Original Paper

Impaired Leukocytes Autophagy in Chronic Kidney Disease Patients

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Key Words

Autophagy · Renal failure · Cardiovascular diseases · Light chain 3

Abstract

Background: Proteins and cytoplasmic organelles undergo degradation and recycling via autophagy; its role in patients with chronic kidney disease (CKD) is still unclear. We hypothesize that impaired kidney function causes autophagy activation failure. **Methods:** We included 60 patients with stage 5 CKD and 30 age- and sex-matched healthy subjects as controls. Patients with conditions that could affect autophagy were excluded. Leukocytes were isolated and analyzed from peripheral blood samples collected after overnight fasting and 2 h after breakfast. **Results:** Overnight fasting induced conversion of microtubule-associated protein-1 light chain 3 I to II (γLC3) and increased mRNA levels of the autophagy-related gene 5 (*Atg5*) and *Beclin-1* in healthy subjects, which were nearly absent in CKD patients ($p = 0.0001$). Moreover, no significant difference in autophagy activation was observed between CKD patients with or without hemodialysis. Correlation studies showed that γLC3 was negatively associated with the left atrium size. Changes in *Atg5* transcript levels were negatively associated with the left ventricular end-diastolic diameter, and changes in *Beclin-1* transcript levels were negatively associated with the mitral inflow E- and A-wave sizes. **Conclusion:** These data suggest that CKD patients have impaired autophagy activation, which cannot be reversed with hemodialysis and is closely related to their cardiac abnormalities.

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Introduction

Autophagy is a self-digestion process of the cell [1]. It involves degradation and reuse of damaged organelles and misfolded proteins, and invading microorganisms to provide nutrients and energy for cellular needs. Under conditions of food or energy shortage, cells are forced to breakdown or redistribute their own components by autophagy to survive during starvation [2]. Autophagy is a tightly regulated process occurring in virtually every cell in the human body in order to maintain basal homeostatic protein or organelle turnover. Fasting is one of the evolutionarily conserved modulators of autophagy. Temperature, oxygen availability, infection, oxidative stress, and circadian rhythm are also important cues in the control of autophagy [3].

Many complex human diseases and their treatments involve autophagy. Defective autophagy or deregulated autophagic activities are found in cardiomyopathies [4], Alzheimer's disease [5], Parkinson's disease [6], systemic lupus erythematosus [7], cancer [8], and diabetes [9]. Kidney diseases also involve autophagy. Animal studies have demonstrated the critical role of autophagy in the pathogenesis of renal fibrosis, diabetic nephropathy, and toxin-related renal damage [10]. However, it is not known if autophagy or autophagy activation is affected in patients with chronic kidney disease (CKD). Patients with CKD have higher cerebrovascular morbidity, increased risk of dementia, and higher mortality due to cancer, infection, or pulmonary disease [11]. The exact mechanisms of the increased risks and mortality due to these diseases in CKD patients are still controversial and probably multifactorial. Recent studies have suggested that autophagy might play an important role in pathogenesis or treatment of these diseases, but the impact of autophagy in CKD patients is unknown.

Currently, there are no standard clinical autophagy measurements available. Guidelines in 2012 for autophagy monitoring suggest to use microtubule-associated protein-1 light chain 3 (LC3)/autophagy-related gene (Atg) quantification, p62 turnover assays, mammalian target of rapamycin (mTOR)/amp-activated protein kinase (AMPK) levels, and transmission electron microscopy (TEM) for monitoring autophagy [12]. LC3 proteins are involved in phagophore formation and characterized as autophagosome markers. The unprocessed proLC3 is converted into LC3-I and then modified into LC3-II. Among these assays, LC3 is most frequently used for monitoring autophagy function. Autophagy-related proteins, such as Atg5 and Beclin-1, are also used for monitoring autophagy. They are involved in different steps of autophagy and can be used to define specific processes in autophagy function. p62 proteins are incorporated into the autophagosomes and degraded in autolysosomes. Therefore, p62 is used as readout of autophagic degradation. AMPK and mTOR are important regulators of autophagy. They can be used for monitoring regulations of autophagy. However, they are not direct measurements of autophagy. TEM provides direct quantitative analysis of changes in various autophagic structures but is prone to misinterpretations due to methodological artifacts. These various methods for measuring autophagy function clinically [13], including tissue biopsies, primary cell isolation, analysis of genetic polymorphisms, and studies in cancer specimens, are either indirect or invasive.

