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Original Article

Serum myostatin level is a positive predictor of endothelial function measured by digital thermal monitoring of vascular reactivity in kidney transplantation patients

Hsiu-Hsien Lin[a], Ching-Chun Ho[a], Yen-Cheng Chen[a,b], Guan-Jin Ho[a,b], Bang-Gee Hsu[b,c], Ming-Che Lee[a,b]**

[a]Department of Surgery, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, [b]School of Medicine, Tzu Chi University, Hualien, Taiwan, [c]Division of Nephrology, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan

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Abstract

Objectives: Myostatin is a myokine predominantly expressed and secreted in skeletal muscle in response to stimulations, including oxidative stress or inflammation. We investigated a potential association between myostatin levels and endothelial function among kidney transplantation (KT) patients. **Materials and Methods:** Fasting blood samples were collected from 64 KT patients. The endothelial function that indicated by vascular reactivity index (VRI) was measured by digital thermal monitoring test. Serum myostatin levels were measured using a commercial enzyme-linked immunosorbent assay kit. All patients were categorized into three groups according to their VRI values: poor vascular reactivity was considered if VRI <1.0; 1.0 ≤VRI <2.0 indicated intermediate vascular reactivity, and VRI ≥2.0 was grouped as good vascular reactivity. **Results:** Seven KT patients (10.9%) were categorized as poor vascular reactivity, 24 KT patients (37.5%) were grouped as intermediate vascular reactivity, and 33 KT patients had good vascular reactivity. Advanced age (r = −0.372, P = 0.002) and serum alkaline phosphate (ALP) level (r = −0.341, P = 0.006) were negatively correlated with VRI. However, serum myostatin level (r = 0.430, P < 0.001) was positively correlated with VRI. In multivariable forward stepwise linear regression analysis, high serum level of myostatin (β = 0.441, adjusted R^2 change = 0.171; P < 0.001), advanced age (β = −0.317, adjusted R^2 change = 0.138; P = 0.003), and serum ALP level (β = −0.270, adjusted R^2 change = 0.060; P = 0.011) were significantly associated with VRI in KT patients. **Conclusion:** Our study showed that fasting myostatin level was positively associated with VRI and endothelial function among KT patients.

Keywords: *Endothelial function, Kidney transplantation, Myostatin, Vascular reactivity index*

Introduction

Endothelial dysfunction is defined as the decrease in capacity in the endothelium to dilate vessels in response to physical and chemical stimulation [1]. This condition is a shift of action of endothelium toward pro-inflammatory and pro-thrombotic status [2]. Endothelial dysfunction contributed to atherosclerosis formation in accordance with the response-to-injury hypothesis that means repeat injuries to endothelial cells will lead to endothelial dysfunction and cause the alternation in the surface of artery and form the atherosclerosis [3]. It is an early event of atherosclerosis and cardiovascular disease (CVD) in end-stage renal disease (ESRD) patients. CVD is a leading cause of mortality in ESRD patients [4] and even common in asymptomatic group [5]. More evidence stands for the effect of kidney transplantation (KT) on endothelial function, despite the possibly remained uremic status and effects of immunosuppressive agents in the post-KT period [6]. Previous study demonstrated an improvement of endothelial function at 6th and 12th month after KT by measuring endothelium-dependent vasodilatation [7]. Another study also illustrated the long-term stabilization of endothelial function

**Address for correspondence:*
Dr. Ming-Che Lee,
Department of Surgery, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, 707, Section 3, Chung-Yang Road, Hualien, Taiwan.
E-mail: mingche1229@gmail.com
Dr. Bang-Gee Hsu,
Division of Nephrology, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, 707, Section 3, Chung-Yang Road, Hualien, Taiwan.
E-mail: gee.lily@msa.hinet.net

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up to 2 years after KT [8]. Although KT might have protective effect in endothelial function, CVD is still the major cause of mortality in KT patients [9].

There are several factors considered to affect endothelial function [10,11]. Myostatin is a secreted myokine of transforming growth factor-beta family that is mainly produced in the skeletal muscle cells and response to stimulations, including inflammation and oxidative stress [12]. Myostatin also had a role in arterial aging and in the progression of aortic atherosclerosis. By studying its tissue level in atherosclerotic lesions, myostatin was found to present in diseased media, intima, and plaque [13]. Myostatin might also have a role in vascular smooth muscle cell (VSMC) action, monocyte chemotaxis, and vascular wall remodeling [12]. An *in vivo* study showed that inactivation of myostatin can protect LDL receptor-deficient mice against the development of atherosclerosis [14].

