Factors Affecting Bone Health in Kidney Transplant Recipients: Klotho Gene Single-Nucleotide Polymorphisms and Other Clinical Features

Hasan H. Yeter,[1] Ozant Helvaci,[2] Berfu Korucu,[1] Sevim Gonen,[3] Galip Guz,[1] Ulver Derici[1]

Abstract

Objectives: **Posttransplant bone diseases are a major cause of morbidity in kidney transplant recipients. We investigated the relationship between klotho gene single-nucleotide polymorphisms and bone diseases after kidney transplant. We also aimed to identify possible risk factors for development of bone disease.**
Materials and Methods: **The study consisted of 251 kidney transplant recipients (164 men and 87 women) with minimum follow-up of 3 years after kidney transplant. Patients with prolonged immobilization, malignancy, parathyroidectomy, glomerular filtration rates less than 30 mL/min/1.73 m², hypo- or hyperthyroidism, and treatment with drugs that affect bone metabolism were excluded. We investigated the relationship between 6 single-nucleotide polymorphisms of the klotho gene (rs480780, rs211234, rs576404, rs211235, rs9536314, and rs1207568) and development of osteoporosis, avascular bone necrosis, and persistent hyperparathyroidism.**
Results: **Longer dialysis treatment (odds ratio, 1.13; *P* = .002) and rs211235 single-nucleotide polymorphism in the klotho gene (odds ratio, 9.87; *P* = .001 for GG genotype) were significantly associated with persistent hyperparathyroidism. A higher magnesium level was detected as a protective factor from development of persistent hyperparathyroidism (odds ratio, 0.19; *P* = .009). Persistent hyperparathyroidism was defined as a risk factor for development of osteopenia/osteoporosis (odds ratio, 2.76; *P* = .003) and avascular bone necrosis (odds ratio, 2.52; *P*= .03). Although the rs480780 (odds ratio, 8.73; *P* = .04) single-nucleotide polymorphism in the klotho gene was defined as a risk factor for development of osteopenia/osteoporosis, none of the klotho single-nucleotide polymorphisms was found to be associated with development of avascular bone necrosis.**
Conclusions: **Persistent hyperparathyroidism could be an important indicator for development of bone disease in kidney transplant recipients. Also, some of the klotho gene single-nucleotide polymorphisms are associated with higher risk for bone disease after kidney transplant.**

Key words: *Avascular bone necrosis, Hyperparathyroidism, Osteoporosis*

Introduction

Kidney transplant is the best renal replacement therapy for end-stage renal disease. Advances in immunosuppressive treatment and surgical techniques have improved graft and patient survival.[1] Prolonged survival leads to increased frequency of long-term complications and morbidities. Posttransplant bone disease is a major cause of morbidity in kidney transplant recipients, with significantly higher risk of fractures and higher costs for hospitalization and health care.[1,2] Preexisting renal osteodystrophy, posttransplant diabetes, and immunosuppressive drugs including glucocorticoids and calcineurin inhibitors contribute to posttransplant bone disease.[3] Also, well-known risk factors for osteoporosis in the general population such as age, sex, race, obesity, smoking, and menopausal status could facilitate development of osteoporosis in kidney transplant recipients. However, the same cumulative glucocorticoid usage in a similar time course causes more bone microarchitecture disorders in some patients. In addition, bone avascular necrosis (AVN) may develop in some patients under similar circumstances. The exact mechanism of this difference is not completely

From the [1]Department of Nephrology, Gazi University, Ankara, Turkey; the [2]Yildirim Beyazit University Yenimahalle Education and Research Hospital, Ankara, Turkey; and the [3]Department of Immunology, Gazi University, Ankara, Turkey

Acknowledgements: *All expenditures of this study were supported by the Gazi University Scientific Research Project Unit. The study was presented as oral presentation No. 21 at the Hypertension and Kidney Disease Congress May 1-5, 2019, Bafra, Cyprus. Other than described above, the authors have not received any funding or grants in support of the presented research or for the preparation of this work and have no further declarations of potential interest.*

Corresponding author: *Hasan Haci Yeter, Department of Nephrology, Gazi University Medical School, Ankara, Turkey*
Phone: *+ 90 554 2397449* ***E-mail:*** *hasanyeter@hotmail.com*

Experimental and Clinical Transplantation (2020)

Copyright © Başkent University 2020
Printed in Turkey. All Rights Reserved.

DOI: 10.6002/ect.2020.0130

understood. Another cause of bone disease after kidney transplant is persistent hyperparathyroidism (PHP). Secondary hyperparathyroidism is typically resolved with the improvement of kidney function. This normalization lasts about 1 year, but high levels of serum parathyroid hormone (PTH) levels persist after 1 year in 15% to 50% of patients.[4]

Recent studies have shown that the homeostasis of bone turnover and PTH depends on other factors such as fibroblast growth factor 23 (FGF-23) and its cofactor, the klotho protein.[5] The klotho gene, first identified in 1997, which is expressed in distal convoluted tubules of kidney and parathyroid cells, has been studied in patients on hemodialysis, and it has been shown that klotho is associated with atherosclerosis, cardiovascular disease, and bone mineral disorders.[6] The klotho gene is located on chromosome 13q2 and encodes a single-pass transmembrane protein that contains 3 members: α-klotho, β-klotho, and klotho-related protein.[6] It is essential for endogenous FGF-23 function. Studies suggest that α-klotho is a circulating hormone that has an effect on FGF-23 stabilization and protection of FGF-23 from degradation; also, α-klotho has a phosphaturic activity independent of FGF-23.[7] The FGF-23+α-klotho+FGF receptor complex inhibits the type 2 Na-P cotransporter in the renal tubules and decreases its expression. This signal pathway also inhibits 1α-hydroxylase and activates 24α-hydroxylase in kidney and directly suppresses PTH synthesis.[8,9] Defects in the klotho-FGF-23 system can cause high serum levels of 1,25-hydroxyvitamin D and PTH.[2]

In the literature, there is only one study that investigated the relationship between klotho gene single-nucleotide polymorphism (SNP) and bone disorders in patients with kidney transplant. This study was conducted by Ozdem and colleagues with 25 kidney transplant recipients and 26 healthy control patients. They investigated the effect of SNP at the 352 amino acid position of the klotho gene (phenylalanine to valine; F352V or rs9536314) on vitamin D status and osteoporosis in kidney transplant recipients. They found an inverse association with F352V polymorphism and vitamin D status but no association with development of osteoporosis.[10] In a study from 2016 in patients treated with hemodialysis, it was shown that the F352V SNP was not associated with serum calcium, phosphorus, and PTH levels.[5] On the other hand, several studies were conducted to define the association between the klotho gene polymorphism and AVN or osteoporosis in other specific disease populations.[11-13] Baldwin and colleagues reported that AVN is associated with the rs480780, rs211234, rs576404, and rs211235 polymorphisms of the klotho gene in patients with sickle cell anemia.[11] However, there is no study in the literature that has reported the relationship between the klotho gene polymorphism and AVN in kidney transplant recipients.

