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The Clinical Impact of an Altered Endogenous Carnitine Profile

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★

This thesis is dedicated to my father who instilled in me the value of education and hard work, and who taught me to always question everything. It is these characteristics that I have inherited from him which have brought me to this point in my life and career.

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Abbreviations

6MWT	Six-Minute Walk Test
AcylLC	Total Acylcarnitines
AcylLC-C2	Non-Acetyl Acylcarnitines
ANOVA	Analysis of Variance
ARR	Arrhythmias
b.i.d.	Bis In Die (Twice Daily)
CoA	Coenzyme A
CV	Coefficient of Variation
Dx	Dialysis Session
ECG	Echocardiogram
ERI	Erythropoietin Resistance Index
ESI MS/MS	Electrospray Tandem Mass Spectrometry
ESRD	End-Stage Renal Disease
Hb	Haemoglobin
HCT	Haematocrit
HD	Haemodialysis
HDL	High Density Lipoprotein
HL	Hyperlipidaemia
HPLC	High-Performance Liquid Chromatography
HT	Hypotensive Episodes
i.v.	Intravenous
ICC	Intraclass Correlation Coefficient
LC	L-carnitine
LDL	Low Density Lipoprotein
LVEF	Left Ventricular Ejection Fraction
MRT	Mean Residence Time
NA	Not Analysed; Not Applicable
NHMRC	National Health and Medical Research Council
NYHA	New York Heart Association
p.o.	Per Os (By Mouth)
REA	Radioenzymatic Assay
rHuEPO	Recombinant Human Erythropoietin
SD	Standard Deviation
SDI	Strength Decrement Index
t.i.d.	Ter In Die (Thrice Daily)
TBC	Total Body Carnitine
TC	Total Carnitine
TRIG	Triglycerides

Summary

Carnitine is an endogenous compound found in all mammalian species. The most important biological function of carnitine is in the transport of fatty acids into the mitochondria for β-oxidation, a process which involves the esterification of carnitine to acylcarnitine derivatives via carnitine palmitoyltransferase. In addition to its role in mitochondrial fatty acid oxidation, it has been established that carnitine has critical functions in the transport of short- and medium-chain acyl groups from the peroxisome to the mitochondria, in the removal of excess acyl groups from the body as well as in the modulation of intracellular coenzyme A homeostasis such that alterations in the ratio of acylcarnitine to (free) L-carnitine is reflective of disturbed mitochondrial metabolism.

Carnitine deficiency has been associated with a number of conditions, either the result of disturbances in the dietary intake, biosynthesis, distribution, elimination or utilisation of carnitine. Despite widespread research examining altered L-carnitine levels in these conditions, relatively few studies have examined the involvement of acylcarnitines. Therefore, the overall aim of this project was to determine the clinical impact of an altered endogenous carnitine profile, in particular with respect to the relative contribution of L-carnitine and its ester derivatives to the composition of the carnitine pool and its involvement in disorders of carnitine insufficiency, specifically end-stage renal disease patients undergoing long-term haemodialysis treatment and patients with chronic fatigue syndrome.

While there has been extensive research which has established that chronic haemodialysis treatment results in low endogenous L-carnitine levels, this project, using data from healthy subjects as a reference, was able to demonstrate that end-stage renal disease patients undergoing long-term haemodialysis treatment also exhibit substantially elevated medium- and long-chain acylcarnitine concentrations. This is due, at least in part, to their limited removal by the haemodialysis procedure, most likely a result of the increased molecular weight, lipophilicity and protein-binding that accompanies increased chain-length of the acyl group. Consequently, the composition of the endogenous plasma carnitine pool is considerably altered in this patient group compared to that observed in healthy controls. These alterations in carnitine pool composition were shown to be significantly related to the severity/incidence of a number of dialysis-related disorders commonly reported in this patient population, including erythropoietin-resistant anaemia, muscle fatigue, cardiac dysfunction, muscle cramps and intradialytic hypotension with a more greatly disturbed carnitine profile associated with more severe symptomology. It is proposed that the accumulation of acylcarnitines and deficiency of L-carnitine in these patients results in decreased activity of carnitine palmitoyltransferase which ultimately leads to impaired acyl group utilisation resulting in symptomology.

The involvement of altered carnitine homeostasis in chronic fatigue syndrome is less well defined. This project was able to demonstrate that while chronic fatigue syndrome is not associated with alterations in endogenous L-carnitine concentrations, patients exhibit significantly lower levels of long-chain acylcarnitines, in particular oleyl-L-carnitine and linoleyl-L-carnitine, than healthy controls. These altered acylcarnitine levels were significantly correlated to fatigue severity, such that patients with lower long-chain acylcarnitine levels exhibited more severe fatigue symptoms. Previous research has established that chronic fatigue syndrome patients exhibit elevated levels of fatty acids that correspond to these deficient acylcarnitines, thus it is proposed that chronic fatigue syndrome is associated with impaired utilisation of fatty acids, most likely due to decreased carnitine palmitoyltransferase activity; the underlying cause of this is unknown.

List of Publications in Support of Thesis

Manuscripts

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.

Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? [Invited Review] *Nephrology* 2008; 13(1): 3-16.

Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.

Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.

Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.

Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.

Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.

Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. [Invited Review] *Curr Drug Metab*; prepared for submission.

Reuter SE, Evans AM. What is the value of the L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.

Conference Presentations

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of a single haemodialysis session on plasma acylcarnitine levels in end-stage renal disease. Clinical Pharmacology and Therapeutics (CPT) Conference, August 2004: Brisbane, Australia.

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Effect of haemodialysis on endogenous plasma acylcarnitine levels in end-stage renal disease patients. Clinical Pharmacology and Therapeutics (CPT) Conference, August 2004: Brisbane, Australia.

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine levels in end-stage renal disease. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2004: Melbourne Australia.

Reuter SE, Evans AM. Development of secondary carnitine deficiency in chronic haemodialysis patients. [Invited Presentation] Adelaide Pharmacology Group (APG) Annual Meeting, November 2005: Adelaide, Australia.

Reuter SE, Evans AM. Possible impact of L-carnitine and acylcarnitines on the treatment of anaemia in end-stage renal disease patients undergoing haemodialysis. Joint Australasian Pharmaceutical Science Association (APSA) and Australian Society for Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) Annual Conference, December 2005: Melbourne, Australia.
Awarded 'Outstanding Presentation in Pharmaceutical Science'.

Reuter SE, Evans AM. Assessment of muscle function and relationship to carnitine levels in chronic haemodialysis patients. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2006: Adelaide, Australia.

Reuter SE, Evans AM. Possible impact of L-carnitine and acylcarnitines on the treatment of anaemia in end-stage renal disease patients undergoing haemodialysis. Australian Society for Medical Research (ASMR) Scientific Meeting, June 2007: Adelaide, Australia.

Reuter SE, Faull RJ, Evans AM. Clinical impact of alterations in endogenous carnitine pool composition in chronic haemodialysis patients. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2007: Sydney, Australia.
Awarded 'Outstanding Presentation in Pharmaceutical Science'.

Reuter SE, Faull RJ, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. XIV International Congress of Nutrition and Metabolism in Renal Disease (ICNMRD), June 2008: Marseille, France.

Reuter SE, Evans AM. Chronic fatigue syndrome: Disruption in carnitine and free fatty acid homeostasis. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2009: Hobart, Australia.

Patents

International (PCT) Patent Application PCT/AU2010/001115 (31 August 2010). Reuter SE, Evans AM, Wigley PL. Methods for diagnosis and treatment of chronic fatigue syndrome.

Declaration

I declare that this thesis presents work carried out by myself and does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university. To the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including joint authored publications, is clearly acknowledged.



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Stephanie Elizabeth REUTER LANGE

24 March 2011

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As is the case with all research, this project would not have been possible without the significant contribution of others.

Words will never be able to express my gratitude to my principal supervisor, Prof Allan Evans, whose support and encouragement over the years has been invaluable. I would not have reached the position I am in today without your guidance and mentorship, and for that I am eternally grateful. I look forward to working with you for many years to come.

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Finally, I would like to acknowledge that the work contained within this PhD thesis was supported by a project grant from the National Health and Medical Research Council (Australia); I am thrilled to have been a part of such a significant project.

Preface

This doctoral thesis is presented as a Thesis by Publication, in accordance with the University of South Australia Academic Regulations for Higher Degrees Research. Specifically, a thesis presented purely by publications typically includes at least 2 - 3 published works depending on the convention for each discipline and depending on the quality of the work and the international standing of the publication outlet. The publications included in a thesis containing published research must be part of an integrated project such that the publications presented in the thesis constitute elements of a connected whole.

The body of knowledge represented within this thesis has been acquired during a part-time PhD candidature from March 2004 to March 2011 which ultimately aimed to investigate the clinical impact of an altered endogenous carnitine profile, in particular with respect to the relative contribution of L-carnitine and its ester derivatives to the composition of the carnitine pool and its involvement in disorders of carnitine insufficiency.

For the purposes of this PhD project, two specific patient groups have been selected; one for which carnitine deficiency has been clearly established (patients undergoing long-term haemodialysis treatment) and one for which the involvement of disturbed carnitine homeostasis is more controversial (patients with chronic fatigue syndrome).

This PhD comprised a series of research studies which resulted in the production of nine manuscripts for publication; at the time of thesis submission five of these manuscripts have been published, one is currently in press, one has been submitted for consideration and two manuscripts have been prepared for submission. Each of these manuscripts forms a chapter within this thesis which are presented under three primary research themes:

Section I examines the composition of the endogenous plasma carnitine pool in healthy adult subjects from which a normal reference range for L-carnitine and acylcarnitines is determined.

Section II presents a series of studies which examined the impact of long-term haemodialysis treatment on the endogenous carnitine pool and the involvement of a disturbance in carnitine homeostasis in a number of common dialysis-related conditions including renal anaemia, muscle fatigue, cardiac dysfunction and intradialytic hypotension.

