Effect of Omega-3 Supplementation on Lipocalin 2 and Retinol-Binding Protein 4 in Type 2 Diabetic Patients

Payam FARAHBAKHSH-FARSI, Abolghassem DZAYERY, Mohammad Reza ESHRAGHIAN, Fariba KOOHDANI, Mahnaz ZAREI, Mohammad Hassan JAVANBAKHT, Hoda DERAKHSHANIAN, *Mahmoud DJALALI

1. Dept. of Cellular and Molecular Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran
2. Dept. of Community Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran
3. Dept. of Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding Author: Email: mjalali87@yahoo.com

(Received 13 May 2015; accepted 21 Sep 2015)

Abstract
Background: Serum levels of lipocalin 2 (LCN 2) and retinol-binding protein-4 (RBP 4), increase in type 2 diabetes mellitus (T2DM). We sought to determine whether serum LCN 2 and RBP 4 change after an intervention with omega-3 fatty acids supplementation in diabetic patients.
Methods: Forty-five type 2 diabetic patients from Iranian Diabetic Association in Tehran, Iran in 2013 were randomly recruited into two groups: one group received 4 g/d omega-3 for 10 wk; and the control group received placebo. Blood samples, food intake records, anthropometric measurements were obtained from all participants at the beginning and end of the study.
Results: Fasting RBP 4 plasma levels significantly changed after 10 wk supplementation (P = 0.01). The LCN 2 concentrations decreased in omega-3 group, but the changes were not statistically significant. Omega-3 supplementation had no noticeable effect on anthropometric factors.
Conclusions: These findings provide a rationale for omega-3 supplements aimed at lowering serum RBP 4 levels in T2DM.
Keywords: Omega-3 fatty acid, Lipocalin, Retinol-binding protein, Diabetes mellitus

Introduction

Lipocalins family encompasses more than twenty different proteins. Theses mall, soluble protein shave different sequences with only 20% similarity to each other. However, all of them composed of 8 antiparallel beta-barrels. Lipophilic substances such as free fatty acids, retinoids, arachidonic acid and steroids pass through a hole inside the barrel (1, 2). Lipocalin 2 is a 25kDa glycoprotein, identified as neutrophil gelatinase associated lipocalin (NGAL), neutrophil lipocalin (NL) and 24P3 oncogene. This protein secreted mainly from the liver and adipose tissue (3). LCN 2 expression is increased by substances that improve insulin resistance and is decreased by thiazolidinediones (TZD) (4). Lipocalin 2 up-regulates peroxisome proliferator-activated receptor gamma (PPARγ) and its target genes including leptin, adiponectin, fatty acid synthase (FAS) and lipoprotein lipase in adipose tissue (5).
Retinol-binding protein 4 (RBP 4) belongs to lipocalins family and transfers small hydrophobic molecules form membranes (6). Its gene locates
on the long arm of chromosome 10, near glucose homeostasis area in European-Caucasian ethnicity and near region of type 2 diabetes in Mexicans-Africans (7, 8). The protein consists of 201 amino acids and its molecular weight is 21kDa (9, 10). The highest expression is seen in the liver and adipose tissue. RBP 4 increased in the early stages of stroke and cardiovascular disease (11-13). Serum RBP 4 might have a negative correlation with insulin resistance and development of T2DM (14, 15). RBP 4 is connected to diabetes through insulin secretion, and pancreas beta cells play a key role in this relationship (15). Significant weight loss by diet, lifestyle modifications and bariatric surgery may reduce RBP 4 levels (16). Improvement in insulin resistance by exercising also reduces RBP 4 concentration (17). RBP4 concentrations increase in humans with obesity and type 2 diabetes. Rosiglitazone, an insulin-sensitizing drug that activates PPARγ, decreases RBP4 concentrations. Lowering RBP 4 might be a novel strategy that helps type 2 diabetic patients (18). Ecological studies have shown that marine foods may negatively associate with insulin resistance in obese subjects. Meta-analysis reports omega-3 fatty acids in the Asian population, unlike the western population, protect against T2DM. However, Asian patients have lower levels of omega-3 than western patients in their plasma and cell membranes (19). EPA correlates with up-regulation of PPARγ and down-regulation of interleukin-6 and tumor necrosis factor-alpha (TNFα). Results suggest that EPA probably change PPARγ activation and expression (20). We carried out this study to compare the effect of omega-3 supplementation versus placebo on LCN 2 and RBP 4 levels in type 2 diabetic patients. We hypothesized that the omega-3 supplementation would reduce these adipokines after 10 weeks as well as TZD.