Number of methods to evaluate the peripheral endothelial function had been introduced over the decades. Digital thermal monitor (DTM), one of the noninvasive measurements, was developed to measure the vascular reactivity index (VRI) that indicated the endothelial function. The change of fingertip temperature during reactive hyperemia test was monitored to assess vascular reactivity when applying a portable self-measureable system [15]. By this finger-based automated assessment of VRI, it provided reproducible and reliable assessment of endothelial function and was considered to be one of the good indices for the evaluation of endothelial function [15].

In this study, we aimed to evaluate the association between serum myostatin and endothelial function measured by DTM test among KT patients.

Materials and Methods

Participants

From September 2015 to February 2016, KT patients older than 20 years of age and who had been on KT for at least 6 months from Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, were invited to participate in this study. A total of 96 participants were invited to participate in this study, and participants were excluded if they had dialysis access fistula or grafts at upper extremity (n = 8), acute infection (n = 1), acute episode of allograft rejection (n = 2), heart failure (n = 1), and malignancy (n = 8) at the time of blood sampling or if they refused to sign consent form for the study (n = 12). Finally, there were totally 64 KT patients enrolled in this study. The Research Ethics Committee, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, approved the study, which conducted under the tenets of the Helsinki Declaration (IRB104-27-B). All patients provided their informed consent before participating in this study. Patients who were diagnosed with hypertension were defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or having received any antihypertensive medication in the previous 2 weeks before enrollment.

Anthropometric analysis

Body weight of the participant was measured in light clothing and without shoes to the nearest 0.5 kg, and body height was measured to the nearest 0.5 cm. Body mass index was calculated as the weight in kilograms divided by the height in meters squared. Body skeletal and fat mass were assessed using a portable whole-body bioelectrical impedance device (Tanita BC 706DB, Tanita Corporation, Tokyo, Japan). This measurement is noninvasive and highly reproducible. The skeletal muscle index (SMI) was calculated as skeletal muscle mass/height^2 (kg/m^2).

Biochemical investigations

The biochemical analysis was processed by procedures that have been previously published [16-18]. In brief, fasting blood samples (approximately 5 mL) from the participants were immediately centrifuged at 3000 *g* for 10 min. Serum levels of blood urea nitrogen (BUN), creatinine, fasting glucose, total cholesterol, triglycerides (TG), calcium, phosphorus, and alkaline phosphate (ALP) were measured using an auto-analyzer (Siemens Advia 1800, Siemens Healthcare GmbH, Henkestr, Germany). Serum myostatin levels (Immundiagnostik AG, Bensheim, Germany) and intact parathyroid hormone levels (iPTH, IBL International GmbH, Hamburg, Germany) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. The detection range of myostatin ELISA is 0.4–32.5 ng/mL and the inter-assay coefficient of variation and the intra-assay coefficient of variation are 12.2% and 7.8%, respectively; the detection range of iPTH ELISA is 7–700 pg/mL and the inter-assay coefficient of variation and the intra-assay coefficient of variation are 5.7% and 4.8%, respectively. The estimate glomerular filtration rate was calculated by Chronic Kidney Disease Epidemiology Collaboration equation.

Endothelial function measurements

After an overnight fast and abstinence from tobacco, alcohol, caffeine, or vasoactive medications, DTM test of endothelial function measurements was obtained using an FDA-approved device (VENDYS-II, Endothelix Inc., Houston, TX, USA). Patients lied down in a supine position in an ambient temperature of 22°C–24°C for 30 min. Blood pressure cuffs were placed on both of the subject's upper arms. Skin temperature sensors were affixed to both of the subject's index fingers. DTM test of both hands was performed during 3 min of stabilization, 2 min of cuff inflation to 50 mmHg greater than SBP, and 5 min of deflation. Once the cuff was released, blood flow rushes into the forearm and hand, causing a temperature rebound in the fingertips which is directly proportional to the reactive hyperemia response. The higher the temperature rebound, the better the vascular reactivity. VRI was determined by taking the maximum difference between the observed temperature rebound curve and the zero reactivity curve during the reactive hyperemia period by the VENDYS software. VRI ranged from 0.0 to 3.5 and was classified as being indicative of poor (0.0 to <1.0), intermediate (1.0 to < 2.0), and good (≥2.0) vascular reactivity [15].