The objective of this study was to answer the question of whether the klotho gene SNPs rs480780, rs211234, rs576404, rs211235, rs9536314, and rs1207568 are associated with osteoporosis, AVN, and PHP in patients with kidney transplant; also, we sought to determine other possible risk factors for bone disease after kidney transplant.

Materials and Methods

Study population and ethical statement

This study was conducted in the Gazi University Nephrology and Transplantation department. Kidney transplant recipients who were older than >18 years of age and fulfilled the necessary criteria were included in the study.

We evaluated the patients who had kidney transplant between 2010 and 2018. Exclusion criteria were as follows: follow-up less than 3 years; prolonged immobilization, systemic illness, or malignancy; parathyroidectomy; glomerular filtration rates (GFR) less than 30 mL/min/1.73 m^2; hypo- or hyperthyroidism; and/or treatment with drugs that affect bone metabolism (except immunosuppressive agents, vitamin D, and calcium supplementation, eg, bisphosphonates and cinacalcet). Prednisolone treatment was initiated at postoperative day 0 in doses of 1000, 500, 250, 160, 80, 40, and 20 mg/day, until the end of first month. Between the first and third months, prednisolone was tapered every 2 weeks and then continued at a maintenance dose of 5 mg/day thereafter. Standard immunosuppressive therapy consisted of glucocorticoids, a calcineurin inhibitor [80% of patients (200/251) received tacrolimus, and 12% (29/251) received cyclosporine] and an antimetabolite [74% of patients (186/251) received mycophenolate mofetil, and 15% (38/251) received azathioprine]. We treated 13% of patients (32/251) with one of two mammalian target of rapamycin (mTOR) inhibitors (28/32 patients received everolimus, and 4/32 received sirolimus), to mitigate calcineurin or antimetabolite side effects. Vitamin D

supplementation was given to all patients who had vitamin D levels below 30 ng/mL.

The study protocol conformed to ethical guidelines of the Declaration of Helsinki, and all participants gave written informed consent and expressed a willingness to participate. The ethics committee of Gazi University approved the study protocol (approval date, December 2, 2018; project No. 87)

Data collection

The hospital's electronic medical records system was used for baseline information such as patients' sex, age, transplant type, dialysis vintage (ie, length of time on dialysis), and cumulative glucocorticoid dose. Total follow-up time and development of AVN in follow-up were determined for all patients. Data for serum creatinine, albumin, PTH, and vitamin D were collected. We used the abbreviated Chronic Kidney Disease Epidemiology Collaboration equation to estimate GFR.[14] The laboratory values of patients in the past 3 years were averaged, and these average values were used for demographic evaluation. Serum PTH level below 65 ng/dL was accepted as normal at the end of first year of follow-up.

For the diagnosis of osteoporosis, we used dual-energy x-ray absorptiometry (Horizon DXA system, Hologic Inc.) to measure bone mineral density (BMD) at the hip (neck of the femur) and lumbar spine. All patients were followed up with measurements of BMD annually according to the KDIGO 2009 guideline in our clinic.[15] We used the World Health Organization classification for osteopenia and osteoporosis, in terms of Z scores and T scores, to determine bone health as follows: group 1, normal (Z or T score, +1 to -1); group 2, osteopenia (Z or T score, -1 to -2.5); and group 3, osteoporosis (Z or T score ≤ 2.5). For premenopausal women and men under age 50 years, the Z score was considered. We used the BMD scores to divide the patients into 2 groups as follows: normal BMD and abnormal BMD (osteopenia or osteoporosis). All patients had multiple BMD assessments, and we used the third year BMD results for evaluation. Cases of symptomatic AVN were diagnosed by standard anterior-posterior x-ray views of pelvis or magnetic resonance imaging of pelvis, hip, knee, or shoulder.

Klotho gene polymorphism analysis

We used the automated magnetic bead method (MagPurix model ZP0200, Zinexts) according to the manufacturer's instructions to prepare genomic DNA extracts from whole blood samples containing EDTA, and all extracts were stored at -80 ºC until analysis. We used specific oligonucleotide primers with real-time polymerase chain reaction to genotype the klotho SNPs rs480780, rs211234, rs576404, rs211235, rs9536314, and rs1207568 (as shown in Table 1). The reaction results were visualized with 2% agarose gel electrophoresis. The polymerase chain reaction pools generated for each sample were purified by NucleoFast 96 (Macherey-Nagel). We used a NanoDrop N-1000 spectrophotometer (Thermo Fisher Scientific) to measure purified samples. To analyze the samples, we used the New Generation Sequencing kit (model v2-300cy, Illumina), a MiSeq benchtop sequencer apparatus (Illumina), and MiSeq Reporter software (Illumina), as well as the Integrative Genomics Viewer software (IGV version 2.3) developed by the Broad Institute.

