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Relationship Between Vitamin D, PTH and Insulin Resistance in the Development of Non-Alcoholic Fatty Liver Disease (NAFLD)

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ABSTRACT

Introduction: Vitamin D plays an important role in the regulation of several genes associated with metabolic disorders. This study was designed to investigate the relationship between serum 25 (OH) D and parathyroid hormone (PTH) levels, and insulin resistance in patients with non-alcoholic fatty liver disease (NAFLD). **Methods:** A case-control study was carried out among patients with NAFLD (n= 80) as cases and age-matched subjects without NAFLD (n= 80) as controls. After 8-12 h of fasting, serum 25 (OH) D, insulin and PTH levels were assessed using Enzyme-Linked Immunosorbent Assay (ELISA) technique. Multivariate logistic regression model was applied to assess the relationship between vitamin D and PTH and insulin resistance in the development of NAFLD by adjusting for the confounders (sex, BMI and waist-to-hip ratio). **Results:** There was no significant difference in sunlight exposure between the two groups (p= 0.274). Patients with NAFLD had significantly lower serum 25(OH) D levels and higher PTH levels compared to subjects without NAFLD ($p< 0.001$). The association between NAFLD and low 25(OH) vitamin D levels was independent of confounders (adjusted OR: 8.78, CI 95%:1.71, 45.03). Homeostatic Model Assessment Insulin Resistance (HOMA-IR) as insulin resistance index was significantly high in the NAFLD group (adjusted OR: 1.80, CI 95%: 1.32, 2.47). **Conclusion:** Our findings showed that lower serum 25 (OH) D levels and higher HOMA indices were independently associated with increased odds of NAFLD and there was a direct but not significant relationship between PTH serum levels and the risk of NAFLD.

Key words: 25(OH) D, insulin resistance, NAFLD, obesity, PTH

INTRODUCTION

Studies about the role of low levels of 25(OH) D and high levels of parathyroid hormone (PTH), in the development of metabolic syndrome and fatty liver have demonstrated that severe vitamin D deficiency contributes to glucose metabolism abnormalities such as insulin resistance, glucose intolerance, metabolic syndrome and type 2 diabetes (Pittas *et al.*, 2007; Hyppönen *et al.*, 2008). Studies in Hawaii, Turkey, India, Iran and Saudi

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Arabia have shown a high prevalence of vitamin D deficiency due to avoidance of sunlight or clothing which prevents adequate sunlight exposure (Guzel *et al*., 2001; Harinarayan, 2005; Lips, 2007; Rahnavard *et al*., 2010). The prevalence of hypovitaminosis D among Middle East adult populations is 20% to 30% (LaBrecque *et al*., 2014), reaching a peak of 75% in patients with metabolic syndrome (MS)(Pinelli *et al*., 2010).

Low levels of 25(OH) D and high levels of PTH have been suggested as markers of metabolic syndrome and fatty liver. Severe vitamin D deficiency results in altered glucose metabolism, such as insulin resistance and glucose intolerance. A common disorder associated with increased inflammatory markers and insulin resistance is non-alcoholic fatty liver disease (NAFLD). It is the most prevalent chronic liver disease characterised by the accumulation of large droplets of triglycerides within hepatocytes, contributing to more than 5% of liver weight, in the absence of chronic alcohol consumption (Targher *et al*., 2007) .

According to the 'multi-hit' theory where a number of diverse parallel processes involving extra-hepatic factors (genetic and nutritional) may contribute to the development and progression of liver inflammation, the conversion and progression of simple steatosis to advanced fibrosis is a separate process. The first hit is insulin resistance which leads to an accumulation of triglycerides in hepatocytes and subsequently, lipid peroxidation. In the second process oxidative stress is involved and results in inflammation and initiation of osteohepatitis; the third hit consists of hepatocyte proliferation progenitors impairment (LaBrecque *et al*., 2014). An important mediator in this process is nuclear factor κ-β (NF-κB) that functions as a pro-inflammatory 'master switch' by up-regulating the transcription of a wide range of inflammatory mediators. Accordingly, in the liver of mice fed a high fat diet, increased NF-κB activity has been shown to be associated with an increased expression of pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β and the activation of Kupffer cells. These cytokines are capable of producing all classical histological features of Nonalcoholic Steatohepatitis (NASH) including hepatocyte necrosis/apoptosis, neutrophil chemotaxis and the activation of hepatic stellate cells. Moreover, human studies have demonstrated increased cytokine gene expression in the liver of patients with NASH compared to obese controls with a normal liver; a correlation between increased expression and severe histological findings was also revealed (Eliades & Spyrou, 2015).