In this context, we hypothesize that CKD patients might have altered autophagy function. We considered that a better clinical autophagy measurement method should take into account the relationship of autophagy activation to the feeding status and time, since autophagy and its activation are greatly affected by feeding status. Therefore, we designed a method to measure autophagy function in peripheral blood leukocytes and used this method to assess whether autophagy in leukocytes was altered in patients with CKD.

Materials and Methods

Study Design

We enrolled 30 healthy volunteers (60% men, mean age 52.6 ± 6.6 years) as the control group. Detailed clinical questionnaires were administered by a physician and blood tests were performed to exclude renal function impairment, diabetes, abnormal liver function test, heart failure, cerebrovascular disease, neurodegenerative disorder, metabolic syndrome, severe obesity with a body mass index (BMI) of more than 35, engagement in shift work with disturbed circadian rhythm, and use of any medications within 3 months in the control group. We then prospectively enrolled 60 consecutive patients (60% men, age 20–79 years) who were sex- and age-matched to within 5 years, and diagnosed with stages 4–5 CKD, according to the National Kidney Foundation Disease Outcomes Quality Initiative (KDOQI) criteria, from the outpatient clinics of Chang Gung Memorial Hospital from 2008 to 2010. Patients without hemodialysis (RF; $n = 30$, 60% men) and with HD ($n = 30$, 60% men) were included, but patients undergoing peritoneal dialysis were excluded. Patients with diabetes, impaired liver function (bilirubin >1.6 mg/dl), uncontrolled hypertension, heart failure, cerebrovascular disease, neurodegenerative disorder, systemic lupus erythematosus, severe obesity (BMI >35), severe hyperlipidemia (total cholesterol >300 mg/dl; triglyceride >400 mg/dl), engaged in shift work, and currently on carbamazepine, statins or immunosuppressive agents were all excluded. All subjects provided written informed consent to participate. The protocol was approved by the institutional review boards of Chang Gung Memorial Hospital and adhered to the Declaration of Helsinki.

Measurement of Autophagy

We thought that autophagy is more accurately assessed if measurements were made after both feeding and overnight fasting [14]. The blood samples from the study subjects were drawn 12 h after overnight fasting and 2 h after breakfast under the supervision of a clinical study nurse to ascertain the fasting status, breakfast intake and accurate timing of the blood sample collection. CKD patients with HD received blood sampling 1 day after HD.

The blood samples were kept on ice and processed within 30 min to isolate leukocytes. Ten milliliters of whole blood were mixed with red blood cell lysis buffer for 6 min at room temperature and then centrifuged at $2,000 g$ and 4°C for 2 min. The pellet containing the leukocytes was resuspended with red blood cell lysis buffer for 30 s and centrifuged again at $2,000 g$ and 4°C for 2 min. After examining the leukocyte viability and yield with the trypan blue exclusion test (average viability $>98\%$, yield $5\text{--}60 \times 10^6$ cells), the leukocyte pellets were divided equally into aliquots of 2×10^6 cells/tube. One hundred microliters of cell lysis buffer (Cell Signaling) with protease inhibitors (Roche) was then added into the tube reserved for protein analysis, and 50 μl of RNeasy lysis buffer (Qiagen) was added into the tube reserved for RNA analysis.

The pellets with cell lysis buffer were then rotated at 4°C for 30 min and centrifuged at $15,000 g$ for 10 min. The leukocyte extracts were analyzed by Western blotting with a rabbit LC3 polyclonal antibody (Abcam), or an anti-actin monoclonal antibody (Sigma). Bands were analyzed with the Image J software package (NIH).