Statistical analysis

Data were expressed as means ± standard deviation and were tested for normal distribution by Kolmogorov–Smirnov statistics. Significant differences in measured values between groups (poor, intermediate, and good VRI) were analyzed using Kruskal–Wallis analysis for parameters that presented with nonnormal distribution (fasting glucose, TG, BUN, and creatinine) followed by Dunn's multiple comparison test or one-way analysis of variance for normally distributed data followed by *post hoc* Bonferroni test for multiple comparisons. Because fasting glucose, TG, BUN, and creatinine levels were not normally distributed, the data were log transformed to achieve normality. Clinical variables that correlated with VRI values were evaluated by simple linear regression analyses. Significant variables in the simple linear regression analyses were included in a multivariable forward stepwise regression analysis. A $P < 0.05$ was considered statistically significant. All statistical analyses were performed on a personal computer with the statistical package SPSS for Windows (Version 19.0, SPSS Inc., Chicago, IL, USA).

Results

The clinical characteristics and usage of immunosuppressive medicines of 64 KT patients are presented in Table 1. Of the 64 KT patients, 7 (10.9%) patients had poor VRI, 24 (37.5%) had intermediate VRI, and 33 (51.6%) had good VRI. Comorbid conditions included diabetes (n = 32; 50.0%) and hypertension (n = 22; 34.4%). The usage of immunosuppressive medicines included tacrolimus (n = 43; 67.2%), cyclosporine (n = 11; 17.2%), mycophenolate mofetil or mycophenolic acid (n = 43; 67.2%), steroids (n = 54; 84.4%), and rapamycin (n = 6; 9.4%). Increased serum ALP (P = 0.019) and decreased serum myostatin levels (P = 0.001) were significantly correlated with VRI cutoff points between groups (poor, intermediate, and good VRI). There was no significant difference between

Table 1: Clinical characteristics according different vascular reactivity index by digital thermal monitoring of the 64 kidney transplantation patients