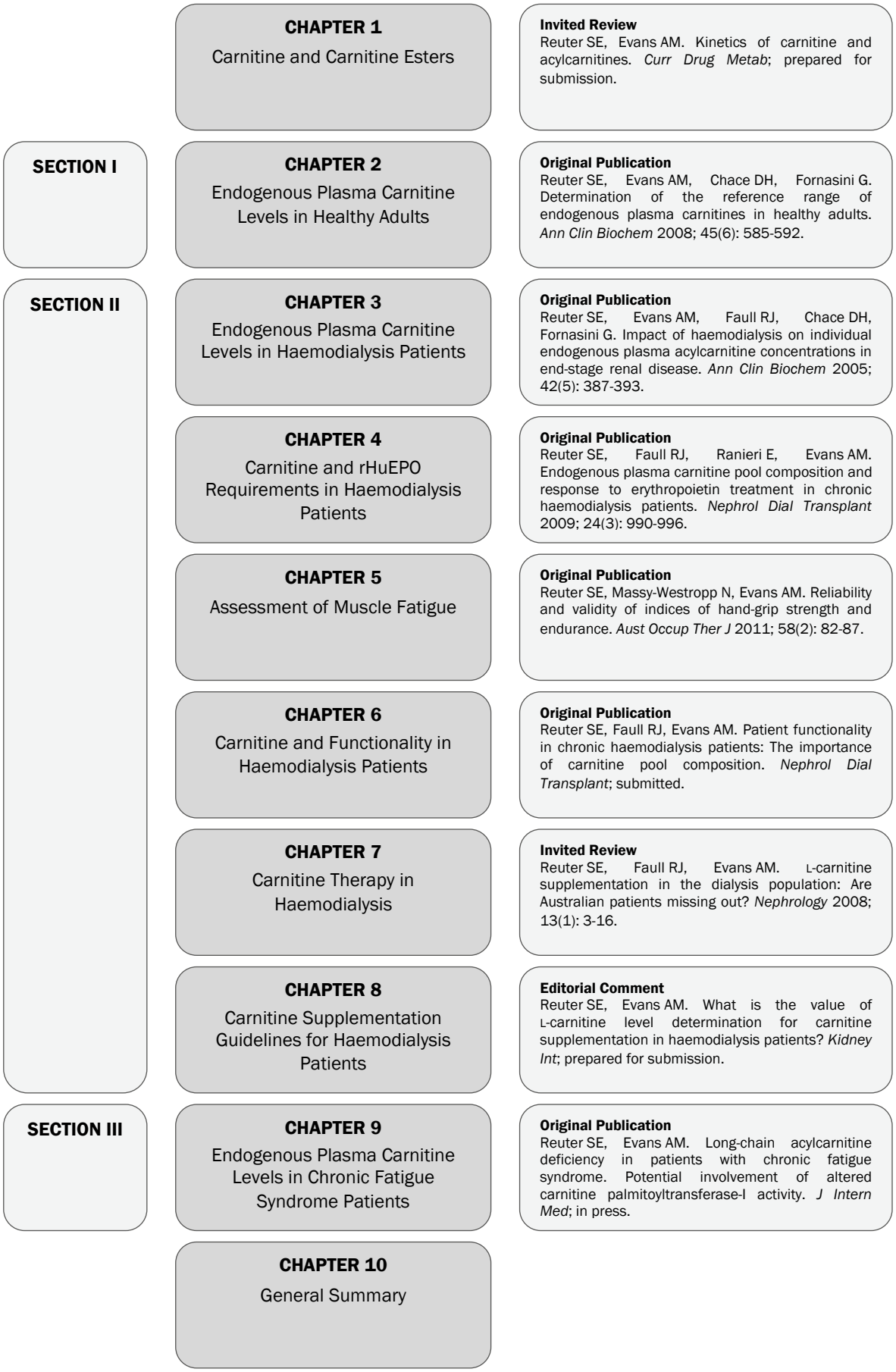
Section III examines alterations in the composition of the endogenous carnitine pool in patients with chronic fatigue syndrome and its relationship to clinical symptomology in these patients.

A summary of the structure of this thesis is presented in Figure 0.1.

The text within each thesis chapter is identical to that contained within the respective manuscript; however, for the purposes of presentation within this thesis, the manuscripts have been reformatted and the sections, figures and tables have been renumbered for continuity throughout the thesis. Copies of the original formatted publications are presented in Appendix 2 for reference.

It should be noted that the order in which the manuscripts are presented within this thesis is not consistent with the order in which they were published. Consequently, in some cases, the thesis chapter refers to results which are presented in subsequent chapters. In addition, as each manuscript is considered a discrete document, the content of which has been preserved, some repetition of figures and data exists throughout the thesis.

Figure 0.1 Thesis Outline



CHAPTER 1

Carnitine and Carnitine Esters

Stephanie E Reuter, Allan M Evans

Kinetics of Carnitine and Acylcarnitines

Current Drug Metabolism; Prepared for Submission

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

This chapter presents an invited review of the literature on the kinetics of endogenous and exogenous L-carnitine and acylcarnitines. Whilst there is considerable research which establishes the physiological importance of L-carnitine, relatively few studies have investigated the contribution of carnitine esters to health and disease. This chapter provides an introduction to the functions of the carnitine family of compounds, the homeostatic mechanisms involved in maintaining carnitine pool composition, as well as the pharmacokinetics of exogenous L-carnitine and carnitine esters. Within this review, the lack of research examining the involvement of the full endogenous carnitine profile in conditions of disturbed carnitine homeostasis is highlighted.

1.1 Summary

L-carnitine, 3-hydroxy-4-*N*-trimethylaminobutyrate, is a naturally occurring compound found in all mammalian species. L-carnitine was first discovered to be a quantitatively important compound in muscle tissue in 1905[1] and its chemical structure was established in 1927[2]. However, the physiological function of L-carnitine was not established until a series of experiments in the 1950s which were able to ascertain the importance of L-carnitine in fatty acid oxidation[3-5].

Carnitine is a low molecular weight, polar molecule that, due to its chiral carbon, exists as two enantiomers: D- and L-carnitine; however, only the L-isomer is physiologically active (Figure 1.1)[6, 7]. Carnitine is able to form high-energy bonds with carboxylic acids at its hydroxyl group which results in the formation of acylcarnitine derivatives. The endogenous carnitine pool is comprised primarily of L-carnitine with short-, medium- and long-chain acylcarnitines forming the remaining component. The levels of L-carnitine and its esters are maintained within relatively narrow limits for normal biological functioning in their pivotal roles in fatty acid oxidation and maintenance of free coenzyme A availability. Disturbances in the relative composition of the endogenous carnitine pool are associated with mitochondrial dysfunction.

Advances in analytical methodology in the last decade have resulted in the ability to quantify not only L-carnitine concentrations but also levels of individual acylcarnitines; however, relatively few studies have utilised this methodology to examine the kinetics of endogenous and exogenous carnitines. Given the importance of L-carnitine as well as acylcarnitines in maintaining normal mitochondrial function, this review seeks to examine previous research associated with the homeostasis and pharmacokinetics of L-carnitine and its esters, and highlight potential areas of future research.

1.2 Carnitine Functions

The most important biological function of L-carnitine is in the transport of fatty acids into the mitochondria for subsequent β-oxidation, a process which results in the esterification of L-carnitine to form acylcarnitine derivatives [6, 7]. As such, the endogenous carnitine pool is comprised of L-carnitine and various acylcarnitines ranging from the short-chain acetyl-L-carnitine (2 carbon length acyl moiety) to the long-chain stearoyl-L-carnitine (18 carbon length acyl moiety) [8]. L-carnitine is the major representative in the carnitine pool such that the normal acylcarnitine to L-carnitine ratio is 0.25; a ratio of 0.4 or greater is considered to be abnormal and representative of disturbed mitochondrial metabolism [9].

In addition to its role in facilitating mitochondrial fatty acid metabolism, it has been established that L-carnitine has critical functions in regulating the cellular to mitochondrial ratio of free coenzyme A (CoA) to acyl-CoA, in the transport of short- and medium-chain acyl groups from the peroxisome to the mitochondria, and in the removal of excess acyl groups from the body via the preferential renal excretion of acylcarnitines [6, 10, 11].

1.2.1 Mitochondrial Fatty Acid Oxidation

The mitochondrial metabolism of cytostolic fatty acids begins with activation via acyl-CoA synthase to form acyl-CoA. The resultant thioester is then able to cross the outer mitochondrial membrane via a voltage-gated anion channel into the membrane space where it undergoes transesterification by carnitine acyltransferase with L-carnitine to form free CoA and the corresponding acylcarnitine [12]. This acylcarnitine is then transported across the inner mitochondrial membrane via a membrane-bound L-carnitine/acylcarnitine translocase, in exchange for a free L-carnitine from within the mitochondria [13, 14]. Once inside the mitochondrial matrix, the acylcarnitine and available free CoA undergo reverse transesterification by carnitine acyltransferase to form L-carnitine and the corresponding acyl-CoA which enters the fatty acid β-oxidation pathway (Figure 1.2) [6, 10].

Three different groups of carnitine acyltransferases have been described which differ in their substrate specificity: carnitine acetyltransferases mediate the transesterification of short-chain acyl groups; carnitine octanoyltransferases utilise medium-chain acyl groups as substrates; and, reactions involving long-chain acyl moieties are facilitated by carnitine palmitoyltransferases [6, 10, 15, 16].

Given that neither free fatty acids, nor acyl-CoA are able to move across the inner mitochondrial membrane alone, the role of L-carnitine and carnitine acyltransferases in fatty acid metabolism is vital.

1.2.2 Modulating Intracellular Coenzyme A Homeostasis

The role of L-carnitine in buffering the ratio of free CoA to acyl-CoA has also been described, a function which is particularly important under conditions of stress. Under normal conditions, short- and medium-chain acyl-CoA, formed as a result of various mitochondria pathways, are further metabolised to generate free CoA. However, under abnormal conditions in which excess quantities of these CoA esters are formed within the mitochondria, acyl-CoA can undergo transesterification with L-carnitine to form acylcarnitines, thereby freeing CoA for use in other mitochondria reactions [6, 10, 11, 17]. This reversible exchange, in combination with the ability of the resultant acylcarnitine to cross the mitochondrial membrane (via L-carnitine/acylcarnitine translocase), means that the intramitochondrial relationship between free CoA and acyl-CoA is reflected in the extramitochondrial ratio of acylcarnitine to L-carnitine [9, 18].

1.2.3 Peroxisomal Fatty Acid Oxidation

Peroxisomal fatty acid metabolism involves the incomplete β-oxidation of very long-chain fatty acids to short- and medium-chain acyl derivatives [19]. Whilst the metabolism of very long-chain fatty acids occurs via a carnitine-independent mechanism, activity of carnitine acetyltransferase and carnitine octanoyltransferase have been established within the peroxisomes [16]. It is thought that chain-shortened acyl-CoA formed via peroxisomal fatty acid oxidation undergoes transesterification by carnitine acyltransferases within the peroxisome to form short- and medium-chain acylcarnitines which are subsequently transported into the mitochondria for complete β-oxidation [20].

1.3 Carnitine Homeostasis

Given the fundamental importance of L-carnitine for mammalian survival via its pivotal role in facilitating mitochondrial and peroxisomal fatty acid oxidation, as well as in the modulation of the intracellular relationship between free CoA and acyl-CoA, it is not unexpected that endogenous plasma and tissue levels of L-carnitine and its ester derivatives are maintained within relatively narrow limits. L-carnitine homeostasis is multifaceted with levels achieved and maintained by a combination of absorption from

dietary sources, *de novo* biosynthesis, carrier-mediated distribution into tissues and extensive, but saturable, renal tubular reabsorption.

1.3.1 Dietary Intake and Absorption

Dietary L-carnitine intake is largely achieved via consumption of animal-based products including red meats, poultry, fish and dairy products, while negligible quantities are available from plant-derived foods [21, 22]. Given the broad range of nutritional choices, dietary L-carnitine intake can vary considerably, with the standard omnivorous diet providing 6 - 15 µmol/kg/day whereas vegans acquire <1 µmol/kg/day from their diet and semi-vegetarians (i.e. those who eat chicken, fish and dairy products but not red meat) consume 1 - 8 µmol/kg/day [21, 23]. However, despite substantial differences in L-carnitine intake, previous research has established that vegetarian diets do not result in a nutritionally significant deficit in endogenous carnitine levels [24]; in fact, on average, endogenous plasma L-carnitine, total carnitine and estimated acylcarnitine concentrations for vegetarian adult subjects were only 10 - 20% lower than those for adult subjects consuming an omnivorous diet. On the other hand, urinary carnitine excretion for L-carnitine and (estimated) acylcarnitine were, on average, 85 - 95% and 40 - 50% lower for vegetarians than for non-vegetarians, respectively. These findings indicate that compensatory mechanisms, including renal conservation in conjunction with biosynthesis, are effective in maintaining carnitine homeostasis when dietary L-carnitine intake is low.