A previous study showed that the LC3-I level is very stable during starvation and that the LC3-II level is reflective of changes in the autophagic function [14]. We thought that LC3-I can, therefore, serve as an ideal control. The ratio of the 14 kDa LC3-II to the 16 kDa LC3-I (LC3-II/LC3-I) can serve as an indicator of autophagy. The ratio of LC3-II/LC3-I after fasting for 12 h (LC3-II/LC3-I-AC) to LC3-II/LC3-I 2 h after breakfast (LC3-II/LC3-I-PC) in the same subject was calculated as γLC3 and regarded as indicator of autophagy flux or activation:

$$\gamma\text{LC3} = \frac{\text{AC } \frac{\text{LC3-II}}{\text{LC3-I}}}{\text{PC } \frac{\text{LC3-II}}{\text{LC3-I}}}$$

Gene Expression

After removal of RNeasy lysis buffer and extraction with Tri Reagent protocol (Ambion), total RNA was reverse transcribed with Superscript[®] III (Invitrogen). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed in a 7200 System (Applied Biosystems) using the SYBR Green PCR master mix with primers for *Atg5* and *Beclin-1* (*Atg5*: forward: 5'-AAAGATGTGCTTCGAGATGTGT-3', reverse: 5'-CACTTTGT-CAGTTACCAACGTCA-3'; *Beclin-1*: forward: 5'-CTGGTAGAAGATAAAACCCGGTG-3', reverse: 5'-CGTGGAC-TATCCGGCAGTT-3'). The amount of RNA was normalized to that of 18S ribosomal RNA.

Echocardiography

M-mode, two-dimensional, and Doppler echocardiography were performed with commercially available instruments (Vivid 7; GE). Variability within and between sonographies was assessed during the examination period. Left ventricular (LV) mass, LV septal thickness, posterior wall thickness, and end-diastolic dimension were all measured at end-diastole. Left ventricular ejection fraction (LVEF) was assessed with the apical four-chamber approach using the modified Simpson's rule. All calculations were performed in accordance with the American Society of Echocardiography guideline [15].

Patient Follow-Up and Data Collection

Patient and control subject characteristics, including age at enrollment, sex, symptoms, kidney disease classification, comorbid disorders, medical history, medication history, and family history were obtained with a case record form. Follow-up visits were conducted at 3-month intervals. All patients were followed up for at least 2 years.

Statistical Methods and Analysis

Effect size and power calculations were based on our pilot experiments in patients without CKD. Power analyses were performed with a significance level of 0.05, effect size of 1.50, and power of 0.8. Continuous variables for normal distribution were tested with the Kolmogorov-Smirnov test. Data with normal distribution (expressed as mean $\pm$ SD) were subjected to one-way ANOVA and post hoc Tukey's tests. For skewed data, such as those for cystatin C, LC3, *Atg5*, or *Beclin-1*, we used non-parametric Kruskal-Wallis tests to compare between groups, and the results are shown as the geometric means with 95% confidence intervals (CI). Receiver-operating characteristic (ROC) analysis was performed to determine the cutoff value of γ LC3 to predict CKD. Since echocardiographic parameters were affected by blood pressure and autophagy markers were not normally distributed, we used partial correlation coefficients, adjusted for blood pressure, to compute the association between autophagy markers and echocardiographic parameters. All reported p values were two-sided, and a p value of <0.05 was considered statistically significant. Statistical analyses were performed with SPSS (IBM).

Results

The baseline demographic and clinical characteristics for healthy subjects (NC), CKD patients without dialysis (RF patients), and CKD patients with dialysis (HD patients) are summarized in table 1. The BMIs, waist circumferences, percentages of smokers, fasting glucose, total cholesterol, and triglyceride levels were similar in CKD patients and NC. Blood pressure, blood urea nitrogen, serum creatinine, cystatin C, and uric acid were all higher in CKD patients, compared to those of the NC. Hemoglobin and estimated glomerular filtration rate were lower in CKD patients, compared to the values in the NC.