Characteristics	All patients (n=64)	Good vascular reactivity (n=33)	Intermediate vascular reactivity (n=24)	Poor vascular reactivity (n=7)	P
Age (years)	45.41±10.92	44.15±11.84	45.46±9.86	51.14±9.17	0.311
KT duration (months)	77.72±50.55	72.58±44.59	88.960±53.96	63.43±64.63	0.358
Height (cm)	161.44±7.36	161.12±7.14	161.13±7.13	164.00±9.71	0.629
Body weight (kg)	65.13±13.15	65.10±13.19	64.58±13.46	67.20±13.72	0.900
Body mass index (kg/m^2)	24.91±4.28	25.08±4.98	24.68±3.26	24.90±4.40	0.941
Body fat mass (%)	29.96±8.91	29.62±10.09	30.98±6.78	28.07±10.29	0.718
Skeletal muscle index (kg/m^2)	16.58±2.65	16.34±2.80	16.40±2.41	18.30±2.43	0.194
Vascular reactivity index	1.96±0.86	2.55±0.59	1.63±0.26‡	0.31±0.30$^{\dagger, a}$	<0.001*
Systolic blood pressure (mmHg)	143.70±18.28	143.94±18.78	141.71±18.31	149.43±17.00	0.621
Diastolic blood pressure (mmHg)	83.03±12.47	84.91±13.84	80.63±10.17	82.43±13.15	0.444
Total cholesterol (mg/dL)	186.23±41.40	184.33±36.16	193.33±50.65	170.86±26.21	0.425
Triglyceride (mg/dL)	124.50 (90.25-165.50)	114.00 (85.00-159.50)	128.50 (03.25-176.75)	152.00 (115.00-166.00)	0.359
Fasting glucose (mg/dL)	95.00 (88.00-109.50)	95.00 (87.00-106.50)	94.50 (88.00-122.75)	96.00 (89.00-162.00)	0.865
Blood urea nitrogen (mg/dL)	24.00 (16.00-36.50)	24.00 (16.50-28.50)	24.00 (14.00-41.00)	23.00 (15.00-50.00)	0.978
Creatinine (mg/dL)	1.35 (1.00-1.80)	1.30 (1.00-1.70)	1.50 (0.90-2.25)	1.30 (1.10-1.60)	0.726
Estimated glomerular filtration rate (mL/min)	58.20±25.74	59.95±22.66	55.75±30.19	58.34±26.18	0.835
Total calcium (mg/dL)	9.16±0.58	9.24±0.51	9.14±0.70	8.86±0.37	0.294
Phosphorus (mg/dL)	3.26±0.71	3.19±0.72	3.29±0.69	3.46±0.78	0.634
Alkaline phosphate (IU/L)	77.94±30.19	70.39±24.88	80.58±33.75	104.43±27.26†	0.019*
Myostatin (pg/mL)	75.73±17.36	81.53±19.88	73.22±10.22	56.99±6.15†	0.001*
Intact parathyroid hormone (pg/mL)	111.39±79.98	98.49±54.99	135.44±106.84	89.69±59.27	0.171
Female, n (%)	31 (48.4)	17 (51.5)	12 (50.0)	2 (28.6)	0.534
Diabetes mellitus, n (%)	32 (50.0)	15 (45.5)	14 (58.3)	3 (42.9)	0.582
Hypertension, n (%)	22 (34.4)	9 (27.3)	10 (41.7)	3 (42.9)	0.466
Living donor, n (%)	11 (17.2)	3 (9.1)	5 (20.8)	3 (42.9) †	0.083
Tacrolimus use, n (%)	43 (67.2)	22 (66.7)	16 (66.7)	5 (71.4)	0.968
Mycophenolate mofetil or MYFORTIC use, n (%)	43 (67.2)	22 (66.7)	17 (70.8)	4 (57.1)	0.791
Steroid use, n (%)	54 (84.4)	27 (81.8)	20 (83.3)	7 (100.0)	0.471
Rapamycin use, n (%)	6 (9.4)	2 (6.1)	4 (16.7)	0 (0)	0.265
Cyclosporine use, n (%)	11 (17.2)	7 (21.2)	3 (12.5)	1 (14.3)	0.675

Values for continuous variables given as mean±standard deviation and test by one-way analysis of variance; variables not normally distributed given as medians and interquartile range and test by Kruskal-Wallis analysis; values are presented as n (%) and analysis after analysis by the Chi-square test. *P<0.05 was considered statistically significant after Kruskal-Wallis analysis or one-way analysis of variance, $^{\dagger}P$<0.05 compared with good vascular reactivity and poor vascular reactivity, $^{\ddagger}P$<0.05 compared with good vascular reactivity and intermediate vascular reactivity, ^{a}P<0.05 compared with intermediate vascular reactivity and poor vascular reactivity after Dunn's multiple comparison test or *post hoc* Bonferroni test

three VRI groups in terms of gender, transplantation type, presence of comorbidity, or type of immunosuppressive medicines.

The correlation of VRI levels and clinical variables by univariate and multivariate linear analyses among 64 KT patients is shown in Table 2. Advanced age (r = −0.372, P = 0.002) and serum ALP level (r = −0.341, P = 0.006) were both negatively correlated with VRI values; however, serum myostatin level (r = 0.430, P < 0.001) was positively correlated with VRI values in KT patients in univariate analysis. Multivariable forward stepwise linear regression analysis of the variables significantly associated with VRI values revealed again that high serum level of myostatin (β = 0.441, adjusted R^2 change = 0.171; P < 0.001), advanced age (β = −0.317, adjusted R^2 change = 0.138; P = 0.003), and serum ALP level (β = −0.270, adjusted R^2 change = 0.060; P = 0.011) were significantly and independently associated with VRI values in KT patients. Two-dimensional scattered plots of VRI values with age, serum ALP level, and serum myostatin level among these KT patients were drawn, which are presented as Figure 1a-c, respectively. Serum myostatin level was positively correlated with SMI [r = 0.266, P = 0.033, Figure 2a]; while myostatin level was not associated with body fat mass [r = −0.190, P = 0.133, Figure 2b] and serum ALP level [r = 0.014, P = 0.915, Figure 2c].

