBP's most important physiological companion, representing a possible explanation for conflicting results.

Retinol abundance influences RBP secretion from the liver (16), the primary storage site of vitamin A. Serum retinol concentration is commonly used to assess vitamin A status in humans because liver biopsy, the gold standard (17), is not feasible. In the fasted state, vitamin A circulates primarily as retinol bound to RBP (i.e., *holo*-RBP) in approximately a 1:1 molar ratio, and to a much lesser extent, as retinyl esters carried by lipoproteins (18,19). Typically retinyl esters make up <5% of the total plasma retinol concentration (20,21). However, during hypervitaminosis A, substantial retinyl esters can be detected in the serum and values >10% of total retinol are clinically relevant [see review (22)]. It is unclear how obesity affects the saturation of RBP with retinol, or the circulation of retinyl esters. No studies have reported the relation between serum retinol and retinyl ester concentrations in fasted obese adults. Nor have both of these retinol forms been correlated with RBP in the same study. Previous failure to include retinol in RBP association analyses has potentially hidden the true relationship between obesity, *holo*-RBP, *apo*-RBP, and altered vitamin A circulation. Thus, we examined the relations between RBP, retinol, retinyl esters, BMI, and measures of insulin sensitivity in a sample of fasted obese adults.

Materials and Methods

Subjects

Obese subject data were pooled from control or baseline groups from a cross-sectional and intervention study, respectively, conducted by our laboratory. A total of 76 (17 men, 59 women) adults (BMI > 30), aged 21–71 y were included in the obese group. Age and sex-matched, nonobese subject data ($n = 41$; 13 men, 28 women) were also pooled from previous studies conducted by our laboratory (21,23,24). All procedures for these studies were approved by the Health Sciences Institutional Review Board at the University of Wisconsin – Medical School.

Blood Collection and Serum Chemistry

Blood samples were drawn after an overnight fast (at least 8 h). Blood from all subjects was analyzed for retinol and RBP, while only blood from obese subjects was analyzed for hematocrit, serum lipids, insulin, glucose, C-reactive protein (CRP), and retinyl esters. For blood samples used to determine hematocrit, study phlebotomists drew whole blood samples into sterile-interior 3 mL Vacutainer® tubes containing 5.4 mg K_2EDTA (Becton Dickinson, NJ). Samples used to determine serum lipid panels, insulin, glucose, CRP, retinol, retinyl esters, and RBP were drawn using 6 mL, sterile interior, Corvac brand serum separator tubes (Tyco Healthcare Group LP, MA). Whole blood samples sat at room temperature for 10 min, then were stored on ice until analysis within 18 h at the contract laboratory (Consultants Laboratory; Fond du Lac, WI). Blood for serum sat at room temperature for 10 – 20 min and then was centrifuged at 2200 × *g* for 10 min at 4 °C. Serum not used for analysis by the contract lab was transferred to cryotubes and stored under argon at −80°C until analysis for retinol, retinyl esters, and RBP.

Surrogate measures of insulin resistance utilizing fasting glucose and insulin concentrations were calculated for the obese group. The homeostasis model assessments of insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were calculated as follows: HOMA-IR = insulin (μU/mL) * glucose (mM)/22.5 and HOMA-β = 20 * insulin (μU/mL)/(glucose (mM) −3.5) (25). The quantitative insulin sensitivity check index (QUICKI) was calculated as follows: QUICKI = 1/[log(insulin (μU/mL) + log(glucose (mg/dL)] (26). For interpretation, HOMA-

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IR and HOMA-β will increase, while QUICKI will decrease with increasing insulin resistance. Logarithmically transformed HOMA and QUICKI models are considered the best and most extensively validated minimal surrogate measures of insulin resistance (27,28).

Serum Vitamin A and Retinol-Binding Protein Assays

Extraction and HPLC analyses were modified from Sowell *et al.* (29) to optimize the sensitivity of detection of retinol and retinyl esters. Briefly, retinol and retinyl esters were extracted from serum using a published procedure (30), with volume alterations. One mL serum was treated with 1 mL ethanol (0.1% butylated hydroxytoluene), and extracted 3 times with 1 mL hexanes. Retinyl butyrate was used as an internal standard to determine extraction efficiency (87 ± 5%). Dried samples were reconstituted in 80 μL methanol:dichloroethane (50:50, v:v) and 60 μL was analyzed by HPLC as published elsewhere (31).

