OBES SURG (2014) 24:219–224
DOI 10.1007/s11695-013-1087-8

ORIGINAL CONTRIBUTIONS

Association Between Liver Vitamin A Reserves and Severity of Nonalcoholic Fatty Liver Disease in the Class III Obese Following Bariatric Surgery

Gabriela Villaça Chaves · Sílvia Elaine Pereira · Carlos José Saboya · Daiane Spitz · Camila Santos Rodrigues · Andréa Ramalho

Published online: 8 October 2013

Abstract
Background Oxidative stress plays a role in nonalcoholic fatty liver disease (NAFLD) pathogenesis and may increase consumption of vitamin A for antioxidant purposes. It is hypothesized that drops in vitamin A concentration induce liver disease progression and increase hepatocellular carcinoma risk. The aim of this study was to assess concentrations of serum and liver retinol in the class III obese and correlate them with the histological diagnosis of NAFLD.

Methods The sample group was composed of 68 class III obese (body mass index, BMI≥40 kg/m^2) males and females who underwent bariatric surgery for treating obesity. Concentrations of serum and liver retinol were determined using high-performance liquid chromatography. The cutoff values used to denote inadequate serum and liver retinol stores were <1.05 μmol/L and ≤20 μg/g, respectively. Anthropometric measurements were taken, and NAFLD was diagnosed via histological assessment.
Results All the patients had some degree of NAFLD. Inadequate concentrations of serum and liver retinol were found in 35.9 and 67.9 % of them, respectively. A significant association was found between liver retinol concentrations and the histological classification of the disease (p <0.001). No such association was found for serum retinol.
Conclusions This study confirms the association between liver retinol and degree of NAFLD, underscoring the need for further research in this area, to identify which patients might benefit from supplementation of vitamin A.

Keywords Vitamin A · Oxidative stress · Nonalcoholic fatty liver disease

G. V. Chaves
National Cancer Institute, Center for Research on Micronutrients, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

S. E. Pereira
Center for Research on Micronutrients, Institute of Nutrition, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: se.pereira@gmail.com

C. J. Saboya · D. Spitz
Surgical Clinic, Center for Research on Micronutrients, Institute of Nutrition, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

C. J. Saboya
e-mail: jcsaboya@gmail.com

D. Spitz
e-mail: daianespitz@hotmail.com

C. S. Rodrigues
National Cancer Institute, Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: camilinha.rodrigues@ig.com.br

A. Ramalho
Department of Social and Applied Nutrition, Center for Research on Micronutrients, Institute of Nutrition, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: aramalho.rj@gmail.com

G. V. Chaves (✉)
Rua General Ribeiro da Costa, 90/604, Leme, Rio de Janeiro, Brazil
e-mail: gabrielavc@gmail.com

Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by triglycerides building up in the liver and making up 5–10 % or more of its weight, without significant alcohol (<20 g/day) ingestion on the part of the sufferer. The histological aspect of the disease is extensive, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis [1].

The most widely accepted hypothesis explaining the mechanism for NAFLD pathogenesis, titled “Two Hits,” was

proposed by Day and James [2], whereby the first step in developing the disease (the "first hit") consists of fats—specifically fatty acids and triglycerides—being deposited in the hepatocyte, characterizing simple hepatic steatosis. At this stage, the disease does not progress, unless additional cellular events (the "second hit") occur, leading to inflammation, cell death, and fibrosis. The factors involved in the disease progression, once hepatic steatosis has set in, can be grouped into two categories: factors that cause a rise in oxidative stress (OS) and factors that promote the expression of proinflammatory cytokines. Insulin resistance (IR) is involved in the two stages in development of the disease, and steatosis itself can only exacerbate the IR, perpetuating the self-destructive cycle.

The drop in serum retinol and carotenoid levels experienced by NAFLD sufferers is well documented in prior research [3, 4]. Considering the role OS plays in NAFLD pathogenesis and the potent effect of vitamin A in combating reactive oxygen species (ROS), it is likely these individuals exhibit low levels of the vitamin because of the increased consumption of substances with antioxidant functions when subjected to OS. There is an apparent association between lipid peroxidation and stellate cell activation. On the other hand, we know that the activation of hepatic stellate cells, with subsequent progression to myofibroblast-like cells, is characterized by a gradual drop in intracellular vitamin A stocks, leading to significant depletion in serum and liver retinol [5, 6].