Orally ingested L-carnitine and its short-chain acyl derivatives are taken up from the small intestinal lumen into the enterocytes via a two-component system: a saturable system mediated via the active, sodium-dependent organic cation/carnitine transporter OCTN2, and a linear component representing passive diffusion [23, 25 - 32]. Research has demonstrated the presence of carnitine acetyltransferase within the intestinal mucosa and has established that L-carnitine undergoes extensive intracellular acetylation (50 - 60%) in rat and guinea pig models; however, these results have not been confirmed in humans [27, 31 - 33]. Given that esterified carnitine can more readily cross the basolateral membrane than L-carnitine, it has been proposed that this esterification process may facilitate the diffusion of carnitine into the circulation [33]. Unabsorbed L-carnitine is degraded in the large intestine by indigenous flora to γ-butyrobetaine, which is subsequently excreted in the

faeces, and trimethylamine which is absorbed, converted to trimethylamine-*N*-oxide and renally excreted [23, 34 - 36].

The dietary bioavailability of L-carnitine has been shown to vary with intake. Rebouche & Chenard [37] demonstrated that the bioavailability of L-carnitine in participants fed a diet low in carnitine content (1.5 - 2.2 μmol/kg/day) was approximately 75%, whereas in participants on a high carnitine diet (8.4 - 11.9 μmol/kg/day) the bioavailability was estimated as 63%. The results of this study indicate that the efficiency of absorption diminishes as the carnitine content of the diet increases, reflecting the saturability of the OCTN2 transporters, even at levels of normal dietary intake. With the observed decrease in bioavailability with increasing carnitine 'dose', the extent of bacterial degradation increases as more L-carnitine reaches the regions of the gastrointestinal tract colonised by bacterial capable of degrading L-carnitine [35 - 37].

1.3.2 Synthesis

Whilst, in omnivorous humans, the majority of total body carnitine levels are achieved from dietary sources, approximately 25% comes from *de novo* biosynthesis [38]. On the other hand, strict vegetarians obtain very little L-carnitine from dietary sources and consequently, in order to maintain carnitine homeostasis, biosynthesis accounts for up to 90% of total body carnitine levels in this group [39]. The amount of L-carnitine produced endogenously (for an omnivorous adult) is approximately 1 - 2 μmol/kg/day [21, 23, 39].

L-carnitine is synthesised from the amino acid precursor's lysine and methionine, with lysine providing the carbon backbone and L-carnitine's 4-*N*-methyl groups originating from methionine (Figure 1.3) [6, 21, 40, 41]. The first step in the biosynthesis of L-carnitine is the *N*-methylation of lysine which, using methionine as the methyl donor, is catalysed by lysine methyltransferase to yield N^6-trimethyllysine. N^6-trimethyllysine is then hydrolysed within the mitochondria to produce 3-hydroxy-N^6-trimethyllysine, a reaction which catalysed by N^6-trimethyllysine dioxygenase in the presence of 2-oxoglutarate, Fe^{2+}, O_2 and ascorbate [21, 38, 42 - 44]. The rate of L-carnitine synthesis is determined by the availability of trimethyllysine for this reaction at the intramitochondrial site [45, 46]. The formed 3-hydroxy-N^6-trimethyllysine subsequently undergoes aldolytic cleavage via 3-hydroxy-N^6-trimethyllysine

aldolase within the cytosol, using pyridoxal 5'-phosphate as a cofactor, to yield 4-*N*-trimethylaminobutyraldehyde and glycine [21, 38, 42, 47]. 4-*N*-trimethylaminobutyraldehyde is then oxidised via 4-*N*-trimethylaminobutyraldehyde dehydrogenase which, using NAD^+ as a hydrogen acceptor, results in the formation of 4-*N*-trimethylaminobutyrate (γ-butyrobetaine) [38, 48]. In the last step, 4-*N*-trimethylaminobutyrate undergoes hydroxylation by 4-*N*-trimethylaminobutyrate dioxygenase, in the presence of 2-oxoglutarate, Fe^{2+}, O_2 and ascorbate, to yield 3-hydroxy-4-*N*-trimethylaminobutyrate (L-carnitine) [38, 49, 50].

The enzymes required for the biosynthesis of L-carnitine are ubiquitous, with the exception of 4-*N*-trimethylaminobutyraldehyde dioxygenase which, in humans, is found only in the liver, kidneys and brain [6, 47]. In adults, the activity of 4-*N*-trimethylaminobutyraldehyde dioxygenase in the kidney is approximately 3-fold higher than that in the liver, and 6-fold higher than that in the brain [47].

1.3.3 Distribution

Carnitine distributes into most tissues within the body, with each tissue exhibiting different kinetics for carnitine transport. The total body L-carnitine content is estimated to be approximately 130 mmol for a healthy 70 kg adult male, the majority of which is located within the muscles [51]; not unexpected given L-carnitine's critical role in muscular fatty acid oxidation. Only a small fraction of total body L-carnitine stores are located within the plasma which results in a steep concentration gradient between the extracellular fluid and the tissues [16, 51]. Given that most tissues lack the necessary enzymes to synthesise L-carnitine [6, 47], the high tissue-to-plasma ratio indicates the presence of an energy-dependent transport system.

Numerous studies have reported endogenous plasma L-carnitine concentrations in healthy adult subjects with varying results [24, 52 - 75]; however, normal levels are generally considered to be 40 – 50 µmol/L. Based on an estimated extracellular volume (including blood plasma) of 13 L for a 70 kg person, this equates to a total extracellular L-carnitine content of 0.7 - 0.9 mmol, of which 0.2 mmol is located within plasma (assuming a volume of 3 L); this equates to approximately 0.7% of total body L-carnitine stores within the extracellular fluid and only 0.2% within plasma.

Research by Marzo et al.[77] has demonstrated that L-carnitine and its short-chain esters (acetyl-L-carnitine and propionyl-L-carnitine) do not bind to plasma proteins; however, the extent of protein binding increases with increasing chain length of the acyl group, with poor-moderate binding for medium-chain acylcarnitines (octanoyl-L-carnitine) and complete binding for long-chain acylcarnitines (palmitoyl-L-carnitine), thereby limiting their availability for uptake into tissues.

Red blood cells have also been shown to contain a considerable amount of L-carnitine, with levels of 100 nmol/gHb reported[16, 59, 62, 73, 78]. Minor levels have also been reported in white blood cells[16, 78]. Studies have demonstrated that there is no correlation between plasma and red blood cell carnitine concentrations and thus erythrocytes can be considered a separate compartment with relatively slow uptake from plasma[59, 79]. Given the lack of mitochondria within the erythrocyte, the role of L-carnitine within red blood cells is unknown; however, it has been proposed that L-carnitine contributes to stabilisation of the red blood cell membrane via the trafficking of acyl groups[80, 81].

Skeletal muscle contains approximately 4200 µmol/kg wet weight of L-carnitine which, assuming a 30 kg muscle mass for a 70 kg adult, equates to an L-carnitine content of 126 mmol, or 97% of total body L-carnitine[51, 82]. Kinetic studies of L-carnitine uptake into skeletal muscle have demonstrated high affinity transport with K_m values of approximately 2 - 60 µM; a second low affinity transporter has also been described[9, 16, 38, 85 - 89]. The turnover of skeletal muscle L-carnitine has been reported as 105 hours[51, 89, 90].

L-carnitine concentrations in cardiac muscle have been reported as 610 - 1300 µmol/kg wet weight or approximately 0.2 - 0.5 mmol for an estimated heart size of 350 g for a 70 kg adult[89, 91]; this constitutes 0.2% of total body L-carnitine stores. As for skeletal muscle, the uptake of L-carnitine into cardiac muscle is via a high affinity transporter with a K_m value of 2 - 60 µM[6, 9, 16, 38, 89, 92, 93]; however, the turnover of L-carnitine within the heart is much more rapid at 21 hours[89, 90].

In light of its vital role in L-carnitine biosynthesis and renal conservation, the concentration of endogenous L-carnitine in the kidney is relatively low (330 - 600 µmol/kg wet weight)[89]. The L-carnitine content of the kidneys is estimated at 0.1 - 0.2 mmol (based on a total organ size of 350 g for a 70 kg

adult)[51, 82, 89, 91], equivalent to 0.2% of total body L-carnitine stores. Kidney turnover of L-carnitine is rapid at 0.4 hours[89, 90], also highlighting its important role in whole-body homeostasis. The uptake of L-carnitine into the kidney is via an intermediate affinity transporter, with reported K_m values of 10 - 200 µM[6, 9, 38, 89].

The liver, another important site of carnitine homeostasis, has a reported L-carnitine concentration of 500 - 1000 µmol/kg wet weight[51, 89]. Based on an organ size of 1.5 kg (for a 70 kg adult)[82, 91], the liver contains approximately 1% of total body L-carnitine stores (0.7 - 1.4 mmol). The turnover of L-carnitine in the liver is rapid (1.3 hours) and uptake is via a low affinity transporter (K_m = 500 µM), although a smaller, high affinity component has also been described[9, 16, 38, 51, 89, 90].

Limited research has been conducted on L-carnitine distribution within the brain; however, uptake is thought to be via a low affinity transported with a K_m value of >1000 µM[9, 16, 38, 89]. L-carnitine turnover within the brain is slow with values of 223 hours reported[90].

The distribution of L-carnitine into human tissues is summarised in Table 1.1.

A number of transporters have been associated with carnitine distribution, including the organic cation/carnitine transporter family (OCTN1, OCTN2 and OCTN3), $ATB^{0,+}$ and recently OAT9; however the involvement of OCTN2 in carnitine transport is the most well described. OCTN2 is a high affinity (3 - 5 µM), sodium-dependent carnitine transporter that is found within most tissues of the body[98 - 101]. It is most highly expressed within the heart, skeletal muscle and kidney but only weakly expressed within the liver and brain[99 - 101]. It binds not only L-carnitine but also acetyl-L-carnitine and propionyl-L-carnitine with comparable affinity[101]. OCTN1 also has broad tissue distribution; however, whilst studies have demonstrated expression in rat liver, OCTN1 is not expressed in human liver[98 - 100]. OCTN1 has a relatively low affinity (~100 µM) and specificity for carnitine[99, 100]. OCTN3 has a higher specificity than OCTN1 and OCTN2, but is only expressed within the testis[98, 99]. $ATB^{0,+}$ is a low affinity (K_m = 1 - 2 mM), low specificity transporter for carnitine which has a distinct tissue distribution with primary expression within the intestinal tract, lung and mammary glands[102, 103]. OAT9 has recently been identified as a carnitine transporter in rat liver and kidney; however, its involvement in carnitine distribution in human tissue is unknown[104].