Materials and Methods

In the present double blind randomized controlled trial in 2013, 45 subjects of both sexes, 40-65 yr old, tested and diagnosed with diabetes mellitus by physician, were enrolled during appointments at the Iranian Diabetic Association Center in Tehran, Iran. Fort four participants successfully completed the study. The inclusion and exclusion criteria are listed in Table 1.

| Inclusion criteria | Exclusion criteria |
|--------------------|--------------------|
| 30-65 year old BMI 18.5- 40kg/m² | Consuming dietary supplements at least 2 weeks before and throughout the intervention; Consuming omega-3 supplements in the last 3 months; Chronic renal, hepatic, gastrointestinal, hematological diseases, thyroid disorder; Using orlistat, sibutramine or any other drug for weight loss; Pregnancy and lactation; Thiazolidinediones insulin therapy |

Randomization was carried out using a random permuted block. All participants (45 T2DM) were randomized to the following groups based on BMI: 1) 23 type 2 diabetic patients constituted the case group; and 2) 22 T2DM patients served as the control group. The two homogeneous groups were prescribed four capsules of omega-3 fatty acids or four capsules of placebo per day. To avoid possible adipokines and progesterone level changes in the luteal phase, premenopausal female patients were asked to begin treatment in the early follicular phase of their menstrual cycles. All participants were requested to maintain their usual exercise and dietary habits. The supplement formulation contained 310 mg eicosapentaenoic acid (EPA), 210 mg docosahexaenoic acid (DHA),

Available at:  [http://ijph.tums.ac.ir](http://ijph.tums.ac.ir)
110mg other polyunsaturated fatty acids and 5mg vitamin E (Maxepa Forte Capsules, Seven Seas, UK). Placebo was composed of paraffin oil that absolutely resembled omega-3 (Zahravi, Iran). The compliance was approximately over 90% for those who remained in study. Researchers were instructed to pay special attention to measure levels of patients’ compliance to ensure the validity of the final data of the investigation. Therefore, subjects were followed each 2 wk after enrolling to study during the 10 wk of follow-up. Blood sample was drawn at the beginning and the end of the study after at least 12 h fasting. All blood samples were centrifuged at 3000 g for 10 min and sera were separated into the clean tube aliquots and were stored at -80 °C.

Dietary intake was assessed using the 3-day food records (comprising two working days and a weekend) at the beginning and end of the intervention. The portion sizes of the consumed foods were explained to all participants by a trained nutritionist. All food items were converted to grams. Modified Nutritionist IV software was used to estimate the energy and nutrient intakes.

Weight was measured using a digital scale (803, Seca Clara, Germany) with an accuracy of 100 g, in light clothes and without shoes. Height was measured without shoes using a stadiometer (206, Seca, Germany) with an accuracy of 0.1 cm. Hip and waist circumferences were measured using a measuring tape (201, Seca, Germany) with an accuracy of 0.1 cm. Body mass index (BMI) was calculated using these recorded values [weight(kg)/hight²(m)]. LCN-2 and RBP-4 were measured by research enzymatic calorimetric ELISA kits (Boster Biological Technology Ltd, China). The tests were conducted according to the company’s instructions.

Data were analyzed using the SPSS 18.0 for Windows (Chicago, IL, USA). The results are explained as mean ± SEM. Student’s t-tests were used to compare the two groups and paired t-tests were used for competing before and after data within each group. P< 0.05 and more than 80% power were considered statistically significant.

**Results**

A total of 45 type 2 diabetic patients were invited to participate in the study. One patient in omega-3 group did not complete the study. Participant characteristics are shown in Table 2. The sample (n=44) of diabetics who completed study had similar characteristics, as there were no significant differences between the group characteristics.

| Variable | Omega-3 (n=22) | Placebo (n=22) | P value‡ |
|----------|----------------|----------------|----------|
| Age (yr) | Before 54.23 ± 1.64 | 53.32 ± 1.45 | 0.68 |
| Height (cm) | Before 162 ± 2.11 | 156 ± 1.37 | 0.02 |
| Weight (kg) | Before 69.21 ± 2.84 | 63.57 ± 2.65 | 0.15 |
| BMI (kg/m²) | Before 26.19 ± 0.78 | 25.93 ± 0.92 | 0.83 |
| WHR | Before 0.84±0.01 | 0.85±0.01 | 0.1 |
| Difference | 0.003±0.002 | -0.005±0.003 | 0.08 |

WHR: waist to hip ratio, All values are expressed as means ±SEM, §paired t-test †two independent sample tests

Available at: http://ijph.tums.ac.ir
At the end of the study, retinol-binding protein-4 decreased significantly in omega-3 group ($P<0.001$). But the changes of lipocalin 2 was not significant following omega-3 supplementation comparing the baseline amount of the same group ($P=0.14$). However, after 10 wk of intervention, there was significant difference in retinol-binding protein 4 and lipocalin 2 between the two groups ($P=0.01$, and $P=0.03$, respectively). Comparing the amount of change between two groups showed a significant difference for RBP 4 but not LCN 2 ($P=0.01$ and $0.08$, respectively (Table 3). There were no significant changes in dietary intake of omega-3 and omega-6 during the study (Table 4).