Serum RBP was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) as developed by Erhardt *et al.* (32). This sensitive ELISA was validated with HPLC-determined serum retinol and a commercially available RBP ELISA kit (ALPCO diagnostics) that has been used in several human studies (3,33,34). Greater serum dilution (1:9664) compared to the original protocol (1:6644) was required to accommodate the relatively higher RBP concentrations of our study population. Additionally, it was necessary to decrease the RBP standard dilutions (from 1:1661 to 1:1158) and use more per plate well (5,10,15,20 and 25 μL) to generate a calibration curve with a higher range. The intra- and inter-assay coefficients of variation were 8.3 and 13.6%, respectively.

Statistical Analysis

Data are presented as means ± SDs. Differences in RBP, retinol, BMI, age, and the retinol to RBP molar ratio (retinol:RBP) between the obese and nonobese groups were determined by one-way ANOVA. Pearson's correlation was performed to determine simple associations between analytes. Fisher's r-to-Z transformation was used to test for significant differences between r values of obese and nonobese subjects. Multiple linear regression (MLR) analysis was performed to identify variables associated with RBP after adjustment for age and retinol. Age was included as a covariate because of the large range of ages in our sample, and retinol was included because of its physiological relationship with RBP. Stepwise MLR with backward selection was used to select the best multivariate models to explain RBP and total retinyl ester concentration. Prior to correlation and regression analyses, non-normally distributed variables (total retinyl ester concentration, glucose, insulin, triacylglycerols, VLDL cholesterol (VLDL-C), HDL cholesterol (HDL-C), CRP, hematocrit, HOMA-IR and HOMA-β) were logarithmically transformed. Bonferroni testing was applied to correct for multiple comparisons. Because hypervitaminosis A causes abnormally high circulating retinyl ester concentrations (>10% of the total circulating vitamin A in the fasted state), regression models with total retinyl ester concentration as the dependent variable were constructed with and without values from potentially hypervitaminotic subjects ($n = 3$). All statistical procedures (PROC CORR, PROC GLM and PROC REG) were performed with SAS software (version 8.2; SAS Institute Inc, Cary, NC). Statistical analyses were 2-sided and $P < 0.05$ was considered significant.

Results

Physical and vitamin A-related biochemical characteristics of obese and nonobese subjects were compared (Table 1). Mean age and serum retinol concentrations were not different between the two groups. Mean BMI and RBP concentrations were higher in obese subjects and retinol:RBP was lower, compared to nonobese subjects. RBP concentrations did not differ by gender in either group alone or in both groups combined. Fasting glucose, LDL cholesterol

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LDL-C), triacylglycerol and total cholesterol levels were slightly higher than recently reported values from a group of overweight and obese subjects without diabetes (11), while insulin, HOMA-IR and HOMA-β were slightly lower (Table 2). The mean QUICKI in our obese sample was similar to previously published values from obese, nondiabetic subjects (26). Importantly, none of the subjects in the current study had blood glucose concentrations above 126 mg/dL, the current diagnostic cut-off for diabetes, established by the American Diabetes Association. Diagnostic cut-off values for HOMA and QUICKI have been proposed, but are not used routinely to diagnose insulin resistance in individuals.

Independent associations between serum RBP and other characteristics related to obesity and insulin sensitivity were determined after control for either age alone, or age and serum retinol concentration in the obese group (Table 3). Total cholesterol, log HDL-C, LDL-C, log CRP, log glucose, and log HOMA-β were not associated with RBP. Log Insulin and log HOMA-IR were positively associated with RBP ($r = 0.26$, $P = 0.04$ and $r = 0.29$, $P = 0.03$, respectively), while QUICKI was inversely associated with RBP ($r = -0.27$, $P = 0.03$) before and after adjusting for age and retinol. BMI was associated with log CRP ($P < 0.005$) after control for age and log CRP was associated with age ($P = 0.02$) after control for BMI. Log VLDL-C, log triacylglycerols, log hematocrit, and log retinyl esters were associated with RBP after control for age, but not after control for age and retinol. BMI and RBP were not associated in the obese group, but a combined analysis of obese and nonobese subjects revealed a significant association before ($r = 0.25$, $P = 0.01$) and after control for age and retinol ($P < 0.001$). RBP was highly correlated with retinol before ($r = 0.71$, $P < 0.0001$) and after control for age ($P < 0.001$) and this association remained significant after Bonferroni correction for multiple comparisons ($P < 0.005$). The age-adjusted association between RBP and VLDL-C was the only other association to survive Bonferroni correction, but the relationship was not significant when retinol was included in the model.