It can thus be assumed that over its progression, the disease will be accompanied by a drop in liver vitamin A reserves. There is growing evidence suggesting that vitamin A plays a major role in the proliferation and differentiation of liver cells and that there may be a link between low concentrations of vitamin A and the development of liver tumors, especially once liver disease has set in, when hepatocytes are undergoing intense regenerative activity, and a drop in the concentration of liver retinol, which helps regulate and maintain the differentiated hepatocytes, can result in the formation of mutated hepatocytes, progenitors for hepatocellular carcinoma cells (HCC) [7]. To date no data have been published on changes in retinol concentrations in the liver tissue of NAFLD sufferers, which would help guide future treatment of the disease.

The aim of this study was to evaluate serum and liver retinol concentrations in the class III obese and correlate them with the histological diagnosis of NAFLD.

Materials and Methods

Study Design and Overview

This is a descriptive cross-sectional study involving 68 male and female patients with class III obesity (body mass index≥ 40 kg/m^2) indicated to undergo bariatric surgery, selected without distinction to color or socioeconomic class, whose progress was accompanied at a private hospital in Rio de Janeiro City between January and October 2012. Excluded from the study were patients who were pregnant or lactating, with malabsorption syndromes and acute or chronic infections or associated endocrinopathies, taking medication or supplementation containing vitamin A during the 6 months preceding data collection, women consuming over 20 g a day and men consuming more than 40 g a day of alcohol, suffering from any other liver disease that was not the NAFLD, and not given a go-ahead to undergo a liver biopsy at the time of bariatric surgery.

The assessment of vitamin A nutritional status (liver and serum retinol concentrations) was case-matched. The determination of serum vitamin A concentration was performed within 30 days before bariatric surgery, and liver biopsies were collected at surgery procedure for subsequent quantification of hepatic retinol.

This study was approved by the Research Ethics Committee of Clementino Fraga Filho University Hospital (Research Protocol no. 011/06-CEP), and patients were admitted to the project only after formally authorizing their participation by signing a consent form after the researcher informed them of the project objectives and procedures, in accordance with National Health Council Resolution no. 196 of Oct. 10, 1996.

Anthropometric Assessment

To classify the degree of obesity, patient weight and height measurements were taken according to the appropriate, described methodology [8]. Body mass index was calculated according to the formula: actual body weight (kg)/height (m^2). The cutoff points used were those recommended by the WHO [9] for classifying normal weight and excess weight, with the study participants qualifying as class III obesity, with BMI≥ 40.0 kg/m^2.

Determination of Vitamin A Nutritional Status

Determination of Plasma Retinol Concentration

Plasma retinol levels were ascertained by obtaining a 5-mL blood sample by venipuncture from patients following a 12-h fast the day before surgery. The method used to quantify serum retinol was the high-performance liquid chromatography (HPLC-UV). Serum retinol levels were compared with the cutoff point for normality proposed by the WHO [10] and were therefore presented in 0.35 μmol/L class intervals. Thus, vitamin A deficiency (VAD) was classified as being severe (<0.35 μmol/L), moderate (≥0.35<0.70 μmol/L), or mild (≥0.70<1.05 μmol/L). In this study, serum retinol values ≥1.05 μmol/L were

deemed to be adequate, while anything below this threshold was considered to indicate VAD [11].

Determination of Liver Retinol Reserves

To analyze liver retinol reserves, a biopsy was taken of the liver during surgery in patients undergoing bariatric surgery and cleared to have a biopsy done. A liver sample weighing approximately 2 g was taken according to the criteria established by Flores et al. [12], so as to quantify liver retinol reserves. The liver samples were stored at a temperature lower than or equal to –20 °C, in individual sterilized tubs protected from contact with light until transported to the laboratory where analysis was performed.

Prior to biochemical analysis, the samples were thawed and homogenized with a 50 % glycerol solution in distilled water totaling 5 mL for each gram of liver [12, 13]. Liver retinol concentrations were determined using the HPLC-UV method, and precautions recommended by the International Vitamin A Consultative Group (IVACG) were adhered to, so as to ensure sample quality was maintained prior to analysis [14].

The prevalence of inadequate liver retinol was estimated based on the criteria of Olson et al. [15], with inadequacy being between 5 and 20 μg/g; critical, <5 and ≥0.6 mg/g; and absent being <0.6 mg/g.