Table 3: Plasma LCN 2 and RBP 4 at baseline and after the intervention

|                      | Omega-3 (n=22)   | Placebo (n=22)   | $P$ value$‡$ |
|----------------------|------------------|------------------|--------------|
| Lipocalin 2 (pg/ml)  | Before 6161.77±207.07 | 6302.59±316.28 | 0.711        |
|                      | After 5635.73±284.73 | 6537.73±298.74 | 0.034        |
| Difference           | -616.95±456.14    | 728.31±595.48   | 0.08         |
| $P$ value$§$         | 0.14             | 0.64            |
| Retinol-binding protein 4 (μg/ml) | Before 28.54±1.16 | 27.51±1.57 | 0.60        |
|                      | After 17.69±0.98  | 24.21±2.27      | 0.012        |
| Difference           | -10.85±1.62      | -3.29±2.6       | 0.01         |
| $P$ value$§$         | 0.000            | 0.21            |

All values are expressed as means ±SEM $§$ paired t-test $‡$ two independent sample tests

Table 4: Omega-3 and omega-6 dietary intakes at baseline and 10th wk of study

|                  | Omega-3 (n=22) | Placebo (n=22) | $P$ value$‡$ |
|------------------|----------------|----------------|--------------|
| omega-3 (gr)     | Before 0.96±0.12 | 1.19±0.11 | 0.18         |
|                  | After 1.07±0.12 | 1.04±0.12 | 0.84         |
| Difference       | 0.11±0.15      | -0.14±0.18   | 0.29         |
| $P$ value$§$     | 0.47           | 0.44          |
| omega-6 (gr)     | Before 20.06±1.37 | 20.53±1.11 | 0.69         |
|                  | After 20.09±1.06 | 22.90±2.39 | 0.33         |
| Difference       | 0.02±1.37      | 2.37±2.75    | 0.45         |
| $P$ value$§$     | 0.86           | 0.46          |

All values are expressed as means ±SEM $§$ paired t-test $‡$ two independent sample tests

**Discussion**

This study for the first time, in our knowledge, investigated the effect of omega-3 supplementation on LCN 2 and RBP 4 concentrations in type 2 diabetic patients. In previous studies changes in the plasma expression of other adipokines such as leptin, adiponectin, and resistin has been reported faced with n-3 fatty acids (21). The results of this double-blind randomized clinical trial demonstrate that intake of 4g omega-3 for 10 wk leads to significant decrease in RBP 4 in T2DM patients. Considering that RBP 4 and LCN 2 come from an analogous family, our hypothesis was their modifications are paralleled. LCN 2 and RBP 4 were decreased in omega-3 group versus placebo group, but mean differences between the two groups only for RBP 4 was statistically significant. Study of the adipocyte cells showed that substances cause insulin resistances; elevate LCN 2 expression while TZD reduce its expression. In addition, insulin resistance might be induced by
exogenous LCN 2. Animal models demonstrate a sharp decline in the level of LCN 2 contribute to insulin action improvement (4). Because of n-3 PUFA is an anti-inflammatory agent and decrease insulin resistance in some studies (22, 23), it seems that omega-3 has a similar mechanism with TZD. Hyperinsulinemic state in human participants definitely increased circulating lipocalin 2 concentrations which indicate the up regulating effect of insulin on lipocalin 2 (5). In a cross sectional study, LCN 2 did not correlate with total body fat or body weight and it could not play a prominent role in prediction of metabolic risk factors (24). Our finding about LCN 2 dealing with body weight was consistent with it. In subjects with impaired fasting glucose, impaired glucose tolerance and type 2 diabetes mellitus, lipocalin 2 concentrations are higher than people with normal glucose metabolism. Glucose metabolism disturbance in T2DM, independently correlates with increased level of LCN 2 (25). In the present study, LCN 2 level was not measured in normal subjects. Various agents with different mechanism can influence on RBP4 such as orlistat, an anti-obesity drug, that promote lipid profile, glycemic control and insulin resistance (26). Acarbose, an anti-diabetic drug, also improve lipid profile, glycemic control and insulin resistance. In addition, acarbose reduce RBP 4 and adiponectin levels (27). Both drugs have local effect, but omega-3 has systematic effects via different pathways. Pan et al. investigated the effects of a flaxseed-derived lignan supplement on RBP 4 in type 2 diabetic patients, so after 12 wk flaxseed oil lignan did not change RBP 4 level (28), this issue could be related to different type of omega-3 applied in the study. Agonists of PPARγ improve RBP 4. Administration of pioglitazone, an insulin sensitizer, for 12 wk in diabetic patients, showed that RBP 4 correlates with insulin resistance and complication of diabetes (29). One study compared the effect of pioglitazone versus gliclazide, another anti-diabetic drug. After approximately 3 months, pioglitazone significantly decreased serum RBP 4 and HOMA-IR values, but gliclazide did not change these variables in type 2 diabetic patients (30). Because omega-3 is also PPARγ agonist, our finding about significant decrease in serum RBP 4 in intervention group was consistent with previous study (30).