RBP and retinol were highly correlated in simple models in both the obese and nonobese groups (Fig. 1). Correlations were significantly different based on obesity status ($P = 0.0035$), according to Fisher's r-to-Z transformation test. In obese subjects, retinol and log total retinyl ester concentration were associated in simple models (Fig. 2). The association was strengthened after removal of three potentially hypervitaminotic A subjects (retinyl ester ≥10% total retinol) from the sample. The association remained significant after adjustment for age, log VLDL-C and LDL-C, the major fasting retinyl ester carriers. Stepwise MLR analysis applying backward selection demonstrated that serum retinol concentration ($P < 0.001$), log VLDL-C ($P < 0.002$) and LDL-C ($P < 0.05$) were the only significant independent variables to explain log total retinyl ester concentration.

The concentration of *apo*-RBP was determined by subtracting the serum retinol concentration from the total RBP concentration. The values for obese and nonobese individuals were 0.90 ± 0.62 and 0.44 ± 0.56 μM, respectively. These means are different ($P < 0.001$) and imply that adipose tissue may be secreting RBP that is not bound to retinol into the general circulation.

Discussion

Serum RBP is predominantly synthesized by hepatocytes, but extrahepatic tissues contain appreciable RBP mRNA (35). Notably, the kidney and adipocytes contain approximately 10 and 20% that of the liver RBP mRNA, respectively (35,36). Yang *et al*. (2) observed that RBP mRNA expression increased in adipose tissue, but not in liver, of insulin-resistant adipose-Glut4$^{-/-}$ mice, suggesting a potential role for adipocyte-derived RBP as a signal in obesity-induced insulin resistance. Soon after, serum RBP, regardless of origin, joined the ranks of leptin, adiponectin, resistin, visfatin, and others as the newest member of the adipokine family. Elevated serum RBP concentrations have since been implicated in a host of comorbidities such

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as Polycystic Ovary Syndrome (37,38), gestational diabetes (39), inflammation (7,34), cardiovascular disease (10), metabolic syndrome (6), and cancer (40). However, much like past associations found between serum retinol concentrations and cancer incidence (41,42), RBP associations with markers of chronic disease have not been consistently observed. For every report of elevated RBP concentrations in obesity or insulin resistance (3–8), there is a report to the contrary (9–15). A partial explanation for equivocal findings may be the past neglect of serum retinol as a covariate.

In the current study, we found that RBP was strongly associated with retinol and weakly or not at all associated with BMI and measures of insulin resistance in fasted obese adults. BMI was not associated with RBP in simple or multivariate models in the obese group, but when obese and nonobese subjects were combined, we saw a weak association after adjusting for age and retinol. RBP was associated positively with insulin and HOMA-IR and negatively with QUICKI after adjustment for age and retinol in the obese group. These associations were far weaker compared to the association between RBP and retinol, based on correlation strength and ability to survive Bonferroni correction. However, simple surrogate measures of insulin resistance such as HOMA and QUICKI lack the sensitivity of the more invasive hyperinsulinemic euglycemic glucose clamp technique, generally considered the gold standard for assessing insulin sensitivity (27). Future studies should utilize more direct measures of insulin sensitivity such as the glucose clamp or intravenous glucose tolerance testing to compare the association strength of RBP and retinol to that of RBP and magnitude of insulin resistance.

