

Original Article

Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients

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Abstract

Background. Anaemia is a common complication associated with haemodialysis and is usually managed by treatment with recombinant human erythropoietin (rHuEPO). However, many patients remain hyporesponsive to rHuEPO treatment despite adequate iron therapy. The effect of L-carnitine administration on rHuEPO dose and/or haematocrit in haemodialysis patients has been previously reported with equivocal results. This study examined the relationship between endogenous carnitine pool composition and rHuEPO requirements in long-term haemodialysis patients.

Methods. Pre-dialysis blood samples were collected from 87 patients and analysed for plasma L-carnitine and individual acylcarnitine levels by LCMS/MS. As an indication of rHuEPO responsiveness, erythropoietin resistance index (ERI) was calculated as rHuEPO dose/kg/week normalized for haemoglobin levels.

Results. A significant negative correlation between L-carnitine levels and ERI was found ($P = 0.0421$). All patients categorized as high ERI ($>0.02 \mu\text{g/kg/week/gHb}$) exhibited subnormal L-carnitine levels ($<30 \mu\text{M}$); conversely, patients with normal L-carnitine levels ($>30 \mu\text{M}$) displayed low ERI values ($<0.02 \mu\text{g/kg/week/gHb}$). More importantly, the ratio of non-acetyl acylcarnitines/total carnitine was significantly positively correlated with ERI ($P = 0.0062$).

Conclusions. These data illustrate the relationship between carnitine levels and response to rHuEPO treatment in haemodialysis patients, in particular, the importance of the proportion of long-chain acylcarnitines within the plasma carnitine pool. This proportion may be more indicative of the response to L-carnitine supplementation than absolute L-carnitine levels alone.

Keywords: acylcarnitine; anaemia; erythropoietin; haemodialysis; L-carnitine

Introduction

Renal anaemia, experienced by the vast majority of chronic haemodialysis patients, is primarily a result of a reduction in the secretion of erythropoietin from the kidney; however, reduced erythrocyte survival caused by haemolysis of red blood cells (RBC) is thought to augment the problem [1]. The treatment of anaemia with recombinant human erythropoietin (rHuEPO) is widespread with most chronic haemodialysis patients receiving rHuEPO or an alternative erythropoiesis-stimulating agent such as darbepoetin [2]. Although rHuEPO has had a dramatic impact on the treatment of renal anaemia, the dose required to maintain target haemoglobin levels varies considerably within the patient population. Numerous patients remain hyporesponsive to treatment (despite adequate iron therapy), and consequently, these patients receive a particularly high dose of rHuEPO to achieve acceptable haemoglobin concentrations, although levels usually remain sub-optimal [3,4]. Hyporesponsiveness to rHuEPO treatment has been associated with a reduction in quality of life and an increase in mortality [5,6]. Adjunctive therapies to reduce rHuEPO requirements, particularly for hyporesponsive patients, have been called for [7–10].

Although the aetiology of renal anaemia is complex, the alteration in the composition of the endogenous carnitine pool associated with chronic haemodialysis, characterized by reduced L-carnitine (free carnitine) levels and elevated medium- and long-chain acylcarnitines [11], has been implicated as a potential contributing factor to rHuEPO hyporesponsiveness in some patients. Consequently, L-carnitine supplementation has been proposed as a potential adjunct to rHuEPO in the treatment of rHuEPO-resistant anaemia.

The impact of L-carnitine supplementation on rHuEPO dose and/or haematocrit levels in haemodialysis patients has been extensively investigated [12–26]. A meta-analysis of 18 clinical trials investigating the effects of L-carnitine treatment on renal anaemia has demonstrated that L-carnitine supplementation results in a significant improvement in the response to erythropoietin treatment; however, the authors also reported on the need for further

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studies [27]. Despite an overall improvement of anaemic symptoms with L-carnitine supplementation, a number of studies have shown that the patient population appears to be separated into 'responders' and 'non-responders' [15,18,19]. Although in all of these studies the division of patients into these subgroups was retrospective, the authors suggest that there was a relationship between carnitine levels and improvement in rHuEPO requirements. In order to investigate this further, this observational study was designed to investigate the relationship between the composition of the endogenous plasma carnitine pool and erythropoietin requirements in chronic haemodialysis patients not receiving L-carnitine supplementation. For the first time, the relationship between a full complement of indices of carnitine pool composition and responsiveness to rHuEPO therapy (taking into account both dose required and effectiveness) was investigated.