Table 1. Real-Time Polymerase Chain Reaction Primers for Klotho Gene Single-Nucleotide Polymorphisms

Primer	Sequence (5′-3′)	Length of DNA Fragment, base pairs
rs480780		269
Forward	ACCTATGTTTGACCCCTCACGATGC	
Reverse	CTCCCATCTCCCGTATTTCTCACTG	
rs211234 and rs211235		
Forward	TTTTCAAATCCCACCTGTTCTTCTG	278
Reverse	TGAAATCAGGCTATTCGGTAAACCAG	
rs576404		335
Forward	TCAGCTGGAGGATCTAATAGATCATGC	
Reverse	AATTTCCCTAAATACAAAGTCCACTGC	
rs9536314		910
Forward	GTGGATAACTACACACTCAACCCAAGG	
Reverse	TTCTACTATTGCCAAAATGAATGTCTCC	
rs1207568		169
Forward	GAGAAAAGTGAGAGCAGGTGCTTCC	
Reverse	GGACGCTCAGGTTCATTCTCTTTGC	

Statistical analyses

We used the Kolmogorov-Smirnov test to determine data distribution, and we used the 1-way analysis of variance homogeneity of variance test for homogeneity of variables. Symmetrically distributed variables in the text and tables are shown as means ± SD. If the distribution was heterogeneous, then the variables are shown as median values (with interquartile range). Categorical variables are expressed as percentages. We used the *t* test or the Mann-Whitney U test to compare continuous variables according to the data distribution; the chi-square test to compare categorical variables; univariate logistic regression analysis to identify variables associated with osteoporosis, AVN, and

PHP; and multivariate logistic regression analysis to identify possible risk factors for osteoporosis, AVN, and PHP. $P \leq .05$ was considered statistically significant. We used the Statistical Package for the Social Sciences software for Windows (SPSS version 20.0.0) to perform the statistical analyses.

Results

A total of 251 patients were evaluated. We retrospectively studied 164 men (65%) and 87 women (35%), with a mean age at transplant of 40.9 years. Median follow-up time was 8.06 ± 5 years. Although 28% of total patients underwent preemptive kidney transplant, median dialysis vintage of other patients was 2 years.

Persistent hyperparathyroidism

Persistent hyperparathyroidism was detected in 34% of total patients (85/251) at the end of first year of follow-up. Patients with PHP had longer dialysis vintage than the patients with normal PTH levels (3 years [interquartile range, 1-7.5 years] vs 1.45 years [interquartile range, 0-3 years]; $P < .001$). In patients who received preemptive kidney transplant, higher rates of remission were achieved than in the patients who received dialysis treatment (52 patients [74%] vs 18 patients [26%]; $P = .05$). Although the mean serum calcium level was higher in patients in the PHP group (9.73 ± 0.76 vs 9.45 ± 0.55 mg/dL; $P = .009$), the mean serum phosphorus levels were similar in both groups (3.09 ± 0.64 vs 3.26 ± 0.64 mg/dL; $P = .07$). Mean serum magnesium level was significantly higher in patients with normal PTH levels versus patients with PHP (1.8 ± 0.25 vs 1.73 ± 0.26 mg/dL; $P = .05$). Other demographic parameters of patients were similar between groups (Table 2). We used logistic regression analysis to analyze risk factors for PHP. Although rs480780 (odds ratio [OR] = 2.13 [95% CI, 1.14-3.97] for GT genotype; and OR = 8.05 [95% CI, 2.96-21.92] for GG genotype) and homozygous polymorphisms of rs211234 (OR, 6.84; 95% CI, 2.61-17.89), rs576404 (OR, 5.48; 95% CI, 2.22-13.55), and rs211235 (OR, 7.90; 95% CI, 3.15-19.77) were found to be associated with PHP, only the homozygous rs211235 SNP (OR, 9.87; 95% CI, 2.42-40.21) was identified as a risk factor for development of PHP by multivariate logistic regression analysis. Also, long-term dialysis treatment (OR, 1.13; 95% CI, 1.05-1.20) was defined as a risk factor for development of PHP by multivariate logistic regression analysis. On the other hand, higher magnesium levels were detected as a protective factor against development of PHP (OR, 0.19; 95% CI, 0.06-0.66) (Table 3).

Table 2. Demographics of Patients According to Parathyroid Hormone Levels

	Total	Normal PTH	High PTH	*P*
No. of patients (% of cohort)	251	166 (66%)	85 (34%)	
Age, y	40.9 ± 12.7	40 ± 13	42 ± 13	.4
Sex, No. (%)				.04
Women	87 (35%)	51 (31%)	36 (42%)	
Men	164 (65%)	115 (69%)	49 (58%)	
Dialysis vintage, y	2 (0-5)	1.45 (0-3)	3 (1-7.5)	< .001*
Donor type, No. (%)				.08
Deceased donor	62 (24.7%)	26 (16%)	36 (42%)	
Living donor relative	0189 (76.3%)	140 (84%)	49 (58%)	
First degree	120 (63%)	98 (70%)	22 (45%)	
Second degree	54 (29%)	10 (7%)	5 (10%)	
Third degree	15 (8%)			
Preemptive transplant, No. (%)	70 (28%)	52 (74%)	18 (26%)	.05
Hypertension, No. (%)	126 (50%)	81 (49%)	45 (51%)	.5
Follow-up time, y	8.06 ± 5	8.1 ± 5	8.7 ± 5	.6
Laboratory parameter				
Creatinine, mg/dL	1.15 ± 0.35	1.15 ± 0.33	1.16 ± 0.37	.7
eGFR, mL/min/1.73 m^2	75.2 ± 24.3	75.4 ± 23.5	74.7 ± 26.3	.7
Calcium, mg/dL	9.52 ± 0.62	9.45 ± 0.55	74.7 ± 26.3	.009
Phosphorus, mEq/L	3.18 ± 0.63	3.26 ± 0.64	3.09 ± 0.64	.07
Albumin, g/dL	4.27 ± 0.39	4.32 ± 0.39	4.24 ± 0.41	.1
Magnesium, mg/dL	1.79 ± 0.25	1.8 ± 0.25	1.73 ± 0.26	.05*
Hemoglobin, g/dL	12.6 ± 2.1	12.7 ± 2	12.5 ± 2.3	.4
ALP, U/L	85.5 ± 39	81 ± 29	95 ± 52	.2
Vitamin D, ng/mL	35 ± 7.7	35.8 ± 7.2	35.02 ± 8	.34

Abbreviations: ALP, alkaline phosphatase; eGFR, estimated glomerular filtration rate
*Statistically significant.