In the NC and CKD patients, LC3-I and LC3-II protein levels showed significant variations among individuals (fig. 1a). In the NC, the levels of LC3-I or LC3-II after fasting or 2 h after breakfast did not show significant statistical changes (fig. 1b, c). In RF or HD patients, the LC3-I or LC3-II levels were also similar after fasting or 2 h after breakfast (fig. 1b, c). The levels of LC3-I and LC3-II in the HD patients were both increased when compared to the NC or RF groups (fig. 1b, c). However, compared with the CKD patients, the NC had significant autophagy activation, as measured by γ LC3 (fig. 1d, e). The γ LC3 in the NC was 1.78 (95% CI 1.34–2.2), while in the RF patients, the γ LC3 was 0.92 (95% CI 0.78–1.06), and in the HD patients, the γ LC3 was 1.22 (95% CI 1.05–1.39, $p < 0.0001$). The cutoff value for γ LC3 that predicts CKD was <1.2 by ROC analysis (area under the curve = 0.79 ± 0.06 , range 0.68–0.90, $p < 0.0001$) (fig. 2). The sensitivity and specificity rates for this ratio were 77 and 60%, respectively. These data suggested that by taking feeding status into consideration, γ LC3 is a better autophagy indicator than isolated LC3-I or LC3-II. To obtain a more complete picture of autophagy, transcript levels of autophagy markers and mediators were measured (fig. 3). After overnight fasting, transcript levels of *Atg5* increased by 80% in the NC group, decreased

Table 1. Demographic and clinical characteristics in NC and CKD patients

	NC (n = 30)	RF (n = 30)	HD (n = 30)
Age, years	52.7±6.6	55.9±16.2	57.3±10.2
Female gender	12 (40)	12 (40)	12 (40)
Weight, kg	67.5±8.9	63.1±14.2	56.2±8.1
Height, cm	165.2±8.9	158.3±8.2	161.5±7.3
Body mass index	24.7±2.1	25.1±5.6	21.5±2.7
Waist circumference, cm	81.7±10.2	89.3±7.8	84.0±10.2
Fasting glucose, mg/dl	91.9±9.0	97.3±14.4	91.9±18.0
Hypertension	0	14 (46.7)	8 (25.8)
Smoking	9 (30)	2 (6.7)	5 (16.1)
BUN, mg/dl	13.4±3.4	77.9±44.8	62.7±16.8
Serum creatinine, mg/dl	0.8±0.2	6.5±4.6	11.5±2.8
MDRD-eGFR, ml/min/1.73 m ²	110.9±32.0	12.2±8.4	–
Cystatin C, mg/l	1.00±0.24	5.51±1.46	8.33±1.19
Total cholesterol, mg/dl	193.1±30.9	216.2±77.2	181.5±38.6
Triglyceride, mg/dl	132.7±97.3	159.3±79.6	123.9±79.6
Uric acid, mg/dl	6.1±1.7	7.7±2.1	7.2±1.4
Hemoglobin, g/l	14.2±1.4	9.3±1.8	10.4±1.3
Patients receiving (drug class)			
ACE inhibitors/ARBs	–	7	2
β-Blockers	–	7	6
Calcium channel blockers	–	14	4
Diuretics	–	6	0

Data are n (%) or mean ± SD unless otherwise specified. MDRD-eGFR = Modification of Diet in Renal Disease Study equation-estimated glomerular filtration rate; ACE = angiotensin-converting enzyme; ARB = angiotensin receptor antagonist.

by 5% in RF patients, and by 8% in HD patients ($p < 0.0001$) (fig. 3a). Changes in the *Beclin-1* transcript were smaller but parallel to changes in γ LC3 and *Atg5*, +33, –28, and –6% in the NC, RF, and HD ($p < 0.0001$) groups, respectively (fig. 3b). When the RF and HD patients were compared, there were no significant differences after overnight fasting and 2 h after breakfast in γ LC3, *Atg5*, and *Beclin-1* levels ($p > 0.05$), implying that HD has no effects on autophagy activation. Taken together, the protein and transcript data strongly suggest that autophagy activation was decreased in CKD patients and that HD has no effect on the autophagy deregulation observed in CKD patients.