Recent studies have come up with inconsistent results. Kayanigil *et al*. (2011) showed that serum 25 (OH) D but not PTH was significantly associated with metabolic syndrome. In the study by Barchetta *et al*.(2011) on patients with normal liver enzymes and NAFLD (confirmed by ultrasonography), low serum 25(OH) vitamin D levels were associated with NAFLD independently of metabolic syndrome, diabetes and an insulin-resistance profile. The association between serum 25 (OH) D and the prevalence of NAFLD was also shown in a retrospective cohort study and the results indicated that the role of serum 25 (OH) D as a NAFLD risk was independent of age, sex, BMI and other studied confounders (Jablonski *et al*., 2013). In another case-control study, *Bhatt et al.* (2013) analysed the association between 25(OH) D and PTH levels, and clinical, anthropometric, biochemical and body composition parameters in Asian Indians with NAFLD; the results demonstrated that low serum 25(OH) D and high PTH levels were independently associated with the presence of NAFLD.

The incidence of abdominal obesity and fatty liver is rising dramatically and necessitates identification of the

risk factors to help work out efficient solutions in this regard. According to the mechanisms mentioned above, and since liver converts vitamin D in its active form and due to the substantial role of vitamin D and PTH in the regulation of many genes associated with metabolic disorders, the present study was designed to investigate the relationship between 25 (OH) D and PTH serum levels and insulin resistance in patients with NAFLD.

METHODS

Study design

In this case-control study, the sample size was calculated using G-power software (power: 80 and OR: 2.49 (12)). One hundred and sixty subjects referred from gastroenterologist to the specialised and subspecialised clinic of Tabriz University of Medical Sciences (governmental center) for nutrition consult, from September 2014 to May 2015 were studied. Those without any history of alcohol consumption, diseases such as diabetes, hepatitis B, hepatitis C, cirrhosis and other chronic liver diseases, consuming hepatotoxic medications, who had abdominal obesity were eligible for the study; pregnant, lactating and post-menopausal women were excluded.

In order to eliminate the impact of latitude, climate, skin colour and race, subjects from the North-west part of Iran, that is, East and West Azerbaijan and Ardebil provinces were included. Fifty percent of female and 50% of male subjects who were referred for nutrition consult were assessed. Since the number of female subjects referred to the nutritionist outnumbered the male subjects, more female subjects participated in this study. Patients with NAFLD were considered as 'cases' (n=80) and those without NAFLD and without any chronic and inflammatory diseases as 'controls' (n=80). Demographic data were collected and anthropometric measurements were performed. A consent form was obtained from the participants. The research protocol was approved by the Ethics Committee of Tabriz University of Medical Science.

Data collection

After 8-12 h of fasting, blood samples (5 cc) were collected and sera samples were stored at -20°C until assays. For all biochemical analyses, the ELISA technique was used. Serum 25(OH) D, the most stable circulating form of this molecule was assessed by Bioactiva 25(OH) D ELISA kit, (bioactiva diagnostic, Germany); intra-assay coefficient of variation (CV) for this kit was 4.6% and inter-assay CV was 5.4%. The sensitivity for the measurement was 0.57 ng/ml. Fasting PTH levels was measured via IBL kit (Parathyroid hormone intact ELISA kit, IBL International GMBH, Germany); intra-assay CV was 14.3% and inter-assay CV was 13.6%, for this kit. The sensitivity for PTH measurement was 1.57 pg/ml. Fasting insulin levels were measured by Monobind kit (Monobind ELISA kit, Accubind, USA); intra-assay CV for this kit was 5.6% while inter-assay CV was 7.3%. The sensitivity was 0.75 μIU/ml for fasting insulin measurement. All clinical tests were conducted by one person and all equipments were calibrated before tests. Fasting serum glucose levels were obtained by referring to patient medical records. Records on fasting serum glucose were based on glucose oxidase method conducted in the laboratory of specialised and subspecialised clinics of Tabriz University of Medical Sciences, for all subjects. HOMA-IR, as an indicator of insulin resistance, was calculated using the following equation:

HOMA-IR= fasting glucose (mmol/L) × fasting Insulin (μIU/ml) / 22.5

Demographic data (age, gender, location, occupation, marital status and education) and medical history of diseases, drugs, alcohol, and tobacco

were recorded. For NAFLD diagnosis, liver ultrasonography technique (US) was applied; all measurements were performed by a single sonographist who was blind to the aims of the study, to prevent bias.

Weight and height were measured by Seca (Germany) stadiometer with precision of 100 g and 0.5 cm, respectively. Waist and hip circumference were measured using a tape to the nearest 0.1 cm. Waist circumferences above 88 cm for women and above 102 cm for men were considered as abdominal obesity. Waist-to-hip (WHR) and waist-to-height ratios were calculated. All measurements were performed three times by a nutritionist and the average of three measurements was recorded. Exposure to the sunlight was recorded as hours per week, using a questionnaire; average hours of sunlight exposure during the day and average number of days with sunlight exposure weekly, were asked. (Mehrangiz *et al*., 2015). Dietary intakes of calcium, phosphors and vitamin D were assessed by three dietary records (one weekend and two week days) (Willett & Stampfer, 1998), considering household serving sizes for each food item and then converting them to gram or milliliters per day; nutritional data were analysed using NUTRITIONIST IV software.

Statistical analysis

SPSS ver.16 was used to analyse data. Kolmogorov Smirnov (K-S) test was used to assess the normality of data. Independent samples *t*-test was conducted to compare means between two groups, when data were normally distributed. Univariate logistic regression was applied to identify confounding factors and then multivariate logistic regression was used to assess the relationship between vitamin D, PTH and insulin resistance in the development of NAFLD by adjusting for the confounders. Pearson and Spearman correlation coefficients were used for the association between symmetric and asymmetric quantitative variables, respectively. P-values less than 0.05 were considered as statistically significant.

RESULTS

A total of 80 NAFLD patients aged 20-50 years (35.82 ±7.76 yrs) and 80 age-matched controls free from NAFLD were studied. There were no missing data. There were significant differences in BMI and waist-to-hip ratio between the groups. Median of sunlight exposure in the two groups is shown in Figure 1. There was no significant difference in sunlight exposure between the groups (p=0.274). Mean serum insulin, HOMA-IR and PTH were significantly higher and serum 25 (OH) D was significantly lower in NAFLD cases compared to healthy subjects (Table 1). There was no significant difference in dietary intake between the two groups; therefore, dietary data were not considered as confounders (Table 2).

After adjusting for age, sex, BMI and WHR, mean serum insulin, HOMA-IR, 25(OH)D were significantly different between the groups; however, mean serum PTH levels were not statistically significant after adjusting for confounders (p= 0.668) (OR: 1.37 CI: 0.35, 6.27) (Table 3).

Multivariate logistic regression showed that serum insulin levels were independently associated with NAFLD after adjusting for age, sex, and BMI and waist/hip ratio (p= 0.001; OR: 1.16, CI 95%: 1.07, 1.25; Table 3). Low serum 25 (OH) D levels increased the risk of NAFLD 8.87 times; and higher HOMA indices increased the risk 1.80 times. After adjusting for confounding factors, serum levels of vitamin D and HOMA-IR index were determined as predictors of NAFLD risk

To investigate the correlation between serum level of 25 (OH) D and PTH, and HOMA-IR scores, Spearman correlation coefficient was used. Serum 25 (OH) D level was reversely associated with HOMA-IR scores in both NAFLD and