Table 3. Risk Factors for Persistent Hyperparathyroidism

	Univariate Logistic Regression		Multivariate Logistic Regression	
	P	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)
rs480780				
GT	.02	2.13 (1.14-3.97)	.07	8.47 (0.84-38.85)
TT	< .001	8.05 (2.96-21.92)	.08	27.05 (0.63-42.59)
rs211234				
GA	.09	1.71 (0.91-3.20)	.6	0.53 (0.02-13.07)
AA	< .001	6.84 (2.61-17.89)	.5	0.29 (0.01-30.75)
rs576404				
CA.06	.19	1.52 (0.81-2.85)	.2	0.25 (0.02-2.67)
AA	< .001	5.48 (2.22-13.55)	.2	0.17 (0.01-3.94)
rs211235				
GT	.06	2.13 (0.94-4.84)	.09	2.42 (0.85-6.84)
GG	< .001	7.90 (3.15-19.77)	.001	9.87 (2.42-40.21)
rs9536314				
TG	.2	1.40 (0.79-2.79)		
GG	.4	2.22 (0.30-16.21)		
rs1207568				
GA	.2	1.45 (0.79-2.69)		
AA	.1	2.33 (0.71-7.62)		
Dialysis vintage	< .001	1.14 (1.06-1.22)	.002	1.13 (1.05-1.23)
Diabetes mellitus	.8	0.94 (0.48-1.82)		
Glomerulonephritis	.7	0.93 (0.59-1.71)		
eGFR	.8	0.99 (0.99-1.01)		
Magnesium	.01	0.27 (0.10-0.76)	.009	0.19 (0.06-0.66)
Preemptive transplant	.09	0.59 (0.32-1.09)		

Abbreviations: eGFR, estimated glomerular filtration rate

Osteopenia or osteoporosis

A total of 240 patients were evaluated. Osteopenia or osteoporosis was detected in 40% of patients (96/240). The incidence of PHP and the mean eGFR were significantly higher in patients with osteopenia or osteoporosis compared with patients with normal BMD. That is, PHP was diagnosed in 46 patients with osteopenia or osteoporosis (48%) versus 31 normal BMD patients (22%) ($P < .001$); and eGFR in patients with osteopenia or osteoporosis was 79.5 ± 25.3 mL/min/1.73 m^2, whereas the eGFR in patients with normal BMD was 71.5 ± 23.3 mL/min/1.73 m^2 ($P = .03$). Also, mean serum creatinine level was significantly higher in patients with normal BMD (1.19 ± 0.37 mg/dL) than in patients with osteopenia or osteoporosis (1.1 ± 0.32 mg/dL) ($P = .05$). Other demographic parameters of patients were similar between groups (Table 4). Univariate logistic regression analysis showed that rs480780 (OR, 5.29; 95% CI, 2.85-9.81), rs211234 (OR, 4.24; 95% CI, 2.30-7.82), rs576404 (OR, 4.72; 95% CI, 2.52-8.84), and rs211235 (OR, 3.13; 95% CI, 1.51-6.49) SNPs of the klotho gene and PHP (OR, 3.09; 95% CI, 1.75-5.46) were associated with osteopenia and osteoporosis in patients with kidney transplant. Multivariate logistic regression analysis revealed that PHP (OR, 2.76; 95% CI, 1.43-5.33) and the rs480780 SNP of the klotho gene (OR, 8.73; 95% CI, 1.03-73.96) were risk factors for the development

Table 4. Demographics of Patients According to Osteopenia and Osteoporosis

	Osteopenia and Osteoporosis			*P*
	Total	No	Yes	
No. of patients (% of cohort)	240	144 (60%)	96 (40%)	
Sex, No. (%)				.8
Women	83 (35%)	49 (34%)	34 (35%)	
Men	157 (65%)	95 (66%)	62 (65%)	
Age, y	40.9 ± 12	42 ± 13	39 ± 12	.1
Donor type, No. (%)				.5
Deceased donor	54 (23%)	32 (22%)	22 (23%)	
Living donor relative	186 (77%)	112 (78%)	74 (77%)	
First degree	118 (63%)	70 (63%)	48 (65%)	
Second degree	54 (29%)	32 (28%)	22 (30%)	
Third degree	14 (8%)	10 (9%)	4 (5%)	
Dialysis vintage, y	2 (0-5)	1.5 (0-5)	2 (0-5)	.3
Laboratory parameter				
Albumin, g/dL	4.31 ± 0.39	4.34 ± 0.4	4.28 ± 0.38	.9
Calcium, mg/dL	9.55 ± 0.6	9.52 ± 0.62	9.6 ± 0.58	.5
Phosphorus, mEq/L	3.2 ± 0.63	3.19 ± 0.61	3.23 ± 0.67	.9
Magnesium, mg/dL	1.78 ± 0.25	1.80 ± 0.25	1.77 ± 0.27	.6
Creatinine, mg/dL	1.15 ± 0.35	1.19 ± 0.37	1.1 ± 0.32	.05
eGFR, mL/min/1.73 m^2	75 ± 24	71.5 ± 23.3	79.5 ± 25.3	.03
Hemoglobin, g/dL	12.7 ± 2.1	12.8 ± 2	12.5 ± 2.2	.1
Total cholesterol, mg/dL	182 ± 39	180 ± 36	185 ± 42	.5
ALP, U/L	86 ± 39	88 ± 41	85 ± 38	.5
Medication, No. (%)				
Statin	15 (6%)	11 (7%)	4 (4%)	.5
Calcium channel blocker	72 (30%)	45 (31%)	27 (28%)	.6
mTOR inhibitor	32 (13%)	20 (14%)	12 (13%)	.5
CNI	219 (91%)	131 (91%)	88 (92%)	.7
Glucocorticoid dose, g	15.5 ± 10	15.8 ± 10	15 ± 11	.5
High PTH (> 65 ng/dL), No.(%)	77 (32%)	31 (22%)	46 (48%)	< .001
AVN, No. (%)	24 (10%)	13 (9%)	11 (11%)	.6
Follow-up time, y	7 (4-11)	7 (4-12)	7 (4-10.5)	.4

Abbreviations: ALP, alkaline phosphatase; AVN, avascular necrosis; CNI, calcineurin inhibitor; eGFR, estimated glomerular filtration rate; mTOR, mammalian target of rapamycin; PTH, parathyroid hormone

of osteopenia or osteoporosis in kidney transplant recipients (Table 5).