Cardiovascular diseases are the major causes of morbidity and mortality in CKD patients [16]. We concluded that the altered autophagy function we found in CKD patients might contribute partly to changes in cardiac structures or functions in these patients. We then examined cardiac structure and function in the NC and CKD patients using echocardiography. Compared to CKD patients, the NC had greater LVEF and a smaller aortic root diameter (AO), left atrial diameter (LA), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and diastolic mitral inflow peak early filling E-wave (E) and late filling A-wave (A) (table 2). Association analysis was performed to determine the correlation of autophagy markers with echocardiographic parameters in the NC and CKD patients. After adjusting for blood pressure, γ LC3 was negatively associated with LA size, changes of the *Atg5* transcript were negatively associated with LVEDD, and changes of the *Beclin-1* transcript were negatively associated with diastolic mitral inflow E- and A-wave values (table 3). These findings suggest that different autophagy markers are associated with different echocardiographic parameters, such as LA size, LV size, or diastolic function.

Fig. 1. LC3-I, LC3-II, and γ LC3 values in NC and CKD patients. **a** Representative immunoblot of LC3-I and LC3-II of leukocytes after overnight fasting (AC) and breakfast (PC) in NC, CKD patients without HD (RF patients), and CKD patients with HD treatment (HD patients). **b, c** Baseline protein expression of LC3-I and LC3-II after PC and AC. **d** Ratio of LC3-II/LC3-I after PC and AC. **e** Autophagy activation, expressed as γ LC3, in NC and CKD patients. * $p < 0.0001$, n.s. at $p > 0.05$.

Discussion

In the present study, we demonstrated that autophagy function could be estimated in patients by taking feeding status into consideration. We have shown that autophagy markers, including γ LC3, Atg5, and Beclin-1, are increased in leukocytes after overnight fasting. This activation is absent in CKD patients irrespective of whether they received HD or not. In addition, the impaired autophagy activation of leukocytes in CKD patients was associated

Fig. 2. ROC analysis to determine the cutoff value of CKD patients. The cutoff value for γ LC3 that predicts CKD was <1.2 (area under the curve = 0.79 ± 0.06 , range 0.68–0.90, $p < 0.0001$). The sensitivity and specificity rates for this ratio were 77 and 60%, respectively.

Fig. 3. Atg5 and Beclin-1 values in NC and CKD patients. **a** Atg5 AC/PC ratio in NC, RF, and HD patients. **b** Beclin-1 AC/PC ratio in NC, RF, and HD patients. * $p < 0.0001$, † $p = 0.0005$, n.s. at $p > 0.05$.

with several abnormalities in cardiac structures, as measured by echocardiography. These findings indicate that impaired autophagy function is a novel mechanism for developing cardiovascular diseases in CKD patients, which cannot be resolved or treated by HD.

The precise mechanisms by which autophagy is impaired after renal function deteriorates in CKD patients are unknown. There are several potential mechanisms that could explain the impaired autophagy activation in these patients. For example, oxidative stress is a common feature and major mediator of complications in CKD. Oxidative stress and reactive oxygen species (ROS) are important regulators of autophagy [17]. H_2O_2 and $O_2 \cdot^-$ mediate the induction of autophagy and modify several autophagy-related proteins, including IKK β , JNK-1, and Atg [18]. ROS induce S-nitrosation of JNK-1, which subsequently inhibits Beclin-1