1.3.4 Renal Conservation and Elimination

As L-carnitine is not systemically metabolised, with the exception of reversible esterification to its acyl derivatives, the primary route of elimination is via renal excretion. Given that L-carnitine is a small, polar molecule that does not undergo protein-binding, it is freely filtered at the glomerulus such that its filtration clearance is equal to the glomerular filtration rate [105]. However, in order to reduce L-carnitine loss and maintain adequate endogenous levels, over 95% of filtered L-carnitine is reabsorbed by the proximal tubule and returned into the bloodstream [51, 105, 106]. L-carnitine tubular reabsorption exhibits typical characteristics of a carrier-mediated process, including saturation kinetics at high plasma concentrations [23, 37, 105, 107 - 112]. The tubular reabsorption of L-carnitine is mediated via a sodium-dependent, active transporter (OCTN2) with an apparent half maximal saturation of 60 μM – in the range of endogenous L-carnitine concentration within the filtrate [110, 113]. The efficiency of the tubular reabsorption of L-carnitine increases as dietary intake decreases in order to maintain circulating L-carnitine concentrations within a narrow normal range. On the other hand, when L-carnitine levels increase, such as with exogenous administration, reabsorption decreases as OCTN2 transporters become partially saturated and greater urinary loss results [23, 37, 105, 107 - 112].

While L-carnitine and its acyl derivatives are both transported via OCTN2, L-carnitine is preferentially reabsorbed over its esters such that renal clearance of acylcarnitine is 4 - 8 times higher than that of L-carnitine. As a result the composition of urine (46% L-carnitine, 29% short-chain acylcarnitines and 16% long-chain acylcarnitines) varies considerably from that of plasma [105]. The preferential reabsorption of L-carnitine by the proximal tubule results in the clearance of acyl compounds that might otherwise lower the rate of fatty acid oxidation and other CoA-dependent reactions [114 - 118].

Interestingly, previous research conducted using an isolated perfused rat kidney model demonstrated that L-carnitine and acetyl-L-carnitine undergo significant interconversion within the kidney and the clearance of these generated compounds was approximately 10 times higher than L-carnitine and acetyl-L-carnitine filtered directly from the perfusate (i.e. circulation) indicating renal secretion of these intracellularly formed compounds [11, 106, 119]. The significance of this renal secretion is not clear; however, it has been suggested

that this may be a mechanism by which fatty acids are removed from the body, in particular when they are in excessive amounts [11].

1.3.5 Carnitine Pool Composition

Considerable research activity in recent decades has clearly established the physiological importance of L-carnitine and its obligatory role in the mitochondrial metabolism of fatty acids. More recently, additional functions of the carnitine system have been described, including the removal of excess acyl groups from the body and the modulation of intracellular CoA homeostasis, such that disturbances in intermediary metabolism, which result in altered free CoA/acyl-CoA homeostasis, are reflected in acylcarnitine levels. In light of this, acylcarnitines cannot simply be considered by-products of the enzymatic carnitine transfer system, but provide indirect evidence of altered mitochondrial metabolism. Consequently, examination of the contribution of L-carnitine and acylcarnitines to the endogenous carnitine pool (i.e. carnitine pool composition) is critical in order to adequately characterise metabolic status.

Acylcarnitines comprise a family of ~40 esters, of which acetyl-L-carnitine is the most abundant. Quantification of the carnitine group of compounds was initially conducted utilising enzymatic methodology [97, 120 - 123]. These analytical methods only allowed for the measurement of L-carnitine and total carnitine levels and consequently (total) acylcarnitine concentrations were only able to be determined by subtraction; although subsequent modifications allowed for the determination of acetyl-L-carnitine levels [124, 125]. Chromatographic methods were also developed for the quantification of L-carnitine and total carnitine as well as short- and medium-chain acylcarnitines [126 - 129]. Whilst complex and time-intensive, these chromatographic methods were able to be applied for the diagnosis of specific inborn errors of metabolism [130]. The most significant advancement in methodology for the quantification of carnitine compounds is in the development of tandem mass spectrometry methods which allow for the accurate and precise measurement of L-carnitine and short-, medium- and long-chain carnitine esters [131]. Given the ability of this methodology to efficiently quantify individual acylcarnitines, thereby fully characterising the composition of the carnitine pool, these methods are routinely utilised in screening for inborn errors of metabolism in newborn infants [132, 133].

The composition of the endogenous carnitine pool varies considerably within the various tissues of the body. Total plasma carnitine levels have generally been reported as 45 – 60 µmol/L, with a corresponding acylcarnitine concentration of 6 – 9 µmol/L [52]. Recently, work by our group has examined the composition of the endogenous plasma carnitine pool, including quantification of individual acylcarnitine concentrations. This work was able to demonstrate that, in healthy adults, the plasma carnitine pool comprises 83% L-carnitine and 17% acylcarnitines, of which acetyl-L-carnitine makes up 75% [52, 76]. The contribution of medium- and long-chain acylcarnitines is minimal. These results are supported by the findings of Harper et al. [73].

Red blood cells have been shown to have a substantially different carnitine pool composition to plasma, with acylcarnitines comprising up to 52% of total red blood cell carnitine levels compared to only 13% in plasma [73].

The composition of the skeletal muscle carnitine pool in healthy subjects has been investigated in a number of studies. Harper et al. [73] reported muscle concentrations of 15.6 µmol/gNCP and 3.5 µmol/gNCP for L-carnitine and acylcarnitine respectively, corresponding to 81% and 19% of total muscle carnitine pool. Similar results were reported by Minkler et al. [83] who demonstrated that the muscle carnitine pool comprised 77% L-carnitine and 23% acylcarnitine, of which 65% was acetyl-L-carnitine. Angsten et al. [84] reported similar carnitine proportions in muscle samples collected from children.

On the other hand, the relative carnitine pool composition within the human heart is unknown; early studies using rat models have indicated that the ratio of acetyl-L-carnitine to L-carnitine is approximately 0.36, higher than that reported for skeletal muscle (0.19) [94]. Similar results have been reported in the dog [95].

Much higher levels of acylcarnitines have been reported for the kidney than other tissues, with a proportion of L-carnitine relative to total carnitine of 50 - 55% [94, 96]; likely reflecting the important role of the kidney in the elimination of excess acyl groups from the body.

The relative carnitine pool composition in the liver has been estimated in a number of studies. Harper et al. [73] investigated endogenous carnitine concentrations in liver samples collected from 29 healthy adults undergoing elective surgery. Reported levels were 3.3 µmol/gNCP, 1.77 µmol/gNCP and

5.0 µmol/gNCP for L-carnitine, acylcarnitine and total carnitine respectively, indicating a carnitine content of 65% L-carnitine and 35% acylcarnitine. Similar results were reported by de Sousa et al.[97] (56% L-carnitine, 31% short-chain acylcarnitines and 13% long-chain acylcarnitines).

Although for the most part, the relative contribution of L-carnitine and acylcarnitines to the total carnitine pool has been well characterised in the various tissues of the body, there is limited research establishing the specific pattern of acylcarnitine distribution. Given that disorders of metabolic dysfunction are generally associated with altered levels of specific acylcarnitines, it is vital to distinguish between L-carnitine and individual acylcarnitines in order to correctly evaluate the state of the carnitine pool.

Conditions of impaired mitochondrial β-oxidation generally give rise to intracellular accumulation of acyl-CoA[134]. By virtue of the dynamic equilibrium between the acylcarnitine/L-carnitine and acyl-CoA/free CoA systems, acylcarnitine profiles reflect the intramitochondrially accumulating acyl-CoAs which are exported out of the mitochondria as their corresponding esters[135 - 138]. Given the difficulties in the determination of acyl-CoA concentrations[135, 136], the quantification of acylcarnitines are currently used in neonatal screening programs as biomarkers for the diagnosis of inherited disorders of mitochondrial β-oxidation[132, 133]. These disorders are associated with elevated levels of specific acylcarnitines depending on the metabolic defect; for example, medium-chain acyl-CoA dehydrogenase deficiency is characterised by elevated octanoyl-L-carnitine levels[139], glutaryl-CoA dehydrogenase deficiency results in increased urinary excretion of glutaryl-L-carnitine[140], and accumulation of decanoyl- and dodecenoyl-L-carnitine has been reported in patients with long-chain acyl-CoA dehydrogenase deficiency[141].

Levels of acyl-CoA have been shown to regulate fatty acid oxidation; this is thought to be mediated via malonyl-CoA, a potent inhibitor of carnitine acyltransferase activity which is the rate-limiting step in fatty acid oxidation[114 - 118]. Malonyl-CoA is synthesised from acetyl-CoA by acetyl-CoA carboxylase, which is regulated by the cytosolic content of citrate[114 - 116, 142 - 148]. Studies have demonstrated that acyl-CoA, in particular long-chain thioesters, inhibit both acetyl-CoA carboxylase and citrate synthase[116, 120, 136, 144, 149]; thus, under conditions of high acyl-CoA, acetyl-CoA carboxylase activity is diminished via both direct inhibition of acetyl-CoA carboxylase and via a reduction in citrate

levels. Consequently, malonyl-CoA levels fall and carnitine acyltransferase activity increases. In contrast, low acyl-CoA levels lead to an increase in acetyl-CoA carboxylase levels (via direct action and an increase in citrate synthase activity) thereby increasing malonyl-CoA levels and thus reducing carnitine acyltransferase activity.

On the other hand, *in vitro* studies have demonstrated that the addition of acylcarnitines to the experimental system has antagonistic effects to the actions of acyl-CoA [118, 144, 149 - 153]. Work by Scholte et al. [149, 152, 153] demonstrated that palmitoyl-L-carnitine and linoleyl-L-carnitine are strong inhibitors of carnitine palmitoyltransferase ($K_{0.5}$ = 250 μM and $K_{0.5}$ = 112 μM, respectively), in direct contrast to the actions of acyl-CoA. In fact, Marquis et al. [144] demonstrated that, while acyl-CoA inhibited acetyl-CoA carboxylase activity, this was opposed by the addition of palmitoyl-L-carnitine; these findings were confirmed by the work of Fritz & Hsu [150]. These experiments established that long-chain acylcarnitines inhibit the action of citrate lysase and increase the activity of citrate synthase, thereby resulting in an elevation in cytosolic citrate levels, enhanced acetyl-CoA carboxylase activity, increased malonyl-CoA levels and thus a reduction in carnitine palmitoyltransferase activity. Therefore, although an increase in acyl-CoA levels results in an increase in the activity of carnitine acyltransferases, a disruption in the homeostatic balance by the addition of long-chain acylcarnitines causes a reduction in carnitine palmitoyltransferase activity. This may account for some of the toxic effects associated with long-chain carnitine esters.