Avascular necrosis

A total of 251 patients were analyzed for AVN. Avascular necrosis was observed in 9.5% of patients (24/251). The cumulative glucocorticoid dose that was used until the development of AVN was found to be significantly lower in patients with AVN compared with patients without AVN (11.4 ± 8 vs 15.6 ± 9 g; $P < .001$). Other demographic parameters were similar in both groups (Table 6). Although no significant association was found between AVN and the klotho gene SNPs, hemoglobin, dialysis vintage, and drugs commonly used in patients with kidney transplant (calcineurin inhibitor, mTOR inhibitor, statins, and acetylsalicylic acid), we found that glucocorticoid dosage and PHP were associated with AVN by univariate logistic regression analysis. However, multivariate logistic regression analysis indicated that only PHP (OR, 2.52; 95% CI, 1.08-5.87) could predict the development of AVN (Table 7).

Discussion

The frequency of complications affecting long-term quality of life may increase with the prolongation of allograft survival in kidney transplant recipients. One of the most important of these complications is the occurrence of bone mineral disorders, and kidney transplant recipients are a unique population with substantial risk factors for bone disease and associated complications. Our study revealed the importance of PTH for bone health in kidney transplant recipients. Despite adequate vitamin D supplementation, we found that 34% of patients had PHP at the end of the first year of follow-up. Persistent hyperparathyroidism has been identified as an important risk factor for osteopenia and osteoporosis in our study. It also appears to be the risk factor for AVN development according to our results. Although the klotho SNPs rs480780, rs211234, rs576404, rs211235, rs9536314, and rs1207568 were not associated with AVN, some of these, including rs211235 for the development of PHP and rs480780 for osteopenia or osteoporosis, were defined as nonmodifiable risk factors for bone disease in patients with kidney transplant.

Parathyroid hormone is an important hormone in calcium, phosphorus, and bone metabolism.[16] High PTH level can still be observed in 30% to 60% of patients 1 year after transplant, and high PTH levels prior to transplant, long dialysis vintage, and nodular hyperplasia are defined as risk factors for PHP in the literature.[16,17] Dialysis vintage was found to be a risk factor for PHP in our study, similar to the

Table 5. Risk Factors for Osteopenia and Osteoporosis in Patients With Kidney Transplant

	Univariate Logistic Regression		Multivariate Logistic Regression	
	P	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)
Age	.07	0.98 (0.96-1.00)		
Sex (women)	.8	0.94 (0.55-1.62)		
rs480780				
GT+TT	< .001	5.29 (2.85-9.81)	.04	8.73 (1.03-73.96)
rs211234				
GA+AA	.001	4.24 (2.30-7.82)	.1	0.10 (0.01-1.61)
rs576404				
CA+AA	.001	4.72 (2.52-8.84)	.06	6.09 (0.90-41.30)
rs211235				
GT+TT	< .002	3.13 (1.51-6.49)	.7	0.84 (0.29-2.45)
rs9536314				
TG+GG	.5	1.19 (0.64-2.2)		
rs1207568				
GA+AA	.1	1.52 (0.85-2.70)		
Creatinine	.06	0.47 (0.21-1.04)		
Preemptive transplant	.5	0.83 (0.47-1.47)		
Diabetes mellitus	.8	1.10 (0.53-2.28)		
Glomerulonephritis	.7	0.88 (0.40-1.90)		
AVN	.5	1.30 (0.56-3.05)		
Glucocorticoid	.7	1.00 (0.99-1.00)		
CNI	.5	1.22 (0.69-2.14)		
mTOR inhibitor	.7	0.91 (0.57-1.47)		
High PTH (> 65 ng/dL)	< .001	3.09 (1.75-5.46)	.003	2.76 (1.43-5.33)

Abbreviations: ALP, alkaline phosphatase; AVN, avascular necrosis; CNI, calcineurin inhibitor; eGFR, estimated glomerular filtration rate; mTOR, mammalian target of rapamycin; PTH, parathyroid hormone

Table 6. Demographics of Patients According to Avascular Necrosis

		Avascular Necrosis		*P*
	Total	No	Yes	
No. of patients (% of cohort)	251	227 (90.5%)	24 (9.5%)	
Sex, No. (%)				.7
Women	87 (35%)	78 (34%)	9 (38%)	
Men	164 (65%)	149 (66%)	15 (62%)	
Age, y	40.9 ± 12.7	41 ± 13	40 ± 12	.8
Donor type, No. (%)				.6
Deceased donor	62 (25%)	57 (25%)	5 (21%)	
Living donor relative	189 (75%)	170 (75%)	19 (79%)	
First degree	120 (63%)	110 (65%)	10 (53%)	
Second degree	54 (29%)	48 (28%)	6 (32%)	
Third degree	15 (8%)	12 (7%)	3 (15%)	
Dialysis vintage, y	2 (0-5)	2 (0-5)	1 (0-6)	.6
Laboratory parameter				
Albumin, g/dL	4.27 ± 0.39	4.3 ± 0.4	4.28 ± 0.36	.8
Calcium, mg/dL	9.52 ± 0.62	9.53 ± 0.64	9.51 ± 0.68	.9
Phosphorus, mEq/L	3.18 ± 0.63	3.21 ± 0.65	3.16 ± 0.63	.9
Magnesium, mg/dL	1.79 ± 0.25	1.78 ± 0.26	1.80 ± 0.26	.9
Creatinine, mg/dL	1.15 ± 0.35	1.14 ± 0.35	1.2 ± 0.33	.4
eGFR, mL/min/1.73 m^2	75.2 ± 24.3	75.5 ± 24	71.8 ± 25	.4
Hemoglobin, g/dL	12.6 ± 2.1	12.6 ± 2.1	12.1 ± 1.6	.3
Total cholesterol, mg/dL	181 ± 39	181 ± 40	176 ± 22	.9
ALP, U/L	85 ± 38	86 ± 39	80 ± 29	.6
Medication, No.(%)				
Statin	16 (7%)	15 (7%)	1 (4%)	.5
Acetylsalicylic acid	22 (9%)	21 (10%)	1 (4%)	.3
Calcium channel blocker	78 (33%)	69 (33%)	9 (37%)	.6
Glucocorticoid dose, g	15 ± 10	15.6 ± 9	11.4 ± 8	< .001
High PTH (> 65 ng/dL), No. (%)	85 (34%)	73 (32%)	12 (50%)	.06
AVN development time, d			691 (61-5869)	

Abbreviations: ALP, alkaline phosphatase; AVN, avascular necrosis; eGFR, estimated glomerular filtration rate; PTH, parathyroid hormone