Subjects and methods

This study was reviewed and approved by the Human Research Ethics Committees of the Royal Adelaide Hospital (protocol number 050208) and the University of South Australia (protocol number P018/05). Patients were fully informed of the study procedures and provided written informed consent prior to study initiation. The study was conducted in accordance with the Declaration of Helsinki and the National Statement on Ethical Conduct in Human Research issued by the National Health and Medical Research Council (Australia).

End-stage renal disease (ESRD) patients undergoing chronic haemodialysis were recruited for study participation from the Royal Adelaide Hospital Dialysis Unit and its associated satellite dialysis centres. Patients were required to be stable on haemodialysis treatment and to have not received carnitine supplementation within the 2 months prior to the study. Patients were to be receiving adequate intravenous iron therapy according to the standard protocol issued by the Royal Adelaide Hospital Dialysis Unit.

On a single occasion, an arterial blood sample was collected from the arteriovenous fistula of each patient prior to commencement of haemodialysis for analysis of plasma carnitine and haemoglobin concentrations. In addition, details of rHuEPO treatment were collected from each patient.

In order to take into account both rHuEPO dose and haemoglobin levels (therefore providing a more reliable indication of responsiveness to rHuEPO treatment), erythropoietin resistance index (ERI) was calculated for each patient as follows:

$$\text{ERI} = \frac{\text{rHuEPO/kg/week}}{\text{gHaemoglobin}}$$

Study patients were prospectively divided into pre-defined Low ERI and High ERI groups based on a cut-off ERI value of 0.02 $\mu\text{g/kg/week/gHb}$. A patient with an ERI value of 0.02 or greater was considered to be rHuEPO resistant based on the National Kidney Foundation practice recommendations for L-carnitine supplementation in which dialysis patients who are unable to maintain a target

haemoglobin level of 110–120 g/L and require rHuEPO doses of at least 300 IU/kg/week (2.55 $\mu\text{g/kg/week}$) are recommended for L-carnitine therapy [28]. An endogenous L-carnitine concentration of <30 μM was defined as sub-normal based on the lower limit of the normal reference range in healthy adults [29].

Analytical methods

Plasma samples were analysed for L-carnitine and individual acylcarnitine concentrations using an MDS-SCIEX API4000 triple quadrupole tandem mass spectrometer (Applied Biosystems Inc., Foster City, CA, USA) with sample delivery using a 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Aliquots (2 μL) of each plasma sample were applied to 3 mm punches of filter paper (Whatman BFC-180, Whatman Inc., Fairfield, NJ, USA) and allowed to dry at room temperature. Once dry, filter papers were shipped to the analytical laboratory for analysis.

Samples were extracted from the filter paper using a solution of pure methanol containing known concentrations of stable isotopically enriched acylcarnitines (synthesized in-house). After a 15-min extraction period, samples were dried under nitrogen. Samples were then esterified using acidified butanol to form the butyl-ester of each acylcarnitine followed by drying under nitrogen to remove excess butanolic HCl. The butyl-esters were determined by a precursor scan of 85.1 amu. The levels of acylcarnitines were determined against the respective deuterated stable isotope using Analyst[®] software (Applied Biosystems Inc.).

As indicators of carnitine pool composition, parameters outlined in Table 1 were calculated using quantified plasma carnitine concentrations.

Statistical methods

Statistical examination of the relationship between carnitine pool composition and responsiveness to rHuEPO treatment was conducted using linear regression of ERI versus carnitine parameters. In addition, statistical comparisons of carnitine parameters between the Low ERI and High ERI groups were conducted using an analysis of variance (ANOVA).

Significance was set at an α -level of 0.05. WinNonlin[®] Professional Version 4.1 (Pharsight Corporation, Mountain View, CA, USA) was used for all statistical analyses.

Table 1. Calculated carnitine parameters

Parameter	Description
LC	L-carnitine
TC	Total carnitine, calculated as the sum of L-carnitine and all acylcarnitines
AcylLC	Total acylcarnitines, calculated as the sum of all acylcarnitines
AcylLC-C2	Non-acetyl acylcarnitines, calculated as the sum of all acylcarnitines excluding acetyl-L-carnitine
LC/TC	Proportion of L-carnitine within the total carnitine pool
AcylLC/TC	Proportion of acylcarnitines within the total carnitine pool
AcylLC-C2/TC	Proportion of non-acetyl acylcarnitines within the total carnitine pool

Results

A total of 87 ESRD patients (56 males, 31 females) undergoing haemodialysis treatment participated in the study. Of these, 75 patients were designated as 'Low ERI' and 12 patients were designated as 'High ERI'. Patient demographic and dialysis details are presented in Table 2. There were no significant differences in patient age, dialysis age or dialysis session duration between patients in the Low ERI and High ERI groups. However, there were a higher proportion of males to females in the Low ERI group than in the High ERI group.