Table 2. Echocardiographic parameters in NC and CKD patients

Echocardiographic parameter	NC	RF	HD	p value
AO, mm	30.1±2.9	31.9±2.9	33.0±3.6	0.002
LA, mm	32.0±3.4	39.0±7.3	38.5±7.2	<0.0001
IVS, mm	11.6±2.2	12.8±2.9	13.0±3.1	0.126
LVPW, mm	10.8±2.0	9.9±3.1	10.0±2.5	0.328
LVEDD, mm	43.7±4.9	49.2±5.6	47.1±8.3	0.007
LVESD, mm	24.9±4.5	30.4±7.4	30.0±6.7	0.001
LV mass, g	179±48.0	228.1±97.5	220.5±122.5	0.065
LVEF, %	75.2±7.4	66.5±12.6	66.4±8.3	0.005
E, cm/s	64.2±12.9	88.7±21.2	71.3±22.4	<0.0001
A, cm/s	68.6±19.2	99.7±24.2	94.0±21.7	<0.0001
E/A	1.1±0.5	1.2±1.2	0.8±0.3	0.131
IVRT, ms	86.3±21.0	87.5±18.9	94.1±22.8	0.361

Data are geometric mean ± SD. IVS = Interventricular septum; LVPW = left ventricular posterior wall; E = diastolic mitral inflow peak E wave; A = diastolic mitral inflow peak A wave; E/A = E wave divided by A wave; IVRT = isovolumic relaxation time.

Table 3. Partial correlation coefficient adjusted for blood pressure between echocardiographic parameters and autophagy markers

Covariate	γLC3		ATG5 AC/PC		Beclin-1 AC/PC	
	Coefficient	p value (two-tailed)	Coefficient	p value (two-tailed)	Coefficient	p value (two-tailed)
AO (mm)	−0.030	0.810	0.055	0.664	−0.120	0.342
LA (mm)	−0.352	0.004	−0.164	0.192	−0.171	0.173
IVS (mm)	0.150	0.235	−0.131	0.300	−0.132	0.295
LVPW (mm)	0.161	0.199	0.108	0.391	0.289	0.060
LVEDD (mm)	−0.177	0.159	−0.254	0.041	−0.146	0.245
LVESD (mm)	−0.159	0.205	−0.187	0.136	−0.092	0.466
LV mass (g)	0.000	0.997	−0.208	0.096	−0.046	0.714
LVEF (%)	0.122	0.331	0.146	0.246	0.063	0.618
E (cm/s)	0.170	0.176	−0.105	0.403	−0.284	0.022
A (cm/s)	−0.149	0.237	−0.053	0.674	−0.430	<0.0001
E/A	−0.009	0.943	−0.006	0.964	0.211	0.091
IVRT (ms)	−0.169	0.178	−0.018	0.888	−0.015	0.904

IVS = Interventricular septum; LVPW = left ventricular posterior wall; E = diastolic mitral inflow peak E wave; A = diastolic mitral inflow peak A wave; E/A = E wave divided by A wave; IVRT = isovolumic relaxation time.

activity and impairs autophagy [19]. Interestingly, ROS stimulation of cells that have abnormal autophagy, due to deficiency or reduction of Atg5, likely results in further accumulation of ROS and more dysfunctional mitochondria, suggesting that impaired autophagy in CKD is part of a vicious cycle of ROS-induced alterations in cell physiology. Another mechanism that could link impaired autophagy and CKD is mTOR. There is substantial evidence for an important role of the mTOR pathway in CKD. Progressive kidney disease and diabetic nephropathy increase mTOR activity within the kidney [20]. Biopsy samples from patients

with diabetic nephropathy indicate increased levels of mTOR mRNA and phosphorylated S6, which is a downstream target of mTOR [21]. Regulation of autophagy by mTOR is evolutionarily conserved from yeast to humans. Starvation induces autophagy through inhibition of mTOR activity. In contrast, conditions of nutrient abundance increase the mTOR activity, which in turn increases the phosphorylation of both ULK1 and Atg13 by the rapamycin-sensitive mTOR complex 1, and inhibits autophagosome formation [22]. It is important to evaluate the association of these mechanisms with autophagy in CKD patients in future studies.