There is evidence to suggest that RBP expression is upregulated in obesity. In our study, the mean serum RBP concentration of the obese group was higher than that of the nonobese group despite identical mean serum retinol concentrations. Elevated RBP concentrations observed in obese subjects may be explained by greater amounts of RBP in circulation derived from adipose tissue (2,3). Perhaps enough retinol is not available in adipose to bind to the RBP and therefore, *apo*-RBP is released from the adipose diluting the *holo*-RBP in plasma. Biochemically, vitamin A researchers have referred to *apo*-RBP as the protein without retinol bound to it. It is unknown if the elevated adipose-derived serum *apo*-RBP we and others have observed in obese individuals is a direct contributor to insulin resistance. Alternatively, *apo*-RBP from adipose may transport an unidentified ligand that is responsible for mediating insulin signaling. There is presently no data to favor one of these hypotheses over the other, thus future investigations are needed to elucidate the mechanism behind the proposed causative role of RBP in insulin resistance.

Retinol:RBP has been in use for decades in the vitamin A field as a more informative variable than either component alone (19). When serum retinol concentrations were taken into account with the use of retinol:RBP, the difference between obese and nonobese subjects was highly significant. In light of this evidence, we propose that retinol:RBP may provide greater utility than RBP concentrations alone in the characterization of abnormal RBP regulation in obese adults. At the very least, we recommend that serum retinol concentration be included as a covariate in association analyses involving RBP, in order to avoid spurious associations. In our analysis, RBP was significantly associated with VLDL-C, triacylglycerol, hematocrit, and retinyl ester concentrations, but after adjustment for retinol, none of these associations remained (Table 3).

RBP was more strongly correlated with retinol in nonobese subjects than in obese subjects (Fig. 1). This supports the notion that abnormal RBP regulation results from obesity-related factors apart from vitamin A status. Utilization of retinol:RBP could help isolate such influences on RBP regulation. In nonobese adults, retinol:RBP has been reported as 0.91 ± 0.24 (43). This is identical to the ratio we observed in nonobese subjects (i.e., 0.90 ± 0.22). In

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obese diabetic adults with and without microalbuminuria, retinol:RBP was significantly lower than in healthy controls (44). In overweight and obese children and adolescents, retinol:RBP was lower compared to lean controls (6). However, values for this ratio have not been reported in nondiabetic obese adults. We observed a significantly lower retinol:RBP in obese subjects without diabetes (0.73 ± 0.13) compared to age-matched nonobese subjects.

In addition to the retinol-RBP complex, vitamin A is found in circulation as retinyl esters in lipoproteins, particularly VLDL-C and LDL-C (18). However, retinyl esters comprise a small percentage of the total vitamin A in circulation and thus are often overlooked (19). Because measurement of post-prandial vitamin A circulation is complicated by chylomicron transport of newly ingested retinyl esters, we collected serum from fasted subjects. We found a significant correlation between total retinyl ester concentration and retinol, independent of VLDL-C, and LDL-C concentrations, suggesting a possible connection between the homeostatic control mechanisms responsible for maintaining serum retinol concentration and the metabolism of circulating retinyl esters. The correlation was strengthened by the removal of potentially hypervitaminotic subjects from the analysis. This observation supports the hypothesis that elevated circulating retinyl esters (>10% of total retinol) are a result of abnormal vitamin A metabolism due to hypervitaminosis A, usually caused by excessive intake. It is interesting to note that total retinyl ester concentration was independently associated with retinol, VLDL-C and LDL-C after backward selection of the best multivariate model to explain total retinyl ester concentration. To the extent of our knowledge, such a relationship has not been reported previously in fasted obese adults.

A novel therapeutic potential has been suggested to lower plasma RBP concentrations in patients with and at risk for type 2 diabetes (45). As etiological partners in the pathogenesis of the metabolic syndrome, obesity and insulin resistance are intimately linked, identifying obese individuals as potential candidates for therapeutic treatment with RBP-lowering drugs. It is unknown how such treatment strategies to lower RBP will affect vitamin A circulation and status. The ramifications, which may include altered vitamin A utilization and changes in liver reserves, of such a drug would need to be carefully monitored and evaluated. Significant weight loss by morbidly obese individuals through stomach banding (5), and by obese adolescents through lifestyle modification (7), resulted in decreased serum RBP concentrations. If the extra *apo*-RBP diluting the plasma pool is adipose derived, decreasing % body fat through diet and exercise may increase retinol:RBP and such possibilities should be investigated.