Parameters calculated as indicators of carnitine pool composition are outlined in Table 1. Mean \pm standard deviation (SD) values for each carnitine parameter for all of the study patients are summarized in Table 3, along with mean \pm SD values for each ERI subgroup. Statistical comparisons of carnitine data between the subgroups indicated that patients classified as Low ERI had significantly higher endogenous plasma L-carnitine and total carnitine concentrations than patients in the High ERI group ($P = 0.0092$ and $P = 0.0210$, respectively). Interestingly, all patients classified as High ERI were noted to have subnormal endogenous plasma L-carnitine levels ($<30 \mu\text{M}$). Similarly, all patients with normal L-carnitine levels had ERI values of $<0.02 \mu\text{g}/\text{kg}/\text{week}/\text{gHb}$ (i.e. Low ERI). In addition, the proportion of endogenous non-acetyl acylcarnitines within the total plasma carnitine pool (AcylLC-C2/TC) was significantly lower in the Low ERI group compared with those in the High ERI group ($P = 0.0158$).

Due to the differences in gender distribution between the ERI groups, statistical comparisons of carnitine parameters

Table 2. Patient demographic and dialysis data; data expressed as mean \pm SD

Parameter	All patients	Low ERI patients	High ERI patients
Subjects (<i>n</i>)	87	75	12
Age (years)	59.1 \pm 13.8	58.6 \pm 13.8	61.8 \pm 14.2
Gender	56 males/ 31 females	53 males/ 22 females	3 males/ 9 females
Dialysis age (years)	2.78 \pm 2.72	2.47 \pm 2.04	4.70 \pm 5.02
Dialysis session duration (hours)	4.10 \pm 0.461	4.09 \pm 0.452	4.17 \pm 0.531

ERI = erythropoietin resistance index.

Table 3. Endogenous plasma carnitine levels for all subjects and each erythropoietin resistance index (ERI) subgroup; data expressed as mean \pm SD

Parameter	All patients	Low ERI patients	High ERI patients
LC	22.8 \pm 8.62 μM	23.8 \pm 8.67 μM	17.0 \pm 5.47 μM *
TC	38.6 \pm 13.7 μM	39.9 \pm 13.9 μM	30.6 \pm 9.29 μM *
AcylLC	15.8 \pm 5.93 μM	16.1 \pm 6.01 μM	13.6 \pm 5.13 μM
AcylLC-C2	7.80 \pm 2.57 μM	7.83 \pm 2.37 μM	7.59 \pm 3.74 μM
LC/TC	0.588 \pm 0.0608	0.593 \pm 0.0585	0.558 \pm 0.0685
AcylLC/TC	0.412 \pm 0.0608	0.407 \pm 0.0585	0.422 \pm 0.0685
AcylLC-C2/TC	0.211 \pm 0.0556	0.205 \pm 0.0504	0.248 \pm 0.0733*

* $P < 0.05$, compared to the Low ERI group.

between the ERI groups were also conducted for female patients alone. Results of statistical comparisons for the female patients were consistent with that observed for the population as a whole. Female patients in the Low ERI group had significantly higher plasma L-carnitine levels ($20.4 \pm 5.13 \mu\text{M}$ c.f. $15.0 \pm 4.28 \mu\text{M}$, $P = 0.0116$) and significantly lower AcylLC-C2/TC values (0.218 ± 0.0406 c.f. 0.268 ± 0.0746 , $P = 0.0310$) than female patients in the High ERI group. Analysis could not be conducted for males due to low subject numbers in the High ERI group. Average L-carnitine and AcylLC-C2/TC values for male patients in the Low ERI group were $25.1 \pm 9.50 \mu\text{M}$ and 0.200 ± 0.0534 , respectively, compared with values of $22.9 \pm 4.62 \mu\text{M}$ and 0.189 ± 0.0180 for male patients classified as High ERI.

A significant negative correlation between ERI and endogenous plasma L-carnitine concentrations was observed ($P = 0.0421$, $R = -0.2184$) (Figure 1). In addition, ERI was shown to be significantly positively correlated with the proportion of endogenous non-acetyl acylcarnitine concentrations within the total plasma carnitine pool ($P = 0.0062$, $R = +0.2911$) (Figure 2); this relationship was found to be independent of dialysis age.