Histological Classification of NAFLD

A histological evaluation was performed by the surgeon, using a needle to puncture (percutaneous biopsy) and remove a 4-mm-thick sample from the left lobe of the liver. The evaluation was carried out by the same pathologist, who had no prior knowledge of the clinical and biochemical data of the patients, via hematoxylin–eosin, Masson's trichrome, and Perl's stains [16].

NAFLD gradation and liver fibrosis staging was done according to the guidelines of Brunt et al. [16]. The gradation was based on the presence of macrovesicular steatosis (simple steatosis) and necroinflammatory activity (presence of NASH), as such:

Macrovesicular steatosis—grade 0, no steatosis; grade 1 (mild), <33 % fat accumulation in hepatocytes; grade 2 (moderate), between 33 and 66 % of hepatocytes affected; and grade 3 (severe), >66 % of hepatocytes affected.

Statistical Analysis

Statistical analyses were performed with the SPSS version 17.0 (SPSS Inc, Chicago, IL) statistical package. All data sets were tested for normality, and skewed data sets were log transformed for data analysis or evaluated by using nonparametric testing procedures. An association test between categorical variables was performed using chi-square test (χ^2). Correlations between continuous variables were performed by Spearman's correlation test. Statistical comparisons for significance were made using the Kruskal–Wallis nonparametric test as appropriate. After significant difference at 5 % level was detected between numerical variables of three or more groups, a Mann–Whitney test for each pair was applied separately to identify where the statistical difference was, by calculating the adjusted p value, in order to control the type I error (α). Statistical significance was assumed if a null hypothesis could be rejected at $p=0.05$.

Results

The sample group consisted of 68 patients with a histological diagnosis of NAFLD and class III obesity, 45 (66.2 %) of whom were female. Average age was 39.6±11.0 years. Of all the patients assessed, more than half had mild steatosis (52.9 %), followed by NASH (29.4 %), moderate and severe steatosis (11.8 %), and necrosis (5.9 %).

Regarding vitamin A nutritional status (VANS), 35.9 % of the sample group was found to have inadequate serum retinol levels, 67.6 % was found to have inadequate liver vitamin A reserves, while 26.5 % was shown to have liver reserves classified as critical (<5≥0.6 mg/g), and the liver reserves of 19.1 % were deemed to be absent (<0.6 mg/g).

Although no significant association ($p=0.214$; $\chi^2=4.486$) was found between NAFLD severity and serum retinol concentration (Table 1), a significant association ($i<0.001$, $\chi^2=30.792$) was found between liver retinol concentration and the histological classification of the disease, showing this parameter to be the earliest indicator of vitamin A deficiency at the various stages of the disease (Table 2, Fig. 1). No association between serum retinol concentration and liver reserves of the micronutrient was found ($p=0.190$, $\chi^2=4.767$). No significant correlation was observed between serum and hepatic vitamin A in the overall group ($r=-0.125$; $p=0.324$). Also, there were no significant correlations between BMI and serum or liver retinol ($p=0.788$ and $p=0.324$, respectively), and no association was found between gender and serum or hepatic vitamin A concentrations ($p=0.370$ and $p=0.274$, respectively).

Discussion

In this study, every individual in the sample group had some degree of NAFLD. Previous studies involving the class III obese have also found a near-total (85–90 %) prevalence of the disease in this population, depending on the method of diagnosis used, and a NASH presence ranging from 25 to 70 % [17, 18]. It is noteworthy that in general, NAFLD diagnosis is accomplished by way of

Table 1 Association between serum retinol levels and histological classification of nonalcoholic fatty liver disease

	Mild steatosis *n* (%)	Moderate to severe steatosis *n* (%)	Nonalcoholic steatohepatitis *n* (%)	Necrosis *n* (%)	*p* value
Adequate retinol (≥1.05 mmol/L)	24 (68.6 %)	3 (42.9 %)	10 (55.6 %)	4 (100 %)	0.214
Inadequate retinol (<1.05 mmol/L)	11 (31.4 %)	4 (57.1 %)	8 (44.4 %)	0 (0 %)	

imaging methods, and this study was conducted using a histological diagnosis of the disease.