Conclusion

Fish oil has several benefits in metabolic syndrome and cardiovascular diseases, but its role in diabetes remains controversial. The present study introduced a new viewpoint about omega-3 supplementation effects on adipokins that relates to insulin resistance in diabetic patients. The results of our study showed beneficial effects of omega-3 on LCN 2 and RBP 4. Further studies are needed to elucidate cellular-molecular pathways, particularly changes in PPARγ gene expression with different doses of omega-3 in diabetics.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

The present double blind randomized controlled trial study was performed under the approval of the Institutional Review Boards of the Tehran University of Medical Sciences (Tehran, Iran). We would like to thank the Iranian Diabetic Society, especially Dr. Assad Allah Rajab for their collaboration and introduction of diabetic patients. This study was supported by Health Services grants from the School of Nutritional Science and Dietetics of Tehran University of Medical Sciences (ID: 15177). Clinical trial.gov registration number: NCT01478776. The authors declare that there is no conflict of interests.

References

1. Kehrer JP (2010). Lipocalin-2: pro- or anti-apoptotic? Cell Biol Toxicol, 26:83-9.

Available at:  http://ijph.tums.ac.ir
2. Miharada K, Hiroyama T, Sudo K, Nagasawa T, Nakamura Y (2005). Lipocalin 2 functions as a negative regulator of red blood cell production in an autocrine fashion. *FASEB J*, 19:1881-3.

3. Stejskal D, Karpek M, Humenanska V, Hanulova Z, Stejskal P, Kusnierova P, Petzel M (2008). Lipocalin-2: development, analytical characterization, and clinical testing of a new ELISA. *Horm Metab Res*, 40:381-5.

4. Yan QW, Yang Q, Mody N, Graham TE, Hsu CH, Xu Z, Houstis NE, Kahn BB, Rosen ED (2007). The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance. *Diabetes*, 56:2533-40.

5. Tan BK, Adya R, Shan X, Syed F, Lewandowski KC, OHare JP, Randeva HS (2009). Ex vivo and in vivo regulation of lipocalin-2, a novel adipokine, by insulin. *Diabetes Care*, 32:129-31.

6. Flower DR (1996). The lipocalin protein family: structure and function. *Biochem J*, 318 (Pt 1):1-14.

7. Duggirala R, Blangero J, Almasy L, Dyer TD, Williams KL, Leach RJ, O’Connell P, Stern MP (1999). Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans. *Am J Hum Genet*, 64:1127-40.

8. Meigs JB, Panhuysen CI, Myers RH, Wilson PW, Cupples LA (2002). A genome-wide scan for loci linked to plasma levels of glucose and HbA1c in a community-based sample of Caucasian pedigrees: The Framingham Offspring Study. *Diabetes*, 51:833-40.

9. Colantuoni V, Romano V, Bensi G, Santoro C, Costanzo F, Raugei G, Cortese R (1983). Cloning and sequencing of a full length cDNA coding for human retinol-binding protein. *Nucleic Acids Res*, 11:7769-76.

10. Jaconi S, Rose K, Hughes GJ, Saurat JH, Siegenthaler G (1995). Characterization of two post-translationally processed forms of human serum retinol-binding protein: altered ratios in chronic renal failure. *J Lipid Res*, 36:1247-53.

11. Mallat Z, Simon T, Benessiano J, Clement K, Taleb S, Wareham NJ, Luben R, Khaw KT, Tedgui A, Boekholt SM (2009). Retinol-binding protein 4 and prediction of incident coronary events in healthy men and women. *J Clin Endocrinol Metab*, 94:255-60.