Table 7. Risk Factors for Avascular Necrosis Development

	Univariate Logistic Regression		Multivariate Logistic Regression	
	P	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)
Age	.7	0.99 (0.96-1.03)		
Sex (women)	.8	1.01 (0.46-2.49)		
rs480780				
GT + TT	.2	0.54 (0.22-1.28)		
rs211234				
GA + AA	.2	0.57 (0.24-1.34)		
rs576404				
CA + AA	.8	0.92 (0.40-2.15)		
rs211235				
GT + TT	.6	1.28 (0.46-3.61)		
rs9536314				
TG + GG	.3	1.61 (0.67-3.87)		
rs1207568				
GA + AA	.9	0.96 (0.39-2.35)		
Hemoglobin	.2	0.90 (0.74-1.08)		
Glucocorticoid	.04	1.00 (1.00-1.01)	.06	1.00 (0.99-1.00)
Diabetes mellitus	.3	0.50 (0.15-1.74)		
Vasculitis	.6	1.28 (0.49-3.36)		
CNI	.3	1.54 (0.65-3.64)		
mTOR inhibitor	.8	0.94 (0.44-1.99)		
Statin	.5	0.57 (0.07-4.48)		
Acetylsalicylic acid	.3	0.39 (0.50-3.05)		
High PTH (< 65 ng/dL)	.04	2.13 (1.01-4.82)	.03	2.52 (1.08-5.87)
Dialysis vintage	.6	1.02 (0.93-1.12)		

Abbreviations: AVN, avascular necrosis; CNI, calcineurin inhibitor, mTOR, mammalian target of rapamycin; PTH, parathyroid hormone

reports in the literature. In addition, we defined hypomagnesemia as a risk factor for PHP. Several in vivo and in vitro studies have demonstrated that magnesium can modulate PTH secretion.[18,19] Also, an

inverse relationship between serum PTH levels and magnesium levels has been shown among patients on dialysis.[20,21] Although there are a few studies that describe this relationship in kidney transplant recipients, Van de Cauter and colleagues demonstrated that hypomagnesemia at year 5 is an independent predictor of PHP in kidney transplant recipients.[19] Therefore, we can speculate that the suppressive effect of hypermagnesemia in the pretransplant period is absent and that development of hypomagnesemia after kidney transplant may contribute to PHP.

Another important finding of our study is that the rs211235 SNP of the klotho gene, which is located in the intron region, increases the risk of PHP development after kidney transplant. α-Klotho is essential for endogenous FGF-23 function, including phosphate homeostasis and suppression of vitamin D and PTH synthesis.[22] Over the past decade, klotho has been one focus for the progression of bone mineral disease in chronic kidney disease.[23,24] However, most of those studies were based on soluble klotho, klotho protein expression, and association with FGF-23.[23-25] Also, klotho gene SNPs have not been well studied. To our knowledge, the present study is the first study to investigate the relationship between klotho gene SNPs and PHP in kidney transplant recipients. The only previous publication to report that the rs211235 SNP is associated with bone mineral diseases is a study by Baldwin and colleagues related to the frequency of AVN in patients with sickle cell anemia.[11] The mechanism by which this polymorphism is related to PHP is probably different, because AVN and PHP develop with different mechanisms. In addition, in our patient population, this SNP was not associated with AVN.

Osteoporosis is one of the major problems for kidney transplant recipients. However, clinical focus has been aimed at allograft function after kidney transplant, and as a result the management of bone disease has often been neglected. Kidney transplant recipients may have legacy bone mineral disease from chronic kidney disease that may be exacerbated by the posttransplant drug regimen. The risk of hip fracture in kidney transplant recipients during the first 6 months of transplant is 34% higher than for patients on dialysis.[26] The prevalence of osteoporosis in kidney transplant recipients ranges between 11% and 56% in the literature, and bone loss occurs at the highest rate in the first year after transplant.[16,27] In our study, the incidence of osteopenia or osteoporosis was found to be 40% at the end of the first year after kidney transplant, which is in accordance with the literature. We showed that PHP is a modifiable risk factor for bone disease independent of age, sex, use of glucocorticoids and calcineurin inhibitors, and calcium supplementation.

In this group of patients, the most important factor that triggers bone disease is the use of glucocorticoids.[1,2,28] With the introduction of modern immunosuppressive drugs, the use of glucocorticoids is substantially reduced. However, it has been shown that discontinuation of glucocorticoid treatment increases rejection rates and frequency of chronic allograft nephropathy.[29,30] Therefore, it is not practicable to interrupt glucocorticoid treatment. In addition, the same cumulative glucocorticoid treatment in similar time courses causes higher incidence of bone disorder in some patients, as in our study. In this case, it is important to control hyperparathyroidism, which could be treatable, for bone health in kidney transplant recipients.

In our study, the nonmodifiable risk factor for osteoporosis in kidney transplant recipients is the rs480780 SNP of the klotho gene. Although we showed the association between osteoporosis and other klotho gene SNPs, including rs211234, rs576404, and rs211235, by univariate regression analysis, it is important to note that all of these SNP associations lost statistical significance when we applied multivariate logistic regression analysis. Also, we showed no association between rs9536314 and osteoporosis; this polymorphism is defined as the SNP of a functional variant of the klotho gene in humans.[31] It has been suggested that the rs9536314 allele influences the trafficking and catalytic activity of the klotho protein.[31] Riancho and colleagues showed that the rs9536314 SNP of the klotho gene is associated with osteoporosis in Spanish postmenopausal women.[32] On the other hand, Ozdem and colleagues found an inverse association between this polymorphism and vitamin D status but no association with development of osteoporosis in kidney transplant recipients; however, that study was conducted with a small number of patients, which included 25 kidney transplant recipients and 26 healthy control patients.[10] Although the rs9536314 SNP seems to be a risk factor for osteoporosis in the general population, it appears to be unassociated with osteoporosis in special patient populations such as kidney transplant recipients. In addition, the rs480780 SNP that we identified as a risk factor for development of bone disorder was determined as a predictor of AVN

development in patients with sickle cell disease in previous studies.[11,33] However, our study is the first study to define the rs480780 SNP, which is located in the intron region of the klotho gene, as a risk factor for development of osteoporosis.