Long chain acylcarnitines have been shown to have detergent effects on membranes and are therefore able to disrupt membrane barriers and produce complete membrane solubilisation [136, 149, 151, 154 - 158]. In addition, long-chain carnitine esters have been shown to increase intracellular calcium and induce electrophysiological alterations in experimental systems [159]. Yamada et al. [159] used ventricular sarcoplasmic reticulum to demonstrate that calcium efflux was increased, in a concentration-dependent manner, by palmitoyl-L-carnitine and stearoyl-L-carnitine but not by short-chain esters. The authors suggested that the long-chain compounds could contribute to the development of ventricular arrhythmias induced during myocardial ischaemia. Studies have also demonstrated that long-chain acylcarnitines have a detrimental effect on red blood cells [160 - 163]. In one such study, incubation of erythrocytes with palmitoyl-L-carnitine resulted in significant alterations in the membrane fluidity and morphology of red blood cells, a finding that was improved with co-incubation

of L-carnitine [160]. Given the disturbances in carnitine homeostasis described in some patient populations, in particular haemodialysis patients (refer Section 1.3.6), these actions of long-chain acylcarnitines may account, in part, for the adverse events commonly experienced within these patient groups.

Products of incomplete mitochondrial fatty acid oxidation, such as acylcarnitines, have been shown to be elevated in obesity [164] and type 2 diabetes [165]. Studies have also demonstrated that abnormal levels of plasma acylcarnitines are associated with insulin resistance, coronary artery disease and cardiovascular events [164, 166 - 172]. Redman et al. [173] demonstrated that, in healthy overweight adults, medium- and long-chain acylcarnitines were positively associated with adiposity and insulin sensitivity. Recent work by Lum et al. [174] has demonstrated that higher levels of endogenous medium- and long-chain acylcarnitines are associated with lower levels of physical performance and function in elderly men. The authors noted that these medium- and long-chain esters are the same metabolites that have been associated with mitochondrial dysfunction in animal models [167, 170].

Whilst long-chain acylcarnitines have been linked to poor clinical status, research has indicated that short-chain carnitine esters are associated with positive effects. Evidence has suggested that propionyl-L-carnitine may be beneficial in the treatment of cardiovascular disorders via increased in myocardial carnitine content coupled with stimulation of the Krebs cycle via an anaplerotic pathway [175]. Similarly, acetyl-L-carnitine has been shown to reverse age-dependent deficits in cellular function thereby improving energy balance, an effect which is thought to be useful in the treatment of Alzheimer's disease [176, 177].

Given the substantial diversity in the roles and effects of the different acylcarnitines in physiological functioning, the characterisation of the full pattern of carnitine pool composition is considered critical in order to suitably assess patient status.

1.3.6 Disturbances in Carnitine Homeostasis

A review of carnitine homeostasis would not be complete without a discussion of carnitine deficiency. Carnitine deficiency is defined as 'a state of carnitine concentrations in plasma or tissues that is below the requirement for the normal function of the organism' [9]. Various disorders of carnitine insufficiency

have been described but ultimately all result in impaired entry of fatty acids into the mitochondria and consequently disturbed lipid oxidation [9, 11, 89, 178 - 180]. Carnitine deficiency disorders can be classified as either primary or secondary.

Primary carnitine deficiency is defined as a clinically significant decrease in endogenous carnitine stores that cannot be attributed to another systemic illness [9, 11, 89, 179, 180]. Primary carnitine deficiency may be further subdivided into systemic or myopathic forms depending on this tissue distribution of the carnitine insufficiency. Systemic carnitine deficiency is characterised by a generalised reduction of L-carnitine within the plasma (<2 - 10% of normal) and tissue compartment (<1% of normal) with a higher than normal excretion of L-carnitine into the urine [9, 11, 89, 180, 181]. This form of the disease is due to a defect in the OCTN2 transporter which results in reduced intestinal L-carnitine absorption, limited uptake into the muscles and other tissues and disturbed renal tubular reabsorption resulting in excessive urinary loss [182 - 186]. On the other hand, patients with myopathic carnitine deficiency exhibit normal plasma and low muscle (<20% of normal) L-carnitine concentrations [9, 11, 89, 180]. Like the systemic form, myopathic carnitine deficiency is a result of a defect in OCTN2 transport; however, in this form the condition is limited to L-carnitine uptake into skeletal muscle [16, 88, 89]. The severe carnitine insufficiency in patients with primary carnitine deficiency results in an energy deficit due to the inability of fatty acids to enter the mitochondria and undergo β-oxidation and the resultant accumulation of fatty acids within the cytosol has a toxic effect on the cell via a detergent action. These patients develop progressive cardiomyopathy, encephalopathy and muscle weakness, leading to death from heart failure unless treated [187, 188].

Treatment of carnitine deficiency consists of high daily doses of L-carnitine (up to 1000 mg/kg p.o.) with presumably ensures adequate absorption and uptake of L-carnitine either via an alternative (non-OCTN2) low affinity transporter or passive diffusion, such that fatty acid oxidation is normalised. However, it should be noted that even at these high doses, tissue L-carnitine stores are generally not restored to normal levels [9, 89, 189].

Carnitine deficiency has also been described as secondary to genetically-determined metabolic disorders, acquired medical conditions or other external factors [9, 11, 179, 180, 190, 191]. Whilst these secondary carnitine deficiencies are less severe than the primary form, their prevalence is more common. Various genetically-determined metabolic errors have been associated with carnitine

deficiency, the most common of which are defects in fatty acid oxidation with either defects in the enzymes required for the transfer of fatty acids into the mitochondria or those required for the β-oxidation process[9, 11, 179, 180]. Ultimately, all of these disorders result in an underproduction of acetyl-CoA and thus dysfunction of the Krebs cycle. This leads to the accumulation of fatty acids and acylcarnitines which cause clinical symptoms.

Numerous medical conditions have been associated with carnitine disturbances, either via decreased biosynthesis (chronic renal failure[69, 192, 193], chronic liver failure[9, 194, 195]) decreased intake (malnutrition[9, 196], malabsorption such as in coeliac disease[9, 56], cystic fibrosis[9], short-gut syndrome[9]), decreased body stores or increased requirements (HIV/AIDS[9, 197], pregnancy[9, 190, 198]), or increased loss (renal Fanconi syndrome[9, 199]). Disturbances in carnitine homeostasis have also been attributed to other conditions including coronary vascular syndrome, peripheral vascular disease, muscular myopathies, male infertility, autism and chronic fatigue syndrome[200 - 203]. Similarly, supplementation with short-chain carnitine esters is potentially beneficial for the treatment of Alzheimer's disease[204 - 206], vascular disorders[207, 208] and fatigue[209, 210].

Secondary carnitine deficiency may also be induced by treatment with certain pharmacologic agents including valproate, pivampicillin and verapamil[9, 211 - 213], either via inhibition of OCTN2 or via the increased utilisation of L-carnitine for urinary excretion of derivatives as acylcarnitines.

The disturbance in carnitine homeostasis associated with haemodialysis treatment has been well described, with numerous studies demonstrating a significant reduction in endogenous L-carnitine concentrations in patients undergoing long-term haemodialysis treatment[51, 63, 66 - 71, 74, 76, 105, 192, 193, 214 - 219]. In fact, kinetic analysis of carnitine levels during the course of a single dialysis session has demonstrated an extraction ratio of 0.74 and 0.71 for L-carnitine and acetyl-L-carnitine into the dialysate[218]. After completion of the dialysis session there was a hyperbolic increase in plasma levels as carnitine is released from tissue stores. Repeated dialysis treatment over an extended period of time has been shown to result in tissue carnitine deficiency[219]. However, more recent studies have established that haemodialysis treatment is not only associated with low endogenous L-carnitine levels, but also accumulation of acylcarnitines, in particular medium- and long-chain compounds due to their inefficient removal during the dialysis

procedure [76, 219]. This alteration in carnitine pool composition has been associated with a number of dialysis-related disorders including anaemia, muscle fatigue and quality-of-life [80, 220].

Given the sensitivity of acylcarnitine levels and the relative carnitine pool composition in reflecting the intramitochondrial acyl-CoA to free CoA ratio (and hence any disturbances in mitochondrial metabolism), it is surprising that relatively few studies have examined full carnitine profiles in conditions of carnitine deficiency, especially in light of recent advances in analytical methods that are able to quantify individual acylcarnitine concentrations. Recent studies by our group have established that long-term haemodialysis treatment is associated with accumulation of medium- and long-chain acylcarnitine levels, as well as reductions in L-carnitine and acetyl-L-carnitine concentrations [76]. In addition, new research has established that patients with chronic fatigue syndrome exhibit significantly lower long-chain acylcarnitines than their healthy counterparts [203], a finding which had not been demonstrated prior to the advent of more advanced tandem mass spectrometry methods. Bene et al. [56] were also able to demonstrate that adult coeliac patients maintained on a long-term gluten-free diet display significantly lower levels of specific short-, medium- and long-chain carnitine esters compared to healthy controls; however, L-carnitine levels were not altered. The involvement of disturbances in acylcarnitine, as well as carnitine, homeostasis in conditions associated with carnitine deficiency requires further investigation.

1.4 Carnitine Pharmacokinetics

Examination of the pharmacokinetics of exogenously administered L-carnitine and acylcarnitines is associated with great complexity. Firstly, given that carnitine homeostasis is maintained via multiple, saturable, interconnected processes, exogenous carnitine displays non-linear pharmacokinetics such that its disposition is dependent on concentration/dose. This is further complicated by the extensive, reversible interconversion between L-carnitine and acylcarnitines. In addition, the quantification of carnitine concentrations in various biological matrices reflects both endogenous as well as exogenous carnitine levels. For the most part, researchers utilise baseline-correction techniques to describe exogenous kinetics whereby 'corrected' carnitine concentrations are calculated as the difference between quantified levels and those observed prior to treatment. Whilst this simple technique is useful, it is assumed that administration of an exogenous carnitine dose does not affect endogenous

carnitine homeostasis – an assumption which is unlikely given the complex homeostatic mechanisms involved.

The pharmacokinetics of carnitine and short-chain acylcarnitines are characterised by multicompartmental kinetics with prolonged absorption and low bioavailability of oral doses, slow distribution into tissues and high, concentration-dependent renal clearance.

1.4.1 Absorption

A number of studies have investigated the pharmacokinetics of orally administered L-carnitine at doses ranging from 500 – 6000 mg [72 112, 221 - 225]. In all cases, the time to maximum plasma concentration (T_{max}) was long, with reported values in the range of 3 – 5 hours after treatment administration. This is in keeping with the findings of Gross & Henderson [27] and Gudjusson et al. [31] who reported that the absorption of L-carnitine is characterised by rapid uptake into the intestinal mucosa, followed by prolonged retention and slow release into the circulation. Slightly lower T_{max} values have been reported for acetyl-L-carnitine and propionyl-L-carnitine after exogenous administration of these carnitine esters (2 – 3 hours) [226, 227], possibly reflecting the more rapid transfer of esterified carnitine across the basolateral membrane which has previously been reported in animal models [33].

Interestingly, Cao et al. [223] investigated the pharmacokinetics of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine after administration of a 2000 mg oral dose of L-carnitine to 12 healthy normal volunteers. In this study, the reported T_{max} values for L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine were 3.4, 2.4 and 3.8 hours respectively, with plasma acetyl-L-carnitine concentrations peaking one hour prior to the parent compound. This finding suggests that the administered dose may have undergone acetylation within the enterocytes during the absorption process, a finding which has been noted in rat and guinea pig models [27, 31 - 33].