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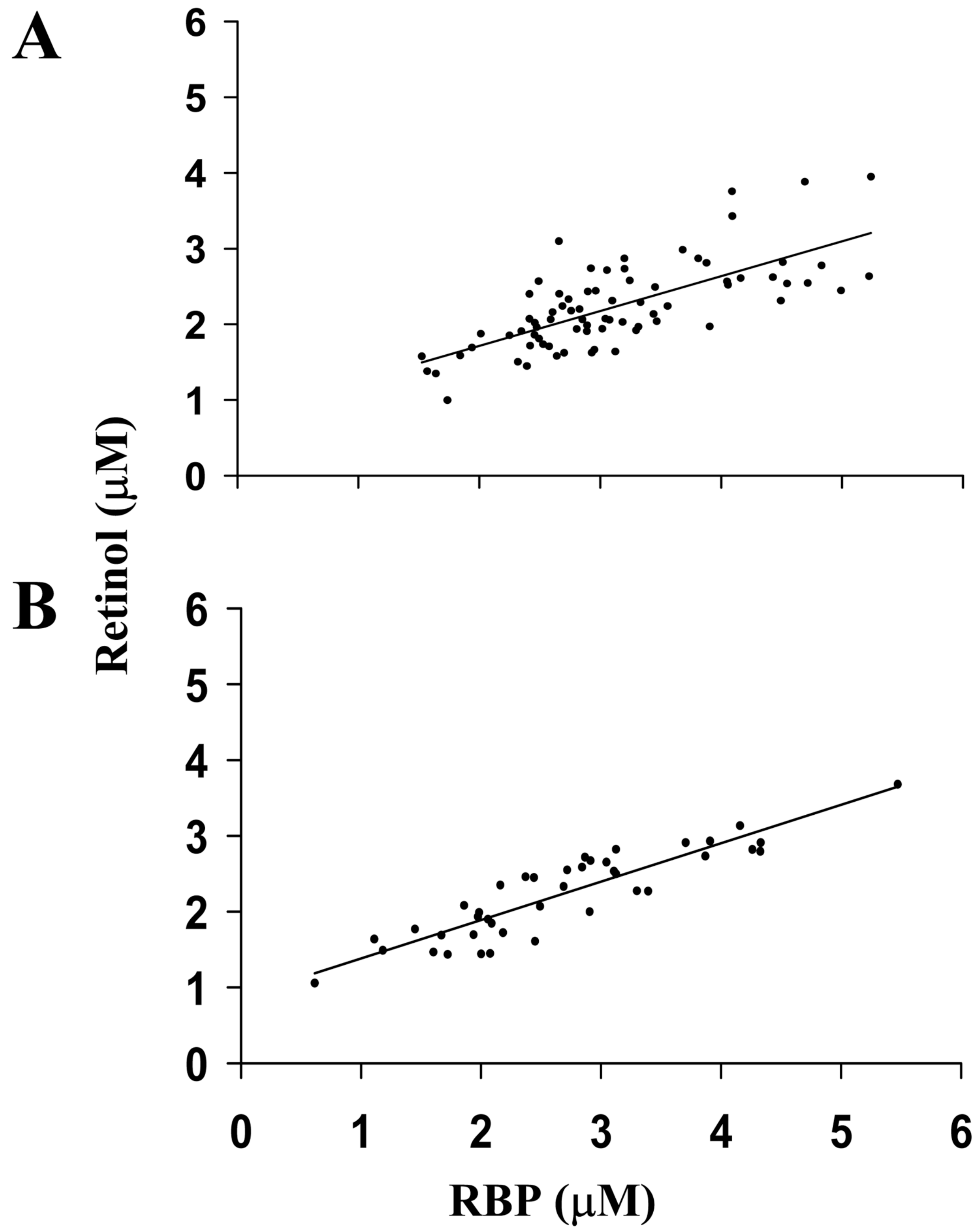

Figure 1.
Correlation between serum retinol-binding protein (RBP) and retinol in fasted obese adults ($n = 76$) ($r = 0.71$, $P < 0.0001$) **(A)** and fasted nonobese adults ($n = 41$) ($r = 0.90$, $P < 0.0001$) **(B)**. Correlations were significantly different by Fisher's r-to-Z transformation ($P = 0.0035$).