Another bone disorder that could be seen after kidney transplant, which decreases in frequency, is AVN, which could adversely affect the quality of life and increase health costs.[2] Prevalence has been reported to be between 3% and 40% in different studies.[2] Although multiple risk factors have been reported in the literature, these risk factors have been largely attributed to the use of glucocorticoids.[34] However, the predictive value of the treatment duration and cumulative doses of glucocorticoids is controversial.[35,36] In our study, the absence of AVN in the majority of patients, despite more cumulative glucocorticoid exposure compared with patients who developed AVN, suggests that mechanisms other than glucocorticoids may be more determinant in the development of AVN. Although the exact mechanism of AVN development remains unclear, some of the proposed mechanisms are glucocorticoid-induced decrease in vascular endothelial growth factor, circulating lipids with resultant fat emboli, increased apoptosis of osteoblast and osteoclasts, and procoagulant state.[37] In our study, the incidence of AVN was found to be 9.5%, similar to other values reported in the literature.

One of the important points in our study on AVN is the time of development. Our data indicate that AVN can be seen very early after transplant and may occur after many years. Therefore, AVN should be considered in patients who present with unexpected joint or bone pain.

Some risk factors that have been defined in the literature include glucocorticoid treatment, presence of antiphospholipid antibodies, alcohol use, dyslipidemia, sickle cell anemia, and HIV infection.[38] However, there is no facilitating factor in 25% of cases. We could not show the relationship of AVN development with glucocorticoids, statin, and antiaggregant therapy and dyslipidemia. However, we defined PHP as a risk factor of AVN. Until now, the only well-known risk factor in kidney transplant recipients is the use of glucocorticoids; in contrast, PHP has not been well studied.[2] Felten and colleagues demonstrated that abnormal BMD and glucocorticoid treatment are risk factors for AVN development in kidney transplant recipients. They also found an association between PTH level > 300 ng/L and AVN development by univariate analysis; however, this association lost its significance in multivariate analysis.[39] It is important to note that the threshold value they used in their study was very high. If lower values were used, then multivariate analysis could be significant. Also, Nehme and colleagues demonstrated that high serum PTH was associated with AVN development in kidney transplant recipients.[40]

This study has some limitations. First, we did not measure serum klotho levels. This was important to determine the effect of polymorphisms on serum klotho level. This could help to explain how these SNPs contribute to serum PTH level and osteoporosis. Second, bone fractures, which represent another important bone mineral problem, could not be evaluated. Because it was observed only in 2 patients, the association of bone fractures with other possible risk factors could not be examined. The strengths of the present study are the long follow-up time, sufficient number of patients, use of average laboratory values of patients in the past 3 years for demographic evaluation, and exclusion of advanced chronic kidney disease, which is an important contributor for bone mineral disorder.

Conclusions

Bone health in kidney transplant recipients is an important issue that affects quality of life in the long-term. Bone metabolism disorders are frequently seen in this patient population. Modifiable risk factors such as PHP should be recognized and treated in a timely manner in cases for which the use of glucocorticoid therapy is mandatory. Also, the klotho gene seems to have important effects on bone metabolism. However, our results are not sufficient to establish a cause-effect association for klotho on bone metabolism, because there is very little information about these SNPs in the literature. Further studies, conducted as randomized controlled trials, are needed to clarify the association between klotho gene and bone metabolism.

References

1. Kulak CA, Borba VZ, Kulak Junior J, Custodio MR. Bone disease after transplantation: osteoporosis and fractures risk. *Arq Bras Endocrinol Metabol.* 2014;58(5):484-492. doi:10.1590/0004-2730000003343
2. Vangala C, Pan J, Cotton RT, Ramanathan V. Mineral and bone disorders after kidney transplantation. *Front Med (Lausanne).* 2018;5:211. doi:10.3389/fmed.2018.00211