Discussion

Renal anaemia is a common complication associated with chronic haemodialysis treatment and is primarily treated with rHuEPO or an alternative erythropoiesis-stimulating agent. The rHuEPO dose required to maintain target haemoglobin levels can vary dramatically within the patient population, and some patients remain hyporesponsive to rHuEPO treatment, despite adequate iron therapy and increases in rHuEPO dose [3,4]. Due to the high costs associated with rHuEPO treatment, a reduction in the prescribed dose of rHuEPO can have enormous financial implications [8]. It is however important to note that the rationale for the improvement of the effectiveness of rHuEPO treatment is not merely economic. Recent research investigating the relationship between rHuEPO requirements and mortality in haemodialysis patients has indicated that the highest mortality rate is observed in patients receiving high doses of rHuEPO with low haematocrit levels (i.e. rHuEPO hyporesponsive) [6]. Perhaps more importantly, examination of patients with haematocrit levels in the desirable range revealed that patients who receive high doses of rHuEPO had a mortality rate twice that of patients receiving low rHuEPO doses, thereby suggesting that, wherever possible, the minimum rHuEPO dose necessary to achieve acceptable haematocrit levels should be prescribed. Consequently, adjunctive therapies to rHuEPO treatment have been called for [7–10].

L-carnitine deficiency has been clearly associated with chronic haemodialysis therapy, with patients exhibiting a reduction in L-carnitine levels and an accumulation of medium- and long-chain acylcarnitines [11,30]. This disruption in carnitine homeostasis has been implicated as a contributing factor towards rHuEPO hyporesponsiveness in haemodialysis patients [7–10]. Numerous studies have demonstrated that supplementation of L-carnitine in

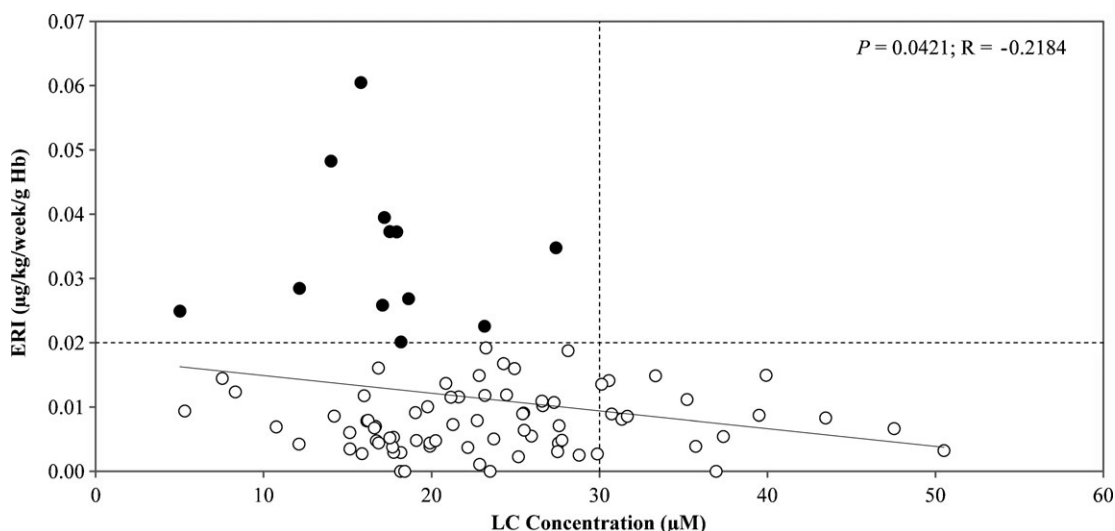


Fig. 1. Relationship between endogenous plasma L-carnitine concentrations and erythropoietin resistance index (ERI) (○ Low ERI patients; ● High ERI patients).