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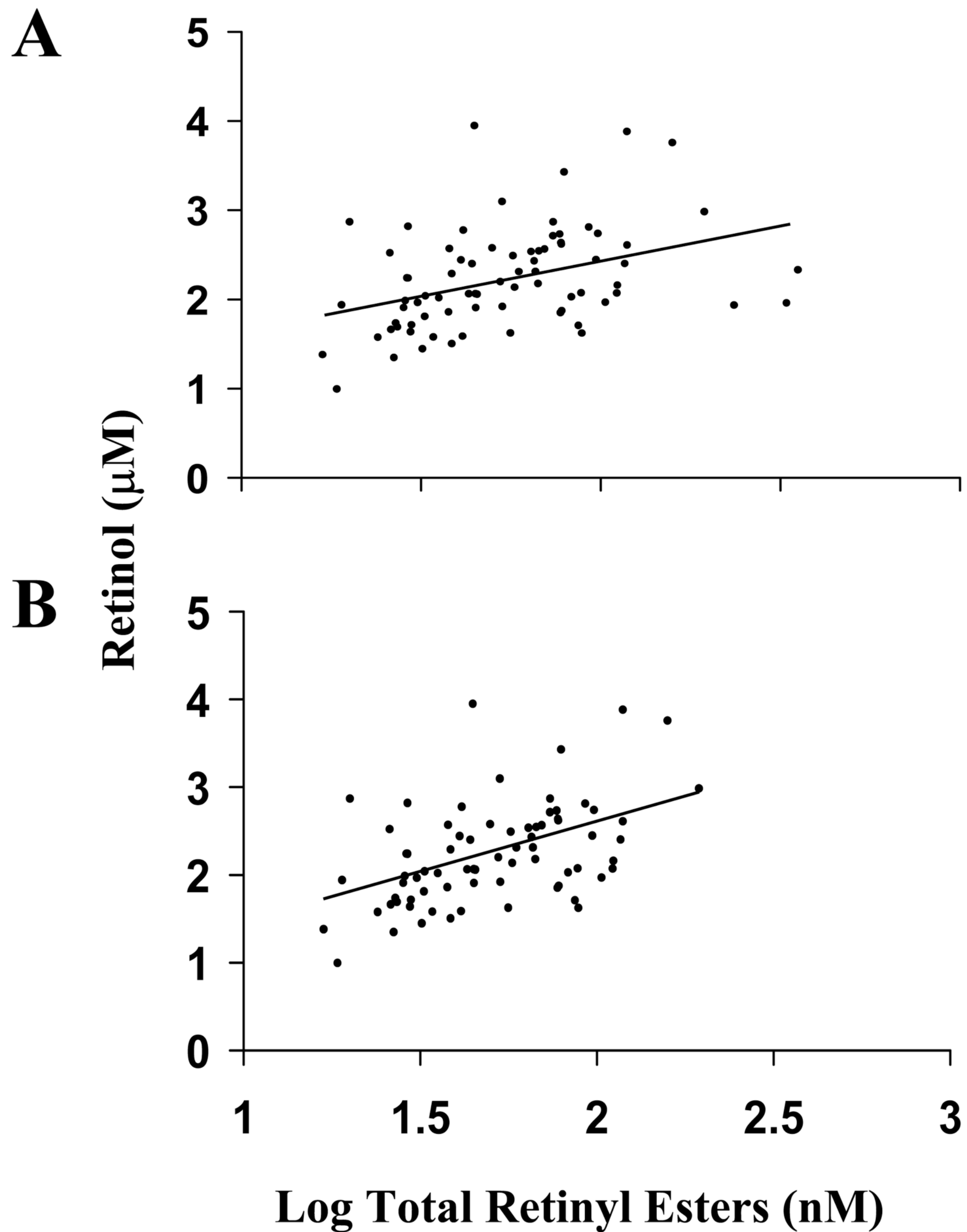

Figure 2.
Correlation between log total serum retinyl ester concentration and retinol in fasted obese adults including potentially hypervitaminotic subjects (>10% of total vitamin A as retinyl esters) ($n = 76$) ($r = 0.34$, $P < 0.003$) **(A)** and excluding potentially hypervitaminotic subjects ($n = 73$) ($r = 0.49$, $P < 0.0001$) **(B)**.