Discussion

This study showed that myostatin was positively and the ALP and age were negatively associated with VRI level measured by DTM in KT patients. After multivariable linear regression analysis, they were all significantly independent factors affecting endothelial function in KT patients.

The capacities of endothelium include promoting vasodilatation, antithrombotic and antiadhesive properties to vascular wall, regulating vascular permeability and smooth muscle proliferation, and maintaining cardiovascular function. [1]. Impaired above functions cause endothelial dysfunction. Meanwhile, several studies have showed the age-related decline of endothelium dysfunction [19-21]. As time goes by, the endothelium would be exposed to more risk factors such as reactive oxygen species and cytokines-inducing chronic inflammation that induce endothelial dysfunction [22]. Moreover, aged endothelium was presented with impaired expression and activity of endothelial nitric oxide synthase. This status would decrease the production of nitric oxide and impair the ability of vasodilatation [23].

Myostatin is negatively regulating skeletal muscle mass and is a biomarker of muscle wasting [24]. Most studies indicate that a higher circulating myostatin concentration can be found in patients with higher muscle mass. However, these findings are not consistent across the literature, and several studies

Table 2: Correlation of vascular reactivity index levels and clinical variables by univariate and multivariate linear analyses among 64 kidney transplantation patients

Variables	Vascular reactivity index				
	Univariate		Multivariate		
	r	*P*	β	Adjusted R^2 change	*P*
Female	0.173	0.171	-	-	-
Diabetes mellitus	−0.062	0.626	-	-	-
Hypertension	−0.161	0.203	-	-	-
Living donor	−0.147	0.246	-	-	-
Age (years)	−0.372	0.002*	−0.317	0.138	0.003*
KT duration (months)	−0.155	0.222	-	-	-
Height (cm)	−0.142	0.264	-	-	-
Body weight (kg)	−0.095	0.456	-	-	-
Body mass index (kg/m^2)	−0.030	0.815	-	-	-
Body fat mass (%)	0.044	0.730	-	-	-
Skeletal muscle index (kg/m^2)	0.244	0.052	-	-	-
Systolic blood pressure (mmHg)	−0.159	0.209	-	-	-
Diastolic blood pressure (mmHg)	0.103	0.418	-	-	-
Total cholesterol (mg/dL)	−0.029	0.822	-	-	-
Log-triglyceride (mg/dL)	−0.219	0.082	-	-	-
Log-glucose (mg/dL)	−0.203	0.100	-	-	-
Log-blood urea nitrogen (mg/dL)	−0.070	0.581	-	-	-
Log-creatinine (mg/dL)	−0.062	0.627	-	-	-
eGFR (mL/min)	0.057	0.654	-	-	-
Total calcium (mg/dL)	0.089	0.484	-	-	-
Phosphorus (mg/dL)	−0.009	0.944	-	-	-
Alkaline phosphate (IU/L)	−0.341	0.006*	−0.270	0.060	0.011*
Myostatin (pg/mL)	0.430	<0.001*	0.441	0.171	<0.001*
Intact parathyroid hormone (pg/mL)	0.064	0.616	-	-	-

Data of triglyceride, fasting glucose, blood urea nitrogen, and creatinine showed skewed distribution and therefore were log-transformed before analysis. Analysis of data was done using the univariate linear regression analyses or multivariate stepwise linear regression analysis (adapted factors were age, alkaline phosphate, and myostatin) *P<0.05 was considered statistically significant. eGFR, Estimated glomerular filtration rate