Table 1. Clinical and biochemical characteristics of subjects

Variables	*NAFLD (n=80)*	*Control (n=80)*	*P**
Sex (M/F)	18/62	4/76	0.001
Age	36.25±8.09	32.18±7.67	0.794
Anthropometric indicators			
Body Mass Index (kg/m2)	35.89±5.34	31.87±6.97	0.001
Waist/hip ratio	0.91±0.08	0.88±0.06	0.001
Waist/height ratio	0.68±0.07	0.64±0.07	0.001
Blood indicators			
25(OH)D (ng/ml)	11.22±7.97	19.24±16.87	0.001
Parathyroid hormone (pg/ml)	59.31±14.31	44.07±11.47	0.001
Fasting Serum Glucose(mg/dl)	96.21±11.42	92.54±9.21	0.027
Insulin (µU/ml)	11.91±5.51	7.34±5.36	0.001
HOMA-IR	2.80±1.42	1.70±1.32	0.001

* Independent samples t-test
For all blood indicators, ELISA technique was used

Table 2. Sunlight exposure, daily intake of vitamin D and micronutrients among the subjects

Variables	*NAFLD (n=80) Median (Min, Max)*	*Control (n=80) Median (Min, Max)*	*p**
Sunlight exposure (hours/week)	3.00(0.00,35.00)	3.50(0.00,28.00)	0.274
Vitamin D (µg)	0.18(0.01,0.77)	2.69(0.04,0.77)	0.052
Calcium (mg)	695.30(219.60,982.35)	318.58(319.70,982.33)	0.129
Phosphorous (mg)	712.35(321.5,1211.00)	686.9(318.91,1239.00)	0.065
Magnesium (mg)	289.36 (120.0, 896.66)	252.9(124.5-733.8)	0.418

* Mann-Whitney test
Sunlight exposure was assessed by questionnaire and nutrient intakes were assessed by three dietary re¬cords

Table 3. Determinants of NAFLD development

Variable	*OR (CI 95%)*	*p*	*Adjusted OR* (CI 95%)*	*p*
25(OH)D (ng/ml)				
10>	11.61 (2.49,54.14)	0.002	8.78 (1.71,45.03)	0.009
10-30	8.00 (1.70,37.58)	0.008	5.58 (1.11,29.14)	0.038
30<	1.00	-	1.00	-
PTH (pg/ml)				
94<	3.25 (0.85,12.50)	0.086	1.37 (0.30, 6.27)	0.688
94>	1.00	-	1.00	-
HOMA-IR				
2<	2.06 (1.48, 2.87)	0.001	1.80 (1.32, 2.47)	0.001
2>	1.00	-	1.00	-

* Multivariate logistic regression applied. * adjusted for sex, BMI and, waist/hip

control groups while this association was statistically significant only in the control group (r: -0.282, p= 0.011). The association between serum PTH and HOMA-IR was significantly positive in NAFLD patients (r: 0.228 p=0.042) while this correlation was reverse (non-significant) among the healthy subjects.

DISCUSSION

This study demonstrated that subjects affected by NAFLD have reduced serum 25 (OH) vitamin D levels compared to age matched individuals without NAFLD. This relationship was independent of sex, BMI, and waist/hip ratio. Lower serum 25 (OH) D levels were independently associated with an increased odds of NAFLD. Previous studies have shown similar results. Targher *et al.(*2007) found an association between serum 25 (OH) D and liver histology in patients with NAFLD. The remarkable differences observed in 25(OH) D concentrations between the groups were significant after adjusting for age, sex, BMI, creatinine, calcium, HOMA score and the presence of metabolic syndrome (Targher *et al*., 2007). Rhee *et al*.(2013) analysed the association of serum 25(OH) D levels with NAFLD and found that those with higher serum 25(OH)D showed a significantly lower risk for NAFLD compared to those with low serum 25(OH)D3 levels, independent of obesity and metabolic syndrome (Rhee *et al*., 2013). In another study, plasma vitamin D concentration, severity of disease and body composition were studied in NAFLD; the results showed that vitamin D concentration was significantly lower in NAFLD patients compared to healthy controls. Low plasma vitamin D concentration seems to be an independent predictor of the severity of NAFLD (Dasarathy *et al*., 2012). In accord with the findings of previous studies, our results demonstrated a strong, inverse relation between NAFLD and serum 25(OH)D levels.