3. Damasiewicz MJ, Ebeling PR. Management of mineral and bone disorders in renal transplant recipients. *Nephrology (Carlton)*. 2017;22 Suppl 2:65-69. doi:10.1111/nep.13028
4. Pihlstrom H, Dahle DO, Mjoen G, et al. Increased risk of all-cause mortality and renal graft loss in stable renal transplant recipients with hyperparathyroidism. *Transplantation*. 2015;99(2):351-359. doi:10.1097/TP.0000000000000583
5. Marchelek-Mysliwiec M, Rozanski J, Ogrodowczyk A, et al. The association of the Klotho polymorphism rs9536314 with parameters of calcium-phosphate metabolism in patients on long-term hemodialysis. *Ren Fail*. 2016;38(5):776-780. doi:10.3109/0886022X.2016.1162062
6. Deng G, Liu D. Klotho: a promising biomarker closely related to kidney transplant. *Exp Clin Transplant*. 2018;16(3):253-258. doi:10.6002/ect.2017.0329
7. Urakawa I, Yamazaki Y, Shimada T, et al. Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature*. 2006;444(7120):770-774. doi:10.1038/nature05315
8. Hardcastle MR, Dittmer KE. Fibroblast growth factor 23: a new dimension to diseases of calcium-phosphorus metabolism. *Vet Pathol*. 2015;52(5):770-784. doi:10.1177/0300985815586222
9. Canalejo R, Canalejo A, Martinez-Moreno JM, et al. FGF23 fails to inhibit uremic parathyroid glands. *J Am Soc Nephrol*. 2010;21(7):1125-1135. doi:10.1681/ASN.2009040427
10. Ozdem S, Yilmaz VT, Ozdem SS, et al. Is Klotho F352V polymorphism the missing piece of the bone loss puzzle in renal transplant recipients? *Pharmacology*. 2015;95(5-6):271-278. doi:10.1159/000398812
11. Baldwin C, Nolan VG, Wyszynski DF, et al. Association of klotho, bone morphogenic protein 6, and annexin A2 polymorphisms with sickle cell osteonecrosis. *Blood*. 2005;106(1):372-375. doi:10.1182/blood-2005-02-0548
12. Nazarian A, Hasankhani M, Aghajany-Nasab M, Monfared A. Association between klotho gene polymorphism and markers of bone metabolism in patients receiving maintenance hemodialysis in Iran. *Iran J Kidney Dis*. 2017;11(6):456-460.
13. Ogata N, Matsumura Y, Shiraki M, et al. Association of klotho gene polymorphism with bone density and spondylosis of the lumbar spine in postmenopausal women. *Bone*. 2002;31(1):37-42. doi:10.1016/s8756-3282(02)00786-x
14. Andrassy KM. Comments on 'KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease'. *Kidney Int*. 2013;84(3):622-623. doi:10.1038/ki.2013.243
15. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Transplant*. 2009;9 Suppl 3:S1-155. doi:10.1111/j.1600-6143.2009.02834.x
16. Taweesedt PT, Disthabanchong S. Mineral and bone disorder after kidney transplantation. *World J Transplant*. 2015;5(4):231-242. doi:10.5500/wjt.v5.i4.231
17. Sirilak S, Chatsrisak K, Ingsathit A, et al. Renal phosphate loss in long-term kidney transplantation. *Clin J Am Soc Nephrol*. 2012;7(2):323-331. doi:10.2215/CJN.06380611
18. Brown EM. Extracellular Ca2+ sensing, regulation of parathyroid cell function, and role of Ca2+ and other ions as extracellular (first) messengers. *Physiol Rev*. 1991;71(2):371-411. doi:10.1152/physrev.1991.71.2.371
19. Van de Cauter J, Sennesael J, Haentjens P. Long-term evolution of the mineral metabolism after renal transplantation: a prospective, single-center cohort study. *Transplant Proc*. 2011;43(9):3470-3475. doi:10.1016/j.transproceed.2011.09.030
20. Cho MS, Lee KS, Lee YK, et al. Relationship between the serum parathyroid hormone and magnesium levels in continuous ambulatory peritoneal dialysis (CAPD) patients using low-magnesium peritoneal dialysate. *Korean J Intern Med*. 2002;17(2):114-121. doi:10.3904/kjim.2002.17.2.114
21. Wei M, Esbaei K, Bargman J, Oreopoulos DG. Relationship between serum magnesium, parathyroid hormone, and vascular calcification in patients on dialysis: a literature review. *Perit Dial Int*. 2006;26(3):366-373.
22. Neyra JA, Hu MC. αKlotho and chronic kidney disease. *Vitam Horm*. 2016;101:257-310. doi:10.1016/bs.vh.2016.02.007
23. Komaba H, Lanske B. Role of Klotho in bone and implication for CKD. *Curr Opin Nephrol Hypertens*. 2018;27(4):298-304. doi:10.1097/MNH.0000000000000423
24. Zheng S, Chen Y, Zheng Y, Zhou Z, Li Z. Correlation of serum levels of fibroblast growth factor 23 and Klotho protein levels with bone mineral density in maintenance hemodialysis patients. *Eur J Med Res*. 2018;23(1):18. doi:10.1186/s40001-018-0315-z
25. Komaba H, Goto S, Fujii H, et al. Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients. *Kidney Int*. 2010;77(3):232-238. doi:10.1038/ki.2009.414
26. Ball AM, Gillen DL, Sherrard D, et al. Risk of hip fracture among dialysis and renal transplant recipients. *JAMA*. 2002;288(23):3014-3018. doi:10.1001/jama.288.23.3014
27. Julian BA, Laskow DA, Dubovsky J, Dubovsky EV, Curtis JJ, Quarles LD. Rapid loss of vertebral mineral density after renal transplantation. *N Engl J Med*. 1991;325(8):544-550. doi:10.1056/NEJM199108223250804
28. Kulak CA, Borba VZ, Kulak J Jr, Custodio MR. Osteoporosis after transplantation. *Curr Osteoporos Rep*. 2012;10(1):48-55. doi:10.1007/s11914-011-0083-y
29. Woodle ES, First MR, Pirsch J, et al. A prospective, randomized, double-blind, placebo-controlled multicenter trial comparing early (7 day) corticosteroid cessation versus long-term, low-dose corticosteroid therapy. *Ann Surg*. 2008;248(4):564-577. doi:10.1097/SLA.0b013e318187d1da
30. Kasiske BL, Chakkera HA, Louis TA, Ma JZ. A meta-analysis of immunosuppression withdrawal trials in renal transplantation. *J Am Soc Nephrol*. 2000;11(10):1910-1917.
31. Arking DE, Krebsova A, Macek M, Sr., et al. Association of human aging with a functional variant of klotho. *Proc Natl Acad Sci U S A*. 2002;99(2):856-861. doi:10.1073/pnas.022484299
32. Riancho JA, Valero C, Hernandez JL, et al. Association of the F352V variant of the Klotho gene with bone mineral density. *Biogerontology*. 2007;8(2):121-127. doi:10.1007/s10522-006-9039-5
33. Driss A, Asare KO, Hibbert JM, Gee BE, Adamkiewicz TV, Stiles JK. Sickle cell disease in the post genomic era: a monogenic disease with a polygenic phenotype. *Genomics Insights*. 2009;2009(2):23-48.
34. Assouline-Dayan Y, Chang C, Greenspan A, Shoenfeld Y, Gershwin ME. Pathogenesis and natural history of osteonecrosis. *Semin Arthritis Rheum*. 2002;32(2):94-124.
35. Abeles M, Urman JD, Rothfield NF. Aseptic necrosis of bone in systemic lupus erythematosus. Relationship to corticosteroid therapy. *Arch Intern Med*. 1978;138(5):750-754.
36. Zizic TM, Marcoux C, Hungerford DS, Dansereau JV, Stevens MB. Corticosteroid therapy associated with ischemic necrosis of bone in systemic lupus erythematosus. *Am J Med*. 1985;79(5):596-604. doi:10.1016/0002-9343(85)90057-9
37. Kerachian MA, Seguin C, Harvey EJ. Glucocorticoids in osteonecrosis of the femoral head: a new understanding of the mechanisms of action. *J Steroid Biochem Mol Biol*. 2009;114(3-5):121-128. doi:10.1016/j.jsbmb.2009.02.007
38. Lafforgue P. Pathophysiology and natural history of avascular necrosis of bone. *Joint Bone Spine*. 2006;73(5):500-507. doi:10.1016/j.jbspin.2006.01.025
39. Felten R, Perrin P, Caillard S, Moulin B, Javier RM. Avascular osteonecrosis in kidney transplant recipients: risk factors in a recent cohort study and evaluation of the role of secondary hyperparathyroidism. *PLoS One*. 2019;14(2):e0212931. doi:10.1371/journal.pone.0212931
40. Nehme D, Rondeau E, Paillard F, et al. Aseptic necrosis of bone following renal transplantation: relation with hyperparathyroidism. *Nephrol Dial Transplant*. 1989;4(2):123-128.