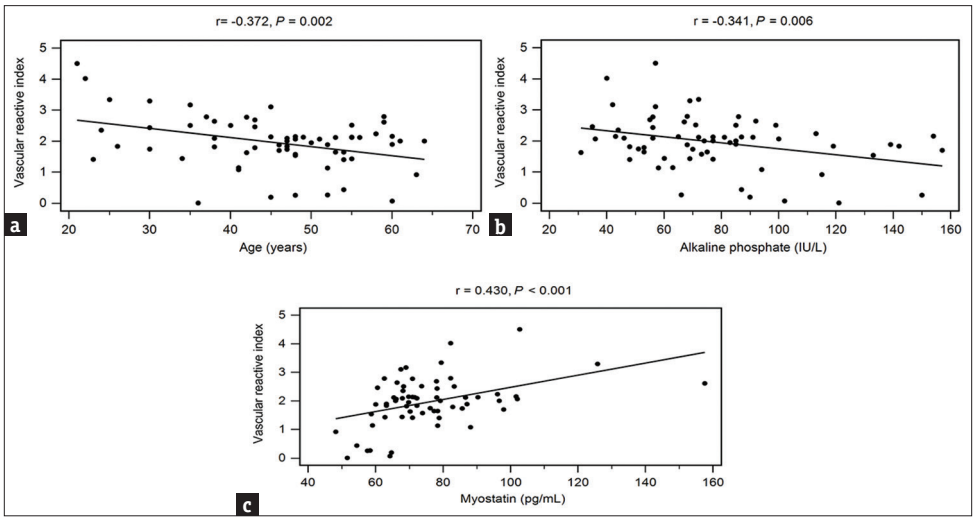

Figure 1: Relationships between vascular reactive index and (a) age, (b) alkaline phosphatase level, and (c) myostatin level among 64 kidney transplantation patients

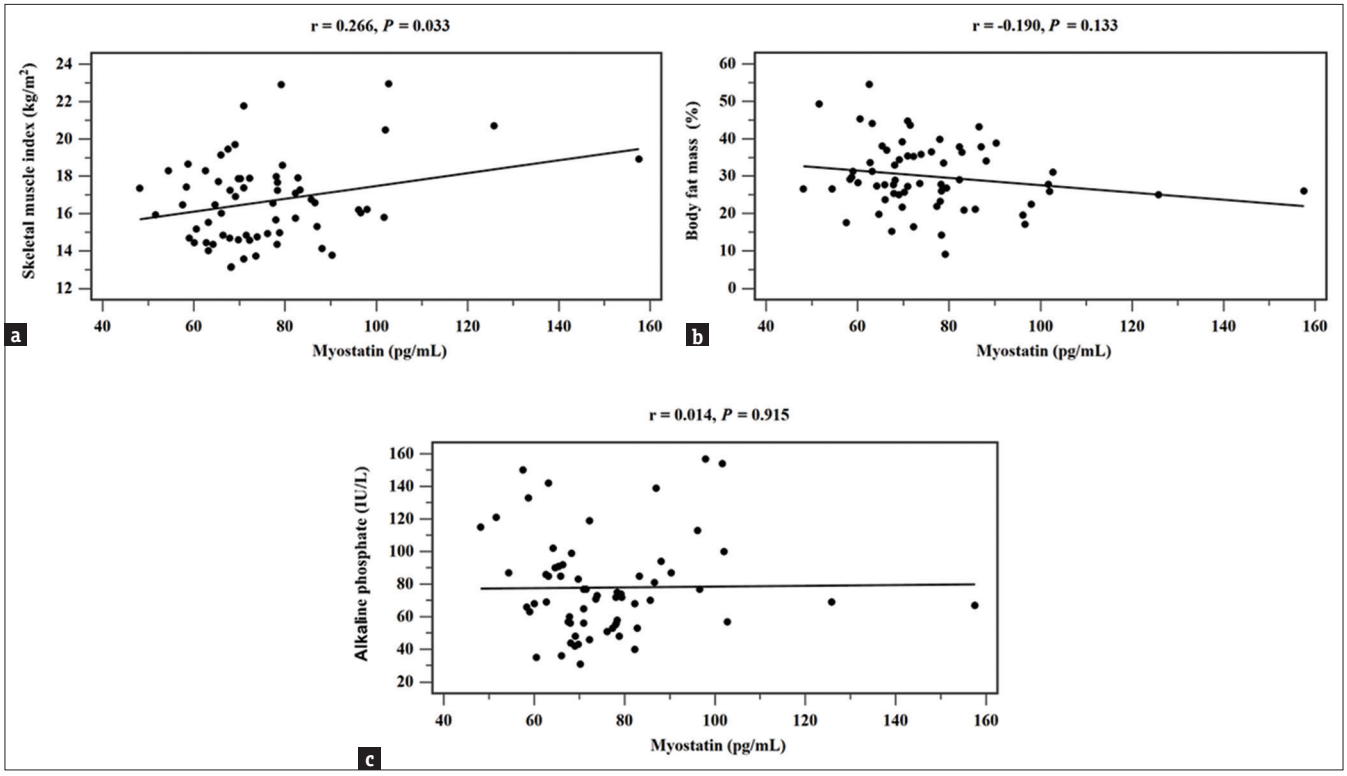

Figure 2: The correlation between serum myostatin level and (a) skeletal muscle index, (b) body fat mass, and (c) alkaline phosphatase level among 64 kidney transplantation patients