A recent systematic review also found that vitamin D levels >25 ng/ml are associated with a 43% lower risk of type 2 diabetes compared to vitamin D levels <14 ng/ml (95% CI 24, 57%) (Mitri, Muraru & Pittas, 2011). Possible mechanisms involved could be that vitamin D deficiency adversely affects insulin sensitivity/secretion and promotes inflammation through increased production of pro inflammatory cytokines. The active form of vitamin D3 possesses anti-apoptotic activity in hepatocytes. Hepatocyte apoptosis has been recognised as a major contributing mechanism for fibrogenesis and cirrhosis. Therefore, induction of the active form of vitamin D3 may be important for prevention of hepatic apoptosis followed by fibrogenesis (Roth *et al*., 2012).

In the present study, PTH serum levels were significantly different between the two groups; but this difference did not remain significant after adjusting for confounding factors. This result was not consistent with recent observations of a similar study. Bhatt *et al.* (2013) analysed the associations of 25(OH) D and PTH levels with clinical, anthropometric, biochemical and body composition parameters in Asian Indians with NAFLD. In that case-control study, 162 cases and 173 age- and sex-matched controls were recruited. Fasting insulin levels, HOMA-IR, serum 25(OH) D, calcium and PTH levels were measured. PTH was assayed by electro-chemiluminescence method (Elecsys 2010, Roche Diagnostics, Mannheim, Germany). Their results demonstrated that low serum 25(OH) D and high PTH levels were independently associated with the presence of NAFLD (Bhatt *et al*., 2013). According to our results, PTH serum levels had a direct relationship with NAFLD but we could not demonstrate this association after adjusting for confounders. Since the elecsys technique is more accurate than ELISA, this difference in results might be attributable to the different measurement methods (Hermsen *et al*., 2001). Other studies investigating the relationship between PTH and metabolic syndrome have reported different results. Reis *et al.(*2008) studied the association of serum 25 (OH) D and PTH with metabolic syndrome. Results showed an inverse association of serum 25 (OH) D with metabolic syndrome, independent of

confounding factors; a direct association of PTH with metabolic syndrome was also observed in men. Lee *et al*. (2009) in a cross-sectional study with 3369 subjects, assessed the association of serum 25 (OH) D, PTH and insulin resistance, and metabolic syndrome. Results showed that levels of 25 (OH) D were inversely associated with metabolic syndrome (independent of confounding factors and PTH) (Lee *et al.*, 2009).

The biological mechanism by which 25(OH) D and PTH may influence metabolic syndrome has not been established, thus far. However, there is accumulating evidence from clinical and experimental studies that vitamin D and PTH may influence glucose homeostasis. Decreased vitamin D and increased PTH levels have both been associated with insulin resistance, including an additional effect for 25(OH) D in optimising b-cell function (Reis *et al.*, 2008). However, evidence of the effect of serum PTH levels exclusively on metabolic syndrome has not been found.

The results of this study showed that HOMA-IR levels and serum insulin levels were significantly different between the two groups; insulin resistance was higher in patients with NAFLD. Higher HOMA indices were independently associated with increased odds of NAFLD. Similar findings have previously been reported. Sesti *et al.* (2013) examined the relationship between insulin resistance indices (liver insulin resistance and HOMA), NAFLD and its related biomarkers. They documented significant cross-sectional associations of NAFLD and liver biomarkers with indices of hepatic insulin resistance. El-Karaksy *et al.* (2015) determined the association between insulin resistance (IR) and both NAFLD and metabolic syndrome in a group of Egyptian overweight/obese children and adolescents. Insulin resistance was calculated using the HOMA-IR, the quantitative insulin-sensitivity check index (QUICKI) and hepatic insulin sensitivity. Results showed that IR was significantly associated with NAFLD (El-Karaksy *et al.*, 2015). In contrast, a recent meta-analysis, on 35 randomised controlled trials with a total of 43,407 patients revealed no significant effect of vitamin D supplementation on prevention of diabetes in individuals without diabetes, or on reduction of insulin resistance and hyperglycemia in those with pre-diabetes or established type 2 diabetes. However, these results could not be generalised because of moderate heterogeneity between the studies, the bias in risk assessment and the short term of follow up (Seida *et al.*, 2014).