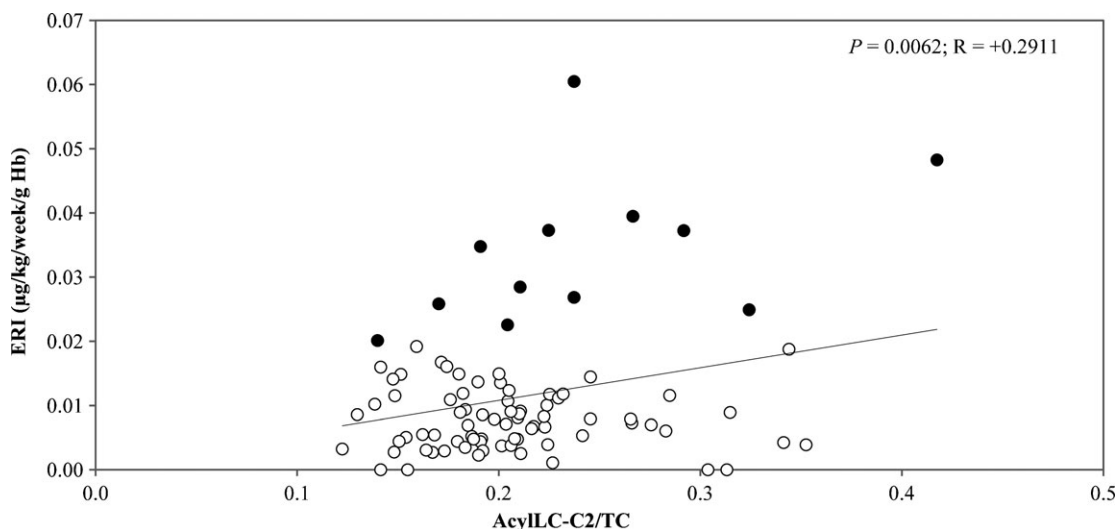


Fig. 2. Relationship between the proportion of non-acetyl acylcarnitines within the total endogenous plasma carnitine pool (AcylLC-C2/TC) and erythropoietin resistance index (ERI) (○ Low ERI patients; ● High ERI patients).

combination with standard anaemia management in dialysis patients results in an increase in haematocrit values and/or a reduction in rHuEPO dose; however, the extent of the effectiveness of L-carnitine treatment has been shown to vary extensively between patients (as reviewed [31]). Previous authors have suggested that the extent of effectiveness of L-carnitine supplementation in improving rHuEPO requirements may be related to carnitine levels [15,18,19]. This study was conducted in order to investigate the relationship between endogenous carnitine profiles and rHuEPO requirements in chronic haemodialysis patients.

In this study, patients exhibited substantial alterations in plasma carnitine pool composition compared to levels observed in healthy control subjects. Patients displayed considerably lower plasma L-carnitine and total carnitine levels and higher concentrations of total acylcarnitines and non-

acetyl acylcarnitines than previously reported for healthy subjects using the same analytical method [11,29]. As a result, the proportion of carnitine within the total carnitine pool was substantially altered; of particular note, in this study, non-acetyl acylcarnitines (i.e. medium- and long-chain acylcarnitines) constituted 20–25% of the total plasma carnitine pool, compared to values of ~5% in the healthy population. These findings are consistent with those previously reported for patients undergoing long-term haemodialysis treatment [11,30,32–37].

Patients classified as erythropoietin-resistant (ERI > 0.02 µg/kg/week/gHb), based on the National Kidney Foundation recommendations, were shown to have significantly lower L-carnitine and total carnitine levels and a higher proportion of non-acetyl acylcarnitines within the total plasma carnitine pool than their Low ERI counterparts,

thereby suggesting that haemodialysis patients with rHuEPO hyporesponsiveness display a more disturbed carnitine profile than rHuEPO responsive patients. This is supported by the finding that all patients classified as High ERI displayed subnormal endogenous plasma L-carnitine levels; on the other hand, the reverse statement does not hold true—not all patients with subnormal L-carnitine levels were classified as High ERI. This therefore suggests that L-carnitine levels alone are not the only factor that contributes to the efficacy of rHuEPO treatment; however, this finding may imply that, in some patients, carnitine levels are important for the effective management of renal anaemia.

Although gender differences in carnitine levels in the haemodialysis population have not been clearly established [18,38], in light of the disparity in the distribution of genders in the two ERI groups, additional statistical comparisons of carnitine levels between the Low and High ERI groups were conducted for female patients. In female patients, results were consistent with those found for the population as a whole, with significantly lower L-carnitine and higher AcylLC-C2/TC levels in erythropoietin-resistant patients than the Low ERI patients. Analysis of male data was not possible due to low subject numbers of males in the High ERI group ($n = 3$).