The relationship between the severity of liver diseases of other etiologies and low plasma concentrations of vitamin A has previously been described [19–21]. However, no prior studies have assessed VANS using the gold standard indicator that is liver retinol reserves, given the technical and ethical difficulty of performing liver biopsies on patients with liver disease, which was made possible for this research by carrying out the biopsy during the act of gastroplasty surgery on patients being treated for obesity.

A high proportion of inadequacy was found in the serum retinol concentrations of the study participants, and the level of inadequacy nearly doubled when liver vitamin A reserves, the earliest indicator of VANS, were assessed. No relationship was found between serum and liver VANS indicators in this study. Serum retinol is considered easy to execute and low cost, when compared to other biochemical markers for diagnosing VAD, and is widely used in diagnosing and monitoring VAD [22]. However, liver vitamin A concentrations are known to decrease steadily for a considerable amount of time before serum levels are affected. That being the case, plasma retinol concentration is subject to homeostatic control, does not correlate linearly with liver vitamin A reserves, and is therefore not a good indicator for identifying marginal VAD [23].

Thus, although serum retinol appears to be a good marker for the progression of liver disease [19–21], there is still a need to discuss what the best cutoff point for classifying VAD in these patients would be, given how a seemingly adequate concentration of serum retinol is often associated with the depletion of stores of the vitamin in the liver, making clear how weak this indicator is for assessing VANS and NAFLD [19].

The dearth of research evaluating VANS in NAFLD in humans shows a clear need for further studies to better characterize the relationship between the vitamin and NAFLD. However, there are hypotheses that can be made regarding this relationship.

The first hypothesis concerning the relationship between drops in the levels of serum and liver vitamin A in patients with NAFLD is based on increased consumption of the vitamin for antioxidant purposes. Chaves et al. [3], evaluating NAFLD sufferers of class 3 obesity and fatty liver disease, found average β-carotene to be significantly less in the group with the disease. The same was not found for serum retinol values, probably because of the greater antioxidant capacity inherent in β-carotene.

A study carried out to assess serum carotenoid concentrations in 350 individuals, classified according to the degree of accumulated fat in the liver (healthy liver, moderate or severe level of steatosis), revealed that serum β-carotene decreased significantly as liver fat increased. The same was not found to be true for the other carotenoids evaluated [4].

Musso et al. [24], by comparing NAFLD patients with a control group matched according to severity of IR, adiposity, and metabolic syndrome, found that reducing the intake of vitamin A correlated independently with the severity of liver disease; oxidative stress, assessed by way of nitrotyrosine concentration, was present throughout all stages of the disease, even in individuals without IR. Therefore, this suggests that adequate intake of vitamin A, particularly carotenoids, is an important means of protection against the oxidative attack of free radicals on cell membranes, reducing oxidative damage and, as a result, avoiding the development of chronic diseases.

Table 2 Association between liver vitamin A reserves and histological classification of nonalcoholic fatty liver disease

		Mild steatosis *n* (%)	Moderate to grave steatosis *n* (%)	Nonalcoholic steatohepatitis *n* (%)	Necrosis *n* (%)	*p* value
Liver retinol reserves	Adequate (≥20 μg/g)	15 (41.7 %)	1 (12.5 %)	5 (25 %)	1 (25 %)	<0.001
	Inadequate (<20 and ≥5 μg/g)	10 (27.8 %)	4 (50 %)	1 (5 %)	0 (0 %)	
	Critical (<5 and ≥0.6 μg/g)	10 (27.8 %)	3 (37.5 %)	5 (25 %)	0 (0 %)	
	Nonexistent (<0.6 μg/g)	1 (2.7 %)	0 (0 %)	9 (45 %)	3 (75 %)	