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Table 1

Physical and vitamin A-related biochemical characteristics of obese (n = 76; 17 men and 59 women) and nonobese fasted adults (n = 41; 13 men and 28 women)[a]

Characteristic	Obese (n = 76)	Nonobese (n = 41)	P[b]
Age (y)	39.1 ± 13.5 (21–71)	37.5 ± 17.7 (20–70)[c]	0.60
BMI (kg/m^2)	34.3 ± 4.0 (30.1–44.4)	24.8 ±3.2(19.7–29.3)	<0.0001
Retinol (μM)	2.23 ± 0.57 (1.00–3.95)	2.23 ± 0.58 (1.06–3.68)	0.96
RBP (μM)	3.13 ± 0.88 (1.52–5.24)	2.67 ± 1.02 (0.61–5.47)	0.012
Retinol:RBP	0.73 ± 0.14 (0.49–1.16)	0.90 ± 0.23 (0.66–1.72)	<0.0001

[a]RBP, retinol-binding protein

[b]Significant differences between nonobese and obese subjects were determined by one-way ANOVA ($P < 0.05$).

[c]Values are mean ± SD; range in parentheses (all such values).

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Table 2

Blood chemistry characteristics of fasted obese adults[a]

Characteristic	Obese [*n*]
Glucose (mM)	5.31 ± 0.90 [64][b]
Insulin (pM)	75.2 ± 48.9 [63]
Total Cholesterol (mM)	5.12 ± 1.01 [75]
Triacylglycerols (mM)	1.68 ± 1.24 [75]
HDL-C (mM)	1.20 ± 0.36 [64]
VLDL-C (mM)	0.53 ± 0.30 [62]
LDL-C (mM)	3.31 ± 0.76 [62]
C-reactive protein (mg/dl)	0.51 ± 0.51 [64]
Hematocrit (%)	42.2 ± 5.2 [63]
HOMA-IR	2.40 ± 1.20 [63]
HOMA-β	129.7 ± 77.1 [63]
QUICKI	0.34 ± 0.03 [63]
Retinyl esters (nM)	64.2 ± 52.0 [76]
Retinyl esters (% of total vitamin A)	2.93 ± 2.34 [76]

[a]HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, HOMA of β cell function; QUICKI, quantitative insulin sensitivity check index.

[b]Values are mean ± SD; *n* in brackets [all such values].

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Table 3

Independent associations between serum retinol-binding protein (RBP) and other characteristics in fasted obese adults after adjustment for age alone or after adjustment for age and retinol[a]

	Age-adjusted		Age and retinol-adjusted	
Characteristic	**β[b]**	***P***	**β**	***P***
BMI (kg/m^2)	−0.019	0.44	0.001	0.94
Retinol (μM)	1.04	<0.0001[c]	NA	NA
Log Glucose (mM)	−0.013	0.99	0.036	0.96
Log Insulin (pM)	0.455	0.031[d]	0.326	0.039[d]
Total cholesterol (mM)	0.002	0.52	−0.001	0.51
Log Triacylglycerols (mM)	0.479	0.0034[d]	0.130	0.33
Log HDL-C (mM)	−0.199	0.59	−0.322	0.24
Log VLDL-C (mM)	0.748	<0.0002[c]	0.310	0.077
LDL-C (mM)	−0.001	0.99	−0.000	0.75
Log C-reactive protein (mg/dl)	0.019	0.87	0.036	0.67
Log Hematocrit (%)	2.26	0.031[d]	1.36	0.089
Log HOMA-IR	0.506	0.021[d]	0.337	0.042[d]
Log HOMA-β	0.366	0.066	0.261	0.080
QUICKI	−8.74	0.028[d]	−6.33	0.034[d]
Log Retinyl esters (nM)	0.314	0.0055[d]	0.066	0.47

[a]RBP, retinol-binding protein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, HOMA of β cell function; QUICKI, quantitative insulin sensitivity check index.

[b]Regression coefficient.

[c]$P < 0.005$ after Bonferroni correction.

[d]Not significant after Bonferroni correction.