Different autophagy markers correlate with different echocardiographic parameters, which may indicate multiple ways in which autophagy impacts the cardiovascular system in CKD. In our study, γ LC3 negatively correlates with LA size, the Atg5 ratio correlates negatively with LV size, and the Beclin-1 ratio correlates negatively with mitral inflow E- and A-wave sizes. The LA size has been consistently shown to be an important predictor of cardiovascular outcome. Increased LA size correlates with increased incidence of atrial fibrillation, stroke, acute myocardial infarction, and congestive heart failure [23]. The LV size also correlates well with cardiac remodeling after myocardial infarction, heart failure, and uremic cardiomyopathy. The mitral inflow E- and A-wave sizes are important parameters for diastolic function. Impaired diastolic function was an important mediator of increased risks in CKD patients in the VALIANT echo study [24]. Previous studies have shown that mechanical unloading after LV assist device in patients with dilated cardiomyopathy decreased mRNA and protein levels of LC3, Atg5, and Beclin-1 [4] and that transaortic banding decreased the LC3-II/LC3-I ratio in mice [25]. It is still unclear which mechanisms are responsible for these different associations. The lack of consistent correlations might be due to the small number of subjects in our study or complexity of autophagy mechanisms. Moreover, LA size, mitral inflow E- and A-wave, and LV end-diastolic diameters could all link to diastolic function. In the future, it will be interesting to determine whether autophagy associates with diastolic dysfunction in CKD.

In this study, despite the observed increase in γ LC3 in the leukocytes of NC, the changes in LC3-I and LC3-II levels were not significant after overnight fasting. In NC, the average value of LC3-I decreased and LC3-II increased after overnight fasting; however, these changes were not statistically significant. This might be due to the wide range of distribution of LC3-I and LC3-II values. Previous studies and our preliminary studies have suggested that LC3-II, detected by immunoblotting after prolonged starvation, is more susceptible to degradation, and this degradation can be partially inhibited with lysosomal protease inhibitors such as E64d and pepstatin A [14]. In our preliminary studies, we have observed that both LC3-I and LC3-II decreased if blood samples were not processed immediately. These findings suggest that the amount of autophagy markers at a certain time point does not indicate the total autophagy activation. Furthermore, autophagy activation measured by γ LC3, Atg5, and Beclin-1 ratios serves as a better autophagy marker for estimating total autophagy activation. Indeed, we have observed much higher LC3-I and LC3-II levels in HD patients (fig. 1b, c), but their autophagy activation was still impaired when measured by γ LC3, Atg5, and Beclin-1 ratios. The exact reasons why HD patients have higher LC3-I and LC3-II levels are unknown. The higher expression levels of LC3 may be associated either with enhanced autophagic flow or with impaired autophagosome maturation. Additional experiments using animal CKD models to examine autophagy in leukocytes and in kidney tissues with acute manipulation of lysosomal activity would be helpful to address this issue [14].

Limitations of the present study are the selection of a restricted set of CKD patients and the case-controlled design. We excluded patients with multiple factors or on medications that could affect autophagy. Therefore, our findings can only be applied to certain CKD patients. The case-control design of the study cannot infer causality, and the study should be considered

as hypothesis generating and proof of concept. Moreover, autophagy markers in leukocytes may not represent whole-body autophagy, as there could be inter-organ variations in autophagy.

Taken together, these results provide evidence that the autophagy function is impaired in CKD patients and that it can potentially be a target for therapeutic intervention for CKD patients with cardiovascular diseases.

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Erratum

In the article by Kudoh Y, et al., entitled ‘Hemodynamic stabilizing effects of L-carnitine in chronic hemodialysis patients’ [*Cardiorenal Med* 2013;3:200–207, DOI: [10.1159/000355016](https://doi.org/10.1159/000355016)], the authors would like to insert a significance mark (*) in figure 3 with the following legend: Changes in hypotensive episodes after L-carnitine supplementation. * $p < 0.05$ (after 3 months of L-carnitine supplementation).

Fig. 3. Changes in hypotensive episodes after L-carnitine supplementation. * $p < 0.05$ (after 3 months of L-carnitine supplementation).