were unable to demonstrate a relationship between serum myostatin and muscle mass [24]. Goossens *et al*. supposed that myostatin has the potential to inhibit VSMC proliferation and the expression of miR-495-3p and therefore reduce vascular stenosis [25]. Myostatin level was negatively associated with abdominal aortic calcification scores on plain radiography and had a positive association with skeletal muscle mass in 71 patients undergoing dialysis [26]. Moreover, reduced skeletal muscle mass is associated with increased arterial stiffness and peripheral resistance in patients with CVD [27]. However, Verzola *et al*. demonstrated that myostatin was overexpressed in abdominal aortic wall deterioration and was contributed to vascular inflammatory change in an *in vitro* study [13]. Butcher *et al*. showed that endothelium-dependent vasodilation was better in those myostatin-knockout mice when compared to control group [28]. Szulc *et al*. reported that serum myostatin levels are inversely correlated with abdominal aortic calcification in older male [29]. In our study, skeletal muscle mass is positively associated with myostatin level and serum myostatin level is positively associated with VRI values in KT patients. It is presumed that myostatin production itself can be decreased when muscle mass is

decreased in KT patients. This indicating preserved skeletal muscle mass that increased myostatin level is associated with endothelial function in KT patients. Despite the fact that such inconsistencies in results might be discouraging for considering serum myostatin as a valid biomarker of vascular function. The complex constellation of physical, metabolic, nutrition, inflammation, and measurement methods and drugs may lead to affect serum myostatin level [24]. There is a need for prospective studies to elucidate mechanisms for this relationship.

The alkaline phosphatase is a powerful predictor of cardiovascular mortality in general population or patients with renal or CVD [30]. It plays a role in pathogenesis of vascular calcification. It hydrolyzes and thus inactivates the inorganic pyrophosphate which is an inhibitor of vascular calcification by binding to microcalcification in the early phase of calcification process [31]. Panh *et al*. showed that, in patients free of CVD, high ALP was positively and independently associated with coronary artery calcification [32]. Perticone *et al*. illustrated that ALP significantly and negatively affected the endothelium-dependent vasodilation in naïve hypertensive patients [33]. In the present study, ALP is negatively associated with VRI values in our KT patients. There did not differ statistically between serum ALP level and myostatin level in our study.

There were several limitations in this study. First, this was a cross-sectional study, and the enrolled subjects were relative small in number. Hence, the results of present study need further large cohort study to investigate the correlation of myostatin and endothelial function in this patient population. Second, the pro-myostatin is cleaved at Golgi apparatus or extracellular space by furin protein convertase to generate N- and C-terminal fragments [34]. The C-terminal that was shown to be capable of forming disulfide-linked homodimers was the mature biologically active form of myostatin. The C-terminal myostatin could be purified from the serum by monoclonal antibody [35]. In the present study, we only measure the total serum myostatin but not the active form. Finally, the dialysis types and dialysis time before KT, potentially influencing the VRI and vascular calcification, did not measure in this study that may affect serum ALP and myostatin level. Further studies are needed to confirm this hypothesis and to evaluate the potential clinical consequences.

Conclusion

Our study showed that serum myostatin level was positively associated with endothelial function measuring by DTM in KT patient. Older age and elevated ALP have negative impact on endothelial function in this patient group. The underlying mechanism of myostatin in this positive association with endothelial function and its potentially clinical implication requires further investigation.

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Conflicts of interest

Dr. Bang-Gee Hsu and Dr. Ming-Che Lee, the editorial board members at *Tzu Chi Medical Journal*, had no roles in the peer review process of or decision to publish this article. The other authors declared that they have no conflicts of interest.

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