The absolute bioavailability of orally administered L-carnitine has been reported as 14 - 16% for doses of approximately 2000 mg. However, in a study conducted by Harper et al. [112], the bioavailability of a 6000 mg dose was reported to be as low as 5%. In this study, as the area-under-the-curve values from the 2000 mg and 6000 mg treatments were equivalent (300 µmol.hr/L c.f. 313 µmol.hr/L), the authors concluded that the intestinal absorption of L-carnitine was completely saturated at a dose of 2000 mg or higher. However,

as the area-under-the-curve of a compound is not only dependent on absorption but also clearance, and given that the non-linearity of clearance has clearly been established, it is more likely that the low area-under-the-curve value for the high dose in this study was due to partial saturation of both renal tubular reabsorption as well as intestinal uptake. In addition, it should be noted that the 6000 mg dose given in this study was administered as 18 separate tablets with only 400 mL of water which would undoubtedly have affected the bioavailability of the dose due to slow or incomplete dissolution of the dosage form. In keeping with this, the bioavailability of a 100 mg/kg (approximately 6000 – 7000 mg) oral dose of L-carnitine in another study was reported as 14% [222].

However, as discussed by Evans & Fornasini [216], these absolute bioavailability values should be interpreted with caution. In the above-mentioned studies the bioavailability of the oral formulations have been determined by comparing the area-under-the-curve values for the oral and intravenous formations for which there is a many-fold difference in values. Given that L-carnitine exhibits non-linear clearance (i.e. clearance increases as plasma levels increase), it is probable that the clearance for the intravenous formulation would be higher than that for the oral treatment and as such the determination of bioavailability is not accurate. A true estimate of bioavailability for a dose of L-carnitine can be determined only by examining oral and intravenous doses which result in comparable plasma profiles, and hence clearances; this has not been established in the literature.

Nonetheless, it can safety be concluded that oral L-carnitine displays low bioavailability. Given that the hepatic extraction of L-carnitine (as well as acetyl-L-carnitine and propionyl-L-carnitine) is low [228], it is unlikely that the liver contributes to the observed low bioavailability and it can be assumed that the absorption of L-carnitine is incomplete primarily due to the saturation of uptake via OCTN2 from the intestinal lumen. This is supported by the findings of studies by Bain et al. [224] and Rebouche [111] in which it was established that increasing oral doses of L-carnitine result in increasing levels of trimethylamine and trimethylamine-*N*-oxide in the urine and γ-butyrobetaine in the faeces, indicating that a greater portion of the administered L-carnitine dose is not absorbed and passes to the lower intestine where it undergoes bacterial degradation to these products.

1.4.2 Distribution

Investigations of intravenously administered L-carnitine indicate that it immediately distributes into a volume of 7 – 14 L[51, 72, 229, 230], equivalent to the volume of the extracellular fluid compartment, after which levels decline in a multiexponential manner[95, 112, 229 - 231].

It has been established that administration of intravenous L-carnitine is also associated with elevations in acylcarnitine concentrations[72, 221, 223, 225], providing evidence that L-carnitine enters the intracellular compartment after dosing. Early work attempted to model L-carnitine and acylcarnitine kinetics using a two compartment model[72]; however, in doing so the model assumed that once L-carnitine is esterified to acylcarnitine it does not undergo de-esterification, and also that the acylation of L-carnitine occurs in the extracellular fluid and not in the extravascular compartment. Given the current knowledge of carnitine homeostasis, this model does not reflect the distribution of L-carnitine accurately. Pivotal work by Rebouche & Engel[95, 231] resulted in the development of a three compartmental model of L-carnitine kinetics, comprising of a central compartment, a slow distribution compartment and a rapid distribution compartment, assumed to represent the extracellular fluid, skeletal & cardiac muscle and the liver & kidneys respectively. The model was initially validated using kinetic data from dogs[95] and was later applied to human data[231]. Importantly, the model demonstrated the prolonged retention of L-carnitine within the muscles with a half-life of release from these tissues into the plasma of approximately 139 hours, with a muscle compartment turnover time of 191 hours. On the other hand, the turnover time for the kidneys and liver was 11.6 hours with a release half-life of 8 hours. The turnover time for L-carnitine within the plasma was reported as 1.13 hours and the whole body turnover time was 66 days. Based on the model data, the muscle, liver & kidney and extracellular fluid compartments comprised 95.5%, 3.5% and 1% of total body L-carnitine, respectively. Given the prolonged distribution of L-carnitine (and most likely acetyl-L-carnitine and propionyl-L-carnitine) into the slow equilibration compartment, it is likely that short-term exogenous administration would have little or no impact on muscle carnitine stores.

The distribution of L-carnitine is summarised in Figure 1.4.

1.4.3 Elimination

Exogenously administered L-carnitine is rapidly eliminated by the kidneys such that plasma concentrations are normalised within 12 - 24 hours after intravenous dose administration [23]. The renal clearance of L-carnitine has been found to increase within increasing dose [112, 119, 224, 230], reflecting the saturability of OCTN2 reuptake of L-carnitine from the renal filtrate. Bain et al. [224] noted renal clearance values of 0.666 L/hr, 0.804 L/hr and 1.752 L/hr after administration of 500 mg, 1000 mg and 2000 mg oral L-carnitine to healthy normal volunteers; this compared to baseline (untreated) renal clearance values of 0.246 L/hr. This finding was supported by Rizza et al. [222] in which intravenous doses of 30 mg/kg and 100 mg/kg resulted in different renal clearance values (6.97 L/hr c.f. 7.89 L/hr, respectively). However, this study was also able to demonstrate that increasing dose is also associated with increased non-renal clearances (1.06 L/hr c.f. 2.05 L/hr); it is presumed that this non-renal clearance can be accounted for by an increase in the metabolic clearance of L-carnitine to its acyl derivatives which are subsequently eliminated.

Interestingly, in a study by Pace et al. [226] in which the pharmacokinetics of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine were examined after intravenous administration of 1000 mg, 2000 mg, 4000 mg and 8000 mg propionyl-L-carnitine, the renal clearance of all three analytes increased with increasing doses of propionyl-L-carnitine. However, the non-renal clearance of propionyl-L-carnitine reduced with increasing dose, suggesting that the de-acylation of propionyl-L-carnitine was saturated. These findings, in conjunction with those of Rizza et al. [222], suggest that, in a state of homeostatic imbalance (such as exogenous administration), there is increased esterification of L-carnitine to acyl derivatives, presumably for their subsequent (preferential) excretion in urine.

1.4.4 Pharmacokinetics in Patients

Whilst there is a considerable amount of research in the literature which examines the various disorders associated with disturbed carnitine homeostasis and the pharmacokinetics of the carnitine group of compounds in healthy subjects, very few studies have investigated carnitine pharmacokinetics in patient groups; in fact, the only studies which have been conducted are in patients with end-stage renal disease undergoing

haemodialysis treatment[218, 232 - 234]. Further research is needed in this area, given the likely impact of the disturbances in the transport, absorption, biosynthesis, distribution, elimination and utilisation of L-carnitine in these conditions on the pharmacokinetics of exogenous doses.

The administration of L-carnitine to patients undergoing haemodialysis treatment presents a unique situation in which the clearance of L-carnitine is 'turned on' and 'turned off' with each dialysis session. This coupled with decreased L-carnitine intake (due to a prescribed low protein diet) and limited biosynthesis (due to impaired kidney function) suggests that the pharmacokinetics of L-carnitine in these patients differs substantially from that in healthy normal volunteers.

Work by Evans et al.[218] investigating the pharmacokinetics of L-carnitine (20 mg/kg i.v. administered after each dialysis session) in haemodialysis patients was able to establish that during the intradialysis interval there is a high extraction of L-carnitine and acetyl-L-carnitine into the dialysate. The data from this study was subsequently fitted to a three compartment mammillary model which incorporated endogenous L-carnitine synthesis[234]. The model comprised a central compartment, a slow equilibrating compartment and a rapid equilibrating compartment. The central compartment had a volume of distribution of 10.09 L, representing the extracellular fluid, from which L-carnitine was intermittently cleared during the dialysis session. The rapid compartment, most likely representing the liver and kidneys, was estimated to have an apparent volume of distribution of 15.42 L with a fast turnover (9.4 hours), whereas the slow equilibrating pool had an apparent volume of distribution of 7207 L and a turnover of 7413 hours. Administration of an exogenous dose was associated with a large increase in plasma concentrations followed by a multiexponential decline as the dose was initially transferred into the liver and kidneys and thereafter taken up by the muscles (Figure 1.5).

A recently published study by Vernez et al.[233] investigated the kinetics of L-carnitine and individual acylcarnitines in haemodialysis patients after administration of intravenous L-carnitine (10 – 20 mg/kg administered after each dialysis session). This study demonstrated that exogenous L-carnitine was associated with increases in plasma L-carnitine, acetyl-L-carnitine, propionyl-L-carnitine and isovaleryl-L-carnitine concentrations, all of which were efficiently removed during the subsequent haemodialysis session. However, the results of this study should be interpreted with caution as the analytical method used did

not have adequate sensitivity to quantify plasma concentrations of acylcarnitines with a chain length of five or more carbons. Given that it has been established that chronic haemodialysis treatment results in the accumulation of these medium- and long-chain acylcarnitines, and the removal of these carnitine esters into the dialysate is limited[76], further studies investigating the impact of exogenous L-carnitine on relative carnitine pool composition are needed.

1.5 Conclusions

L-carnitine has an obligatory role in fatty acid oxidation and maintaining mitochondria function. Critically, under conditions of stress, L-carnitine is able to buffer the intracellular free CoA to acyl-CoA ratio by forming acylcarnitines; as such the intramitochondrial relationship between free CoA and acyl-CoA is reflected in the extracellular L-carnitine to acylcarnitine ratio. The relative contribution of L-carnitine and acylcarnitines within the total carnitine pool is therefore considered critical in the identification of mitochondria dysfunction.

Although there is considerable research in the literature focussed on disorders of carnitine insufficiency, relatively few have examined relative carnitine pool composition in these conditions. Consequently, the complexity of these disorders may not be fully understood; for example, while absolute L-carnitine levels may be normal, or even borderline normal, these conditions may be associated with substantial disturbances in the L-carnitine to acylcarnitine ratio or alterations in the levels of specific acylcarnitines. Studies investigating the relative carnitine pool composition and its potential involvement in clinical symptoms in patients with these disorders are required.

Whilst important studies have been conducted establishing the kinetics of exogenous carnitine and carnitine esters in healthy normal volunteers, few studies have examined carnitine pharmacokinetics in patient groups. Furthermore, the impact of L-carnitine administration on the kinetics of acylcarnitines has not been established. It is proposed that studies examining the whole body kinetics of L-carnitine and its esters are needed.

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Table 1.1: Carnitine tissue distribution

The distribution of carnitine into the primary sites of carnitine storage in the human body for a 70 kg adult [6, 9, 16, 38, 51, 82, 85 – 91].