Previous studies have investigated the relationship between endogenous carnitine levels and rHuEPO requirements with inconsistent results. Matsumura *et al.* [39] described a significant negative correlation between L-carnitine and total carnitine levels and rHuEPO dose, whereas Kooistra *et al.* [40] was only able to demonstrate a significant correlation for total carnitine. On the other hand, Kletzmayer *et al.* [18] was unable to show any significant relationships between rHuEPO requirements and carnitine levels measured. However, it should be noted that these studies only examined relationships between rHuEPO requirements and carnitine levels alone; more complex indicators of carnitine pool composition have not been examined until now. In keeping with the results of Matsumura *et al.* [39], the present study has shown a significant correlation between rHuEPO requirements and L-carnitine levels. However, perhaps more importantly, this study has demonstrated a stronger relationship between ERI and the proportion of non-acetyl acylcarnitines within the total carnitine pool. This ratio is likely to be more indicative of carnitine pool status as it takes into account both the accumulation of non-acetyl acylcarnitines and the reduction in L-carnitine levels associated with haemodialysis treatment. Estimates of total acylcarnitine levels, commonly used in previous studies, include acetyl-L-carnitine levels that have been shown to decline with dialysis treatment, and therefore the accumulation of medium- and long-chain acylcarnitines is effectively ‘cancelled out’ by the reduction in acetyl-L-carnitine levels [30]. This may also explain the lack of a significant correlation between total carnitine levels and rHuEPO in some studies as the accumulation of medium- and long-chain acylcarnitines is balanced by the reduction in L-carnitine and acetyl-L-carnitine levels with a net result of relatively normal total carnitine levels.

The specific mechanism by which changes in carnitine levels affect rHuEPO requirements is not yet fully understood. It has been suggested that L-carnitine may

act by stabilization of the erythrocyte membrane with studies demonstrating improvements in membrane fluidity, osmotic fragility and RBC deformability with L-carnitine supplementation [23,39,41]. Based on the findings of previous studies, we have hypothesized that alterations in the AcylLC-C2/TC ratio may have an effect on erythrocyte membrane by regulation of RBC carnitine palmitoyltransferase (CPT). Acylcarnitines, in particular, long-chain acylcarnitines, have been shown to inhibit CPT activity [42] and conversely L-carnitine increases CPT activity [43–45]; accordingly, accumulation of medium- and long-chain acylcarnitines and a deficiency in L-carnitine levels (characterized by a high AcylLC-C2/TC ratio) would result in a decrease in RBC CPT activity. As CPT has been shown to have a critical role in RBC membrane acyl trafficking, decreases in CPT activity would lead to a reduction in RBC membrane repair and turnover thereby resulting in reduced membrane stabilization and decreased life-span of the erythrocyte. On the other hand, in patients with lower AcylLC-C2/TC values (either by lower non-acetyl acylcarnitine or higher L-carnitine levels), RBC CPT activity is more normal due to a better ‘balance’ between acylcarnitine inhibition and L-carnitine activation. Previous studies have demonstrated that long-chain acylcarnitines have a detrimental effect on RBC [41,46–48]. In one such study, incubation of erythrocytes with palmitoyl-L-carnitine resulted in significant alterations in the membrane fluidity and morphology of RBC, a finding that was improved with co-incubation of L-carnitine, suggesting that L-carnitine counteracts the detrimental effects caused by long-chain acylcarnitines [41]. This is in keeping with our findings that show that as AcylLC-C2/TC increases (i.e. as the proportion of medium- and long-chain acylcarnitines increased compared to L-carnitine), rHuEPO requirements increase.

The study has demonstrated a clear relationship between the composition of the endogenous carnitine pool in chronic haemodialysis patients and rHuEPO requirements. Whilst no specific conclusions can be made from this study with relation to rHuEPO dosing, we suggest that patients who have a high ERI (i.e. $\geq 0.02 \mu\text{g}/\text{kg}/\text{week}/\text{gHb}$), and hence a more greatly disturbed carnitine profile (as indicated by a high AcylLC-C2/TC ratio), are more likely to ‘respond’ to L-carnitine supplementation administered for an improvement in rHuEPO requirements. In support of this hypothesis, re-examination of data from a previous study conducted by our group [49] has been performed. In this study, patients classified as High ERI ($n = 2$) had an average reduction in ERI of 24% after 10 weeks of intravenous L-carnitine supplementation; on the other hand, Low ERI patients ($n = 10$) had, on average, a 22% worsening in ERI values. In fact, both High ERI patients had an improvement in ERI with L-carnitine treatment, whereas all of the Low ERI patients had an increase in ERI after treatment. A randomized, double-blinded, placebo-controlled study is now being conducted to investigate this hypothesis.

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Conflict of interest statement. None declared.

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