12. Sasaki M, Otani T, Kawakami M, Ishikawa SE (2010). Elevation of plasma retinol-binding protein 4 and reduction of plasma adiponectin in subjects with cerebral infarction. *Metabolism*, 59:527-32.

13. Ingelsson E, Sundstrom J, Melhus H, Michaelsson K, Berne C, Vasan RS, Riserus U, Blomhoff R, Lind L, Arnlов J (2009). Circulating retinol-binding protein 4, cardiovascular risk factors and prevalent cardiovascular disease in elderly. *Atherosclerosis*, 206:239-44.

14. Promitzer M, Krebs M, Todoric J, Lugger A, Bischof MG, Nowotny P, Wagner O, Estebauer H, Anderwald C (2007). Insulin resistance is unrelated to circulating retinol binding protein and protein C inhibitor. *J Clin Endocrinol Metab*, 92:4306-12.

15. Broch M, Vendrell J, Ricart W, Richart C, Fernandez-Realt JM (2007). Circulating retinol-binding protein-4, insulin sensitivity, insulin secretion, and insulin disposition index in obese and nonobese subjects. *Diabetes Care*, 30:1802-6.

16. Haider DG, Schindler K, Prager G, Bohdjalian A, Lugger A, Wolzt M, Ladvik B (2007). Serum retinol-binding protein 4 is reduced after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab*, 92:1168-71.

17. Graham TE, Yang Q, Bluhm M, Hammarstedt A, Ciaramdi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB (2006). Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med*, 354:2552-63.

18. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB (2005). Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature*, 436:356-62.

19. Zheng JS, Huang T, Yang J, Fu YQ, Li D (2012). Marine N-3 polyunsaturated fatty acids are inversely associated with risk of type 2 diabetes in Asians: a systematic review and meta-analysis. *PLoS One*, 7:e44525.

20. Magee P, Pearson S, Whittingham-Dowd J, Allen J (2012). PPARgamma as a molecular target of EPA anti-inflammatory activity during TNF-alpha-impaired skeletal muscle cell differentiation. *J Nutr Biochem*, 23:1440-8.

21. Drevon CA (2005). Fatty acids and expression of adipokines. *Biochim Biophys Acta*, 1740:287-92.
22. Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L, Lee WH, Fitzgerald KA, Hwang DH (2004). Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. J Biol Chem, 279:16971-9.

23. Serhan CN, Chiang N, Van Dyke TE (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol, 8:349-61.

24. Liu X, Harnvik OP, Petrou M, Gong H, Chamberland JP, Christophi CA, Kales SN, Christiani DC, Mantzoros CS (2011). Circulating lipocalin 2 is associated with body fat distribution at baseline but is not an independent predictor of insulin resistance: the prospective Cyprus Metabolism Study. Eur J Endocrinol, 165:805-12.

25. Huang Y, Yang Z, Ye Z, Li Q, Wen J, Tao X, Chen I, He M, Wang X, Lu B, Zhang Z, Zhang W, Qu S, Hu R (2012). Lipocalin-2, glucose metabolism and chronic low-grade systemic inflammation in Chinese people. Cardiovasc Diabetol, 11:11.

26. Derosa G, Cicero AF, D’Angelo A, Fogari E, Maffioli P (2012). Effects of 1-year orlistat treatment compared to placebo on insulin resistance parameters in patients with type 2 diabetes. J Clin Pharm Ther, 37:187-95.

27. Derosa G, Maffioli P, D’Angelo A, Fogari E, Bianchi L, Cicero AF (2011). Acarbose on insulin resistance after an oral fat load: a double-blind, placebo controlled study. J Diabetes Complications, 25:258-66.

28. Pan A, Demark-Wahnefried W, Ye X, Yu Z, Li H, Qi Q, Sun J, Chen Y, Chen X, Liu Y, Lin X (2009). Effects of a flaxseed-derived lignan supplement on C-reactive protein, IL-6 and retinol-binding protein 4 in type 2 diabetic patients. Br J Nutr, 101:1145-9.

29. Takebayashi K, Suetsugu M, Wakabayashi S, Aso Y, Inukai T (2007). Retinol binding protein-4 levels and clinical features of type 2 diabetes patients. J Clin Endocrinol Metab, 92:2712-9.

30. Lin KD, Chang YH, Wang CL, Yang YH, Hsiao PJ, Li TH, Shin SJ (2008). Thiazolidinedione addition reduces the serum retinol-binding protein 4 in type 2 diabetic patients treated with metformin and sulfonylurea. Transl Res, 151:309-14.