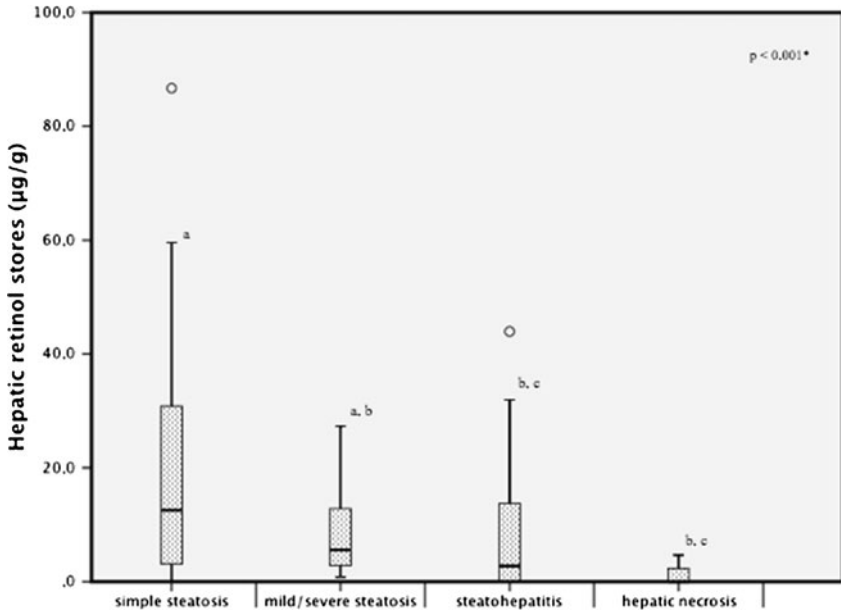

Fig. 1 Distribution of hepatic retinol reserves according to the histological classification of nonalcoholic fatty liver disease

Another associated factor of VAD and NAFLD is the aforementioned relationship between IR and VANS. In class 3 obese NAFLD sufferers, the HOMA-IR index, used to assess insulin resistance, correlated negatively with insufficient β-carotene. Moreover, almost all the individuals with low plasma β-carotene and retinol concentrations had IR [3]. Sugiura et al. [25] found an inverse association between plasma carotenoid concentrations and HOMA-IR-estimated IR, which supports the hypothesis that carotenoids may have a protective effect on IR pathogenesis, probably for their role as a protective agent against OS, given how it has been suggested that an increase in OS implies a decrease in insulin action.

Lastly, an increased expression of retinol metabolism-related proteins and enzymes has been demonstrated in NAFLD, suggesting that the process of retinol oxidation to all-*trans*-retinoic acid (ATRA) is accelerated in the presence of the disease. We also found an increase in the expression of CYP26A1, which is most responsible for ATRA degradation, which may represent an important mechanism in the progression of the disease. Moreover, greater degradation of ATRA leads to lower vitamin A stocks in the stellate cells, which is linked to loss of retinoid signaling, with increased OS and progression of the disease [26]. This explains the association between lower liver retinol concentrations and severity of the disease in these findings.

It is suggested that ATRA supplementation promotes oxidation of triglycerides in the liver. The proposed mechanisms are the following: (1) There is an increase in the hepatic expression of the genes that encode proteins that promote fatty acid oxidation (PPAR-α, RXR-α, liver-type carnitine palmitoyltransferase 1, carnitine/acylcarnitine carrier, uncoupling protein 2) and (2) there is a reduction in the hepatic expression of genes that encode proteins involved in lipogenesis (SREBP-1c, fatty acid synthase). This reduction in liver fat stores may be a contributing factor in the previously demonstrated improved insulin sensitivity of rats treated with ATRA, showing that vitamin A may be a protective agent in the development of steatosis in situations where there is an increased flow of free fatty acids to the liver, as is the case with fat-rich diets, abdominal obesity, and rapid weight loss [27]. However, there is no research that has tested the efficacy of vitamin A supplementation alone in the treatment of NAFLD, and issues such as effective dose still have to be determined.

Conclusion

The high rate of retinol inadequacy in the liver of obese sufferers of NAFLD and its association with the severity of the disease suggest a need for research that establishes a causal relationship between VAD and NAFLD. Serum was not shown to be a reliable indicator for assessing VANS in these individuals, given its lack of association with severity of the disease, and although it is not yet possible to implement intervention protocols involving vitamin supplementation for the class III obese, in order to prevent the disease from progressing, clinicians should attempt to ensure adequate dietary intake of vitamin A.

Conflict of Interest The authors Gabriela Chaves, Silvia Pereira, Carlos Saboya, Daiane Spitz, Camila Rodrigues, and Andréa Ramalho certify that there is no conflict of interest.