Tissue Compartment	Compartment Size	Carnitine Concentration	Carnitine Content	% Total Body Carnitine	K_m Value	Turnover Time
Plasma	3 L / 70 kg	40 µmol/L	0.2 mmol	0.2%		
Extracellular Fluid (including Plasma)	13 L / 70 kg	40 µmol/L	0.9 mmol	0.7%		
Skeletal Muscle	30 kg / 70 kg	4200 µmol/kgww	126 mmol	97%	2 - 60 µM	105 hours
Heart	350 g / 70 kg	1300 µmol/kgww	0.5 mmol	0.4%	2 - 60 µM	21 hours
Kidney	350 g / 70 kg	600 µmol/kgww	0.2 mmol	0.2%	10 - 200 µM	0.4 hours
Liver	1.5 kg / 70 kg	1000 µmol/kgww	1.4 mmol	1%	500 µM	1.3 hours
Brain					>1000 µM	223 hours

Figure 1.1 Chemical structure of L-carnitine

Structure of L-carnitine, with the site of esterification illustrated in bold.

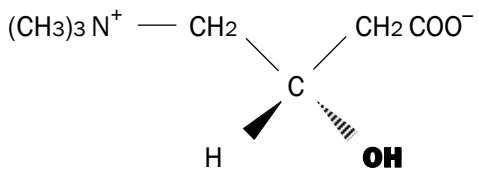


Figure 1.2 Role of L-carnitine in fatty acid metabolism

Schematic diagram of the function of L-carnitine in the transport of fatty acids across the inner mitochondrial membrane. CoA = coenzyme A.

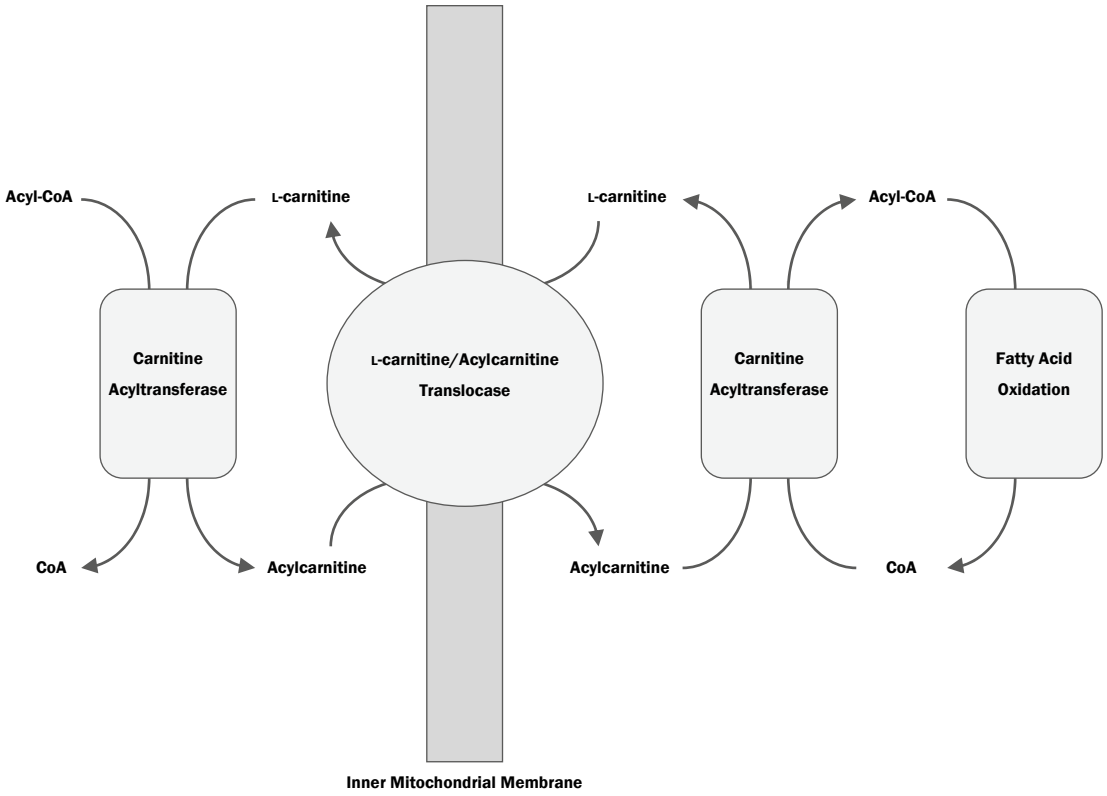


Figure 1.3 Biosynthesis of L-carnitine

Pathway of the biosynthesis of L-carnitine from lysine and methionine precursors.

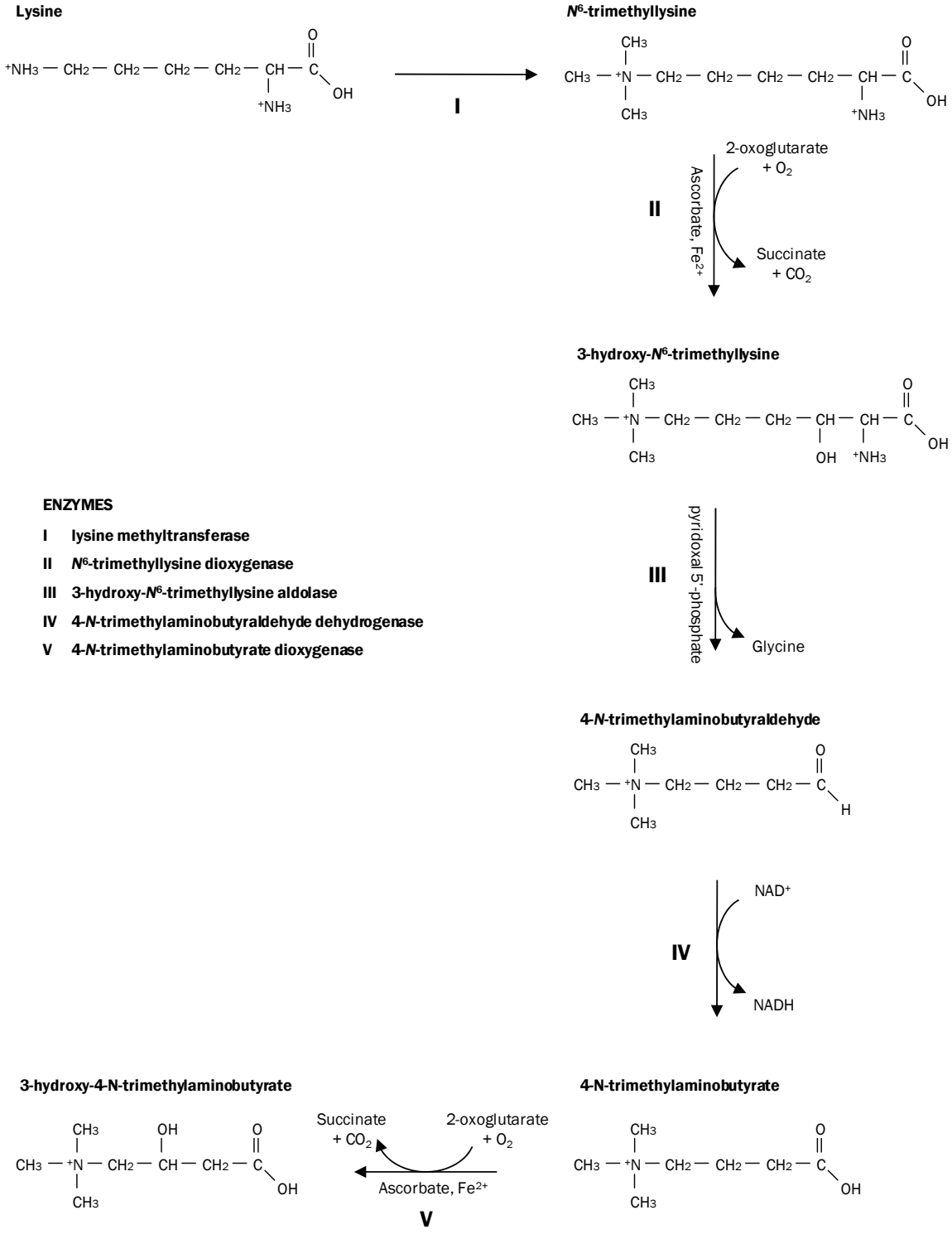


Figure 1.4 Distribution kinetics of carnitine

Schematic diagram of the three compartment model of L-carnitine pharmacokinetics [168]. MRT = mean residence time (turnover time); TBC = total body carnitine.