Insulin resistance, a key risk factor in the pathogenesis of NAFLD, is linked to the development of oxidative stress and lipotoxicity. It is now recognised that proinflammatory cytokines such as TNF-α and IL-6, and adipokines such as leptin and adiponectin play a major role in the progression from steatosis to steatohepatitis (Eliades & Spyrou, 2015).

The dietary intake of vitamin D, calcium, phosphorous and magnesium revealed no significant differences between the two groups. In a case-control -led study conducted by Gibson *et al.*(2015) in which dietary intakes were assessed by 24-h dietary recall and 7-day food diary in NAFLD patients under 18 years old, no differences in micronutrient intakes were reported. A similar study showed that the intakes of calcium, vitamin D and antioxidant micronutrients were lower in NAFLD patients than in controls (Alavian *et al.*, 2013). The results of these studies have been inconsistent; this may be due to the diverse methods adopted for dietary intakes assessment.

In the present study, the association between 25(OH) D, PTH and insulin resistance was assessed. It was observed that Serum 25(OH) D levels were reversely associated with HOMA-IR scores in the control group while the correlation between serum PTH and HOMA-IR was

positive in NAFLD patients. Pirgon *et al*. (2013) performed a study to investigate the relationships between 25(OH) D and insulin resistance in obese adolescents with NAFLD. Eighty-seven obese adolescents (mean age: 12.7 ± 1.3 years) were studied. The authors demonstrated an association between insufficient vitamin D status and low insulin sensitivity in obese adolescents with NAFLD (Pirgon *et al*., 2013). In a similar study, a different method (euglycemic insulin clamp with 3-(3) H-glucose) was used to measure insulin resistance. The results suggested that plasma vitamin D levels were not associated with insulin resistance, the amount of liver fat accumulation, or the severity of NASH (Bril *et al.*, 2015). The different methods might have caused this difference in results; however in a clinical trial by Foroughi, Maghsoudi & Askari (2016), patients with NAFLD were randomly assigned to consume vitamin D supplements (n = 30) or placebo (n = 30) for 6 weeks, In this study, vitamin D supplementation was inversely associated with IR. Vitamin D supplementation may have beneficial effects on controlling the glycemic indicator (Foroughi *et al*., 2016). Our results were consistent with those of most studies with similar designs. Chiu *et al.* (2000) hypothesised that plasma intact parathyroid hormone (iPTH) level is a determinant of either insulin sensitivity or β-cell function. Their study included 52 normotensive healthy subjects with glucose tolerance. Relationships between iPTH level and insulin sensitivity index and β-cell function were examined. Results indicated that plasma iPTH level is inversely correlated with insulin sensitivity index (Chiu *et al.*, 2000). Our study included healthy subjects and we failed to find a study on NAFLD patients in this regard.

Our study had some limitations. First, matching our groups for sex was not possible and this might have influenced our results. Second, due to financial constraints, serum levels of calcium and phosphorus were not measured. Third, although ultra sonography is a practical approach commonly used to detect liver steatosis, it is not the gold standard technique for quantitative liver fat assessment. Fourth, a 3-day dietary record was used to assess dietary intakes and there might have been errors related to this method. Fifth factors known to affect serum levels of vitamin D, PTH and, insulin, were considered as confounders in this study; however, it is probable that some unknown factors also affect these results.

Our study had some strengths. This case-control study was the first to investigate the relationship between both vitamin D and PTH, with NAFLD; similar previous studies had not examined these two factors simultaneously in association with fatty liver. Moreover, in our study, exposure to sunlight, as a source of vitamin D, was assessed.

CONCLUSION

According to the results of this study, after adjusting for confounding factors, serum levels of vitamin D, insulin and HOMA-IR index were determined as predictors of NAFLD risk.

Lower serum 25 (OH)D levels and higher HOMA indices were independently associated with increased odds of NAFLD. Serum PTH levels had direct but non-significant association with NAFLD. Further interventional studies are required to assess the effect of vitamin D supplementation on NAFLD development or improvement.

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Conflict of interest

Authors of the present paper declare no conflict of interest. All authors confirmed the final version of the manuscript.

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