References

1. Festi D, Colecchia A, Sacco T, et al. Hepatic steatosis in obese patients: clinical aspects and prognostic significance. Obes Rev. 2004;5(1):27–42.
2. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology. 1998;114:842–5.
3. Chaves GV, Pereira SE, Saboya CJ, et al. Non-alcoholic fatty liver disease and its relationship with nutritional status of vitamin A in individuals with class III obesity. Obes Surg. 2008;18: 378–85.
4. Park SK, Lee HJ, Lee DH, et al. Associations of non alcoholic fatty liver disease with the metabolic syndrome and serum carotenoids. J Prev Med Public Health. 2008;41:39–44.
5. Yadav D, Hertan HI, Schweitzer P, et al. Serum and liver micronutrient antioxidants and serum oxidative stress in patients with chronic hepatitis C. Am J Gastroenterol. 2002;97:2634–9.
6. Friedman LS. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev. 2008;88:125–72.
7. Shirakami Y, Gottesman ME, Blaner WS. Diethylnitrosamine-induced hepatocarcinogenesis is suppressed in lecithin:retinol acyltransferase-deficient mice primarily through retinoid actions immediately after carcinogen administration. Carcinogenesis. 2012;33(2):268–74.
8. Cuppari L. Guias de Medicina Ambulatorial e Hospitalar. Nutrição Clínica no Adulto. UNIFESP. São Paulo: Manole, 2002
9. World Health Organization. Obesity: preventing and managing the global epidemic. Report of a WHO Consultation on Obesity. WHO: Geneva, 1998
10. World Health Organization. Indicators for assessing vitamin A deficiency and their application in monitoring and evaluating intervention programmes. Micronutrient Series, WHO/NUT. 10. World Health Organization, Geneva, Switzerland, 1996
11. IVACG (International Vitamin A Consultative Group). Improving the vitamin A status of populations. USAID-ILSI. 2003
12. Flores H, Ramalho RA, Ribeiro AR. Intrahepatic distribution of vitamin A in humans and rats. Int J Vit Nutr Res. 1988;58(3): 276–80.
13. Araujo CR, Flores H. Improved spectrophotometric vitamin A assay. Clin Chem. 1978;24(2):386.
14. Arroyave G, Chichester CO. Biochemical methodology for the assessment of vitamin A status: a report of International Vitamin A Consultative Group. The Nutrition Foundation: Washington; 1982.
15. Olson JA, Gunning D, Tilton R. The distribution of vitamin A in human liver. Am J Clin Nutr. 1979;32(12):2500–7.
16. Brunt EM, Janney CG, Di Bisceglie AM, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94(9):2467–74.
17. Angulo P. GI epidemiology: nonalcoholic fatty liver disease. Aliment Pharmacol Ther. 2007;25:883–9.
18. Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol. 2006;40:s5–s10.
19. Peres WA, Chaves GV, Gonçalves JCS, et al. Vitamin A deficiency in patients with hepatitis C virus-related chronic liver disease. British J Nutr. 2011;6(11):1724–31.
20. Paula TP, Ramalho A, Braulio VB. The effectiveness of relative dose response to retinol intake as an evaluation of vitamin A status of cirrhotic patients. J Hum Nutr Diet. 2010;23(6):583–9.
21. Clemente C, Elba S, Buongiorno G, et al. Serum retinol and risk of hepatocellular carcinoma in patients with Child-Pugh class A cirrhosis. Cancer Lett. 2002;178:123–9.
22. Tanumihardjo SA. Vitamin A: biomarkers of nutrition development. Am J Clin Nutr. 2011;94:658S–65S.
23. IOM (Institute of Medicine). Food and nutrition board. Vitamin A. In: Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, vanadium, manganese, molybdenum, nickel, silicon, vanadium and zinc. Washington: National Academy Press; 2001. pp. 82–161.
24. Musso G, Gambino R, De Micheli F, et al. Nitrosative stress predicts the presence and severity of nonalcoholic fatty liver at different stages of the development of insulin resistance and metabolic syndrome: possible role of vitamin A intake. Am J Clin Nutr. 2007;86:661–71.
25. Sugiura M, Nakamura M, Ikoma Y, et al. The homeostasis model assessment-insulin resistance index is inversely associated with serum carotenoids in non-diabetic subjects. J Epidemiol. 2006;16:71–8.
26. Ashla AF, Hoshikawa Y, Tsuchiya H, et al. Genetic analysis of expression profile involved in retinoid metabolism in non-alcoholic fatty liver disease. Hepatol Res. 2010;40:594–604.
27. Amengual J, Ribot J, Bonet L, et al. Retinoic acid treatment enhances lipid oxidation and inhibits lipid biosynthesis capacities in the liver of mice. Cell Physiol Biochem. 2010;25:657–66.