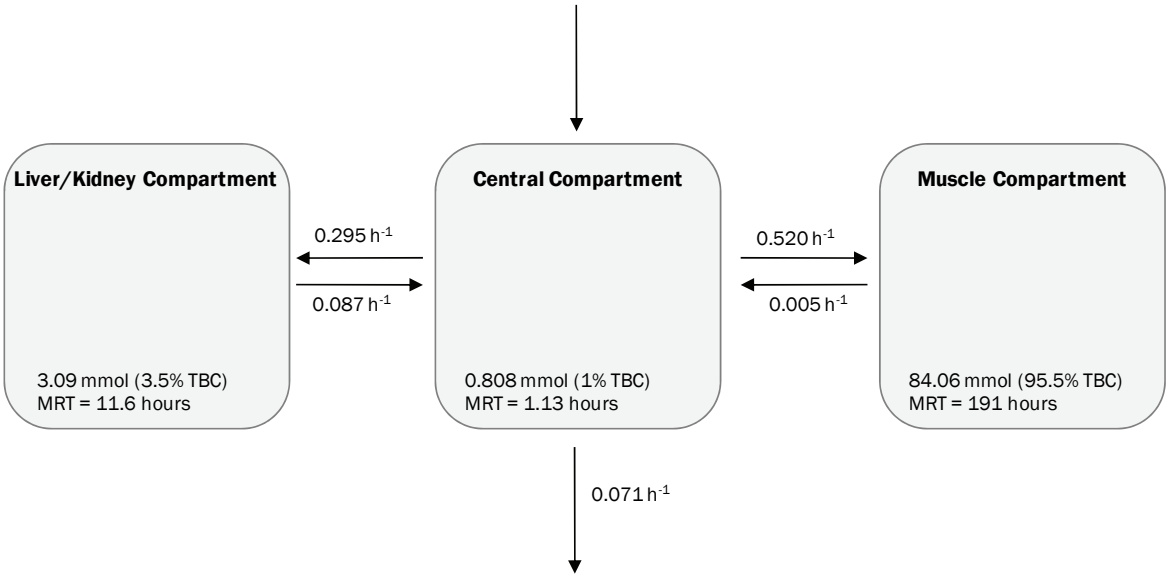
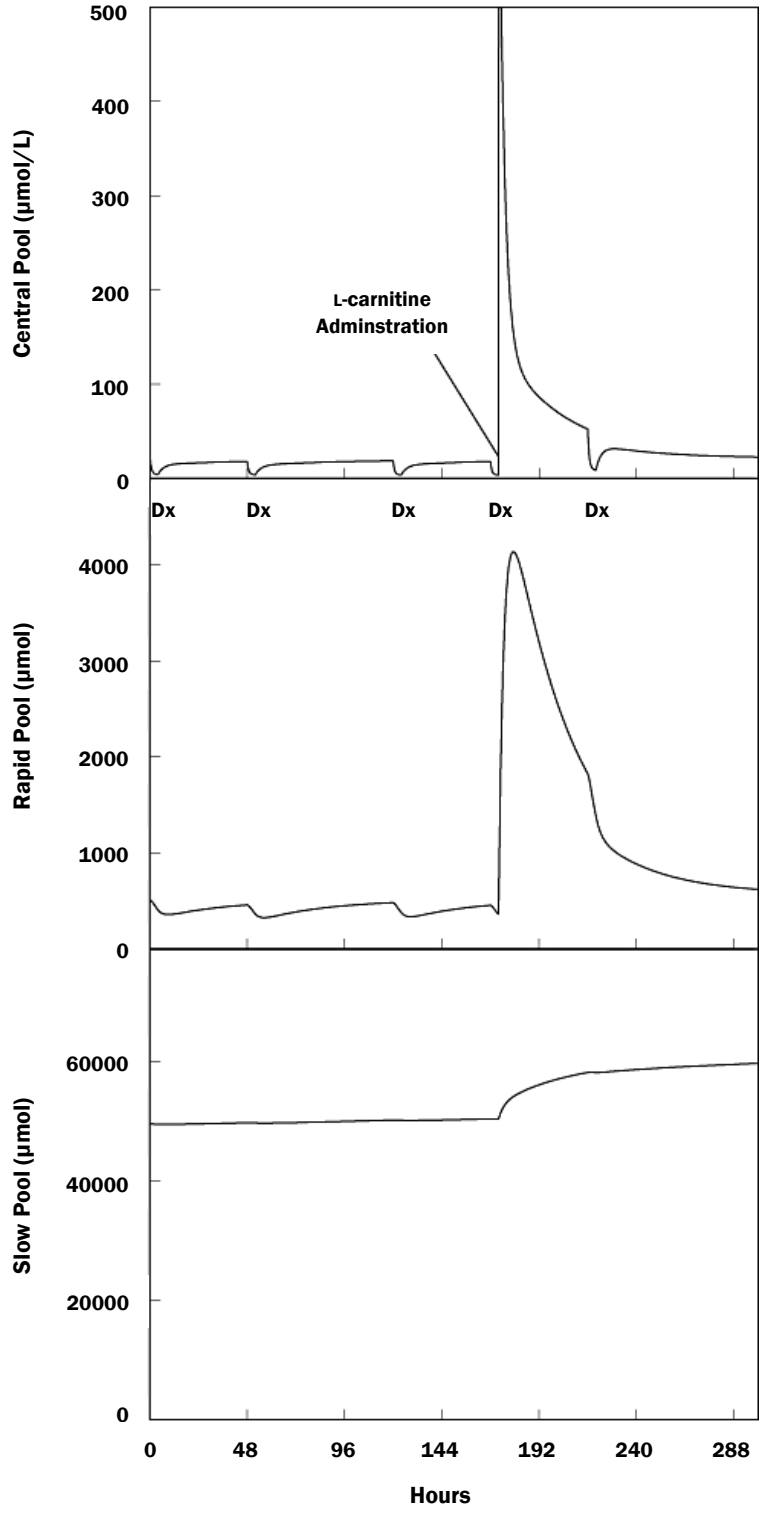


Figure 1.5 Simulated compartmental carnitine concentrations

Simulated levels of carnitine within the three compartments of the developed pharmacokinetic model for L-carnitine in patients receiving haemodialysis treatment [234]. Administration of a single dose of L-carnitine (20 mg/kg i.v.) is associated with a large increase in central (plasma) compartment concentrations, followed by a multiexponential decline as the dose initially transfers into the rapid equilibrating compartment (i.e. liver & kidneys) and thereafter taken up by the slow pool (i.e. muscles). Dx = dialysis session.



CHAPTER 2

Endogenous Plasma Carnitine Levels in Healthy Adults

Stephanie E Reuter, Allan M Evans, Donald H Chace, Gianfranco Fornasini

Determination of the Reference Range of Endogenous Plasma Carnitines in Healthy Adults

Annals of Clinical Biochemistry 2008; Volume 45(6): Pages 585 - 592

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

This chapter forms the basis of Section I of the thesis and sought to characterise normal endogenous concentrations and reference intervals for L-carnitine, total carnitine and acylcarnitines in a healthy adult population. Whilst numerous studies have reported concentrations of these carnitines in healthy adult subjects, the values reported in the literature vary considerably. This is further complicated by utilisation of different analytical methods and a lack of clarity regarding the effect of age and gender on carnitine levels. The quantification of endogenous carnitine concentrations and the examination of the impact of analytical method, gender and age on these levels are vital in order to adequately examine the potential involvement of carnitine pool composition in disorders associated with disturbed carnitine homeostasis.

This study demonstrated that average endogenous concentrations were in the range of 38 - 44 µmol/L, 6 - 7 µmol/L and 49 - 50 µmol/L for L-carnitine, acetyl-L-carnitine and total carnitine, respectively. However, it should be noted that there were small but significant differences in the reference intervals obtained from the three analytical methods used, highlighting the importance of developing laboratory-specific reference intervals for use in the diagnosis of carnitine deficiency disorders. In doing so, gender should be taken into consideration in the determination of reference intervals for L-carnitine and total carnitine; however, age was shown to have no impact on carnitine pool composition.

It should be noted that, although not presented as part of this publication, this study also characterised normal endogenous plasma concentrations of individual acylcarnitines utilising electrospray tandem mass spectrometry methodology; these results are presented in Chapter 3.

2.1 Summary

L-carnitine is an endogenous substance, vital in the transport of fatty acids across the inner mitochondrial membrane for oxidation. Disturbances in carnitine homeostasis can have a significant impact on human health; therefore, it is critical to define normal endogenous concentrations for L-carnitine and its esters to facilitate in the diagnosis of carnitine deficiency disorders. This study was conducted to determine the normal concentrations of a number of carnitines in healthy adults using three analytical methods. The impact of age and gender on carnitine concentrations was also examined. Blood samples were collected from 60 healthy subjects of both genders and various ages. Plasma samples were analysed for endogenous carnitine concentrations by radioenzymatic assay, high performance liquid chromatography and electrospray tandem mass spectrometry. Precision and accuracy of results obtained for each assay were within acceptable limits. Average endogenous concentrations obtained from the

three analytical methods in this study were in the range of 38 - 44 µmol/L, 6 - 7 µmol/L and 49 - 50 µmol/L for L-carnitine, acetyl-L-carnitine and total carnitine, respectively. Comparisons of results between the genders indicated that males had significantly higher endogenous plasma L-carnitine and total carnitine concentrations than females. Age was found to have no impact on plasma carnitine concentrations. These results are useful in the evaluation of biochemical or metabolic disturbances and in the diagnosis and treatment of patients with carnitine deficiency.

2.2 Introduction

L-carnitine (3-hydroxy-4-*N*-trimethylammonium butyrate) is an endogenous substance present in all mammalian species. A wide range of biological activities of L-carnitine have been described, including buffering of the ratio of free coenzyme A (CoA) to acyl-CoA and as a scavenger of unwanted acyl groups; however, the most important role of L-carnitine is in the transport of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation [1]. Mitochondrial fatty acid oxidation is a fundamental source of cellular energy, particularly in cardiac and skeletal muscle; therefore it is not surprising that approximately 98% of the total carnitine concentrations within the body are located in these tissues [2, 3]. Despite the fact that plasma comprises only approximately 1% of the total body carnitine pool, it is commonly used in studies as an indication of the total body carnitine status in patients and subjects [4, 5].

Disturbances in carnitine homeostasis have been shown to impact greatly on human health. Metabolic disorders resulting in low plasma L-carnitine concentrations are associated with cardiomyopathy, encephalopathy, muscle weakness and even early death if left untreated [6, 7]. The less severe, but more common, secondary carnitine deficiency may be the result of impaired renal or hepatic function, or may be induced by administration of certain therapeutic agents such as pivampicillin and valproic acid [6, 8, 9]. It is therefore critical to properly define normal endogenous values for L-carnitine and its esters so that these carnitine deficiency disorders may be correctly and easily diagnosed.

Analytical methods used for the measurement of plasma carnitine concentrations can be categorised as enzymatic, chromatographic and mass spectrometric assays. Enzymatic assays represent the more traditional approach and most published papers of L-carnitine pharmacology have utilised this type of assay. High performance liquid chromatography potentially has a wider application as it allows for the quantification of specific short-chain acylcarnitines, in addition to L-carnitine, which may be used as markers of metabolic diseases. Mass spectrometry methods are established in the

diagnosis of metabolic disorders and have the added advantage of allowing the measurement of L-carnitine and an entire range of its esters in one analytical run. These assays have been reviewed in more detail elsewhere [10].

While a large number of studies have reported a range of plasma L-carnitine levels in healthy adults (Table 2.1), most of these published reports have used modifications of early enzymatic assays [11 - 14]. With the advent of newer high performance liquid chromatography and mass spectrometry methods, it is not known whether utilisation of these methods has affected the interpretation of normal values. Consequently, the aim of this study was to determine normal concentrations and reference intervals for a number of endogenous carnitines in the healthy adult population using three analytical approaches - radioenzymatic assay, high performance liquid chromatography and electrospray tandem mass spectrometry. In addition, the impact of gender and age on endogenous carnitine concentrations was examined.

2.3 Methods

This study was reviewed and approved by the Human Research Ethics Committee of the University of South Australia. Subjects were completely 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).

Sixty healthy adult subjects were recruited for study participation and were divided into four categories according to gender and age, as outlined in Table 2.2. A single blood sample (20 mL) was collected via venepuncture from each subject and transferred into heparinised collection tubes. Samples were stored on ice and centrifuged within 30 minutes of collection for separation of plasma. Plasma was divided into three aliquots for sample analysis, one each for analysis by radioenzymatic assay, high performance liquid chromatography and electrospray tandem mass spectrometry. An additional aliquot was taken from each plasma sample and combined to form a pooled plasma sample for use as a quality control.

All plasma samples were stored at -20°C until sample analysis. Previous preliminary studies have demonstrated that L-carnitine and its esters are stable for 4 weeks under the collection and storage conditions that were used.

All analytical laboratories were blinded to sample identification codes.

2.3.1 Quality Control Samples

Identical quality control samples were prepared for each of the three analytical methods and submitted for analysis with the subject samples. In order to test precision, 10 aliquots of a pooled plasma sample (QCP) were included in the sample set for each method. For assessment of accuracy, quality control samples with known concentrations were prepared with dialysed plasma and designated as QCA (L-carnitine 7.50 µmol/L, acetyl-L-carnitine 1.50 µmol/L, total carnitine 9.75 µmol/L), QCB (L-carnitine 30.0 µmol/L, acetyl-L-carnitine 6.00 µmol/L, total carnitine 39.0 µmol/L) and QCC (L-carnitine 150 µmol/L, acetyl-L-carnitine 30.0 µmol/L, total carnitine 195 µmol/L). Five aliquots of each of these quality control samples were included for analysis by each analytical method.

2.3.2 Radioenzymatic Assay

Plasma samples were analysed for L-carnitine (free carnitine) concentrations by the Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, South Australia. Samples were analysed by radioenzymatic assay based on the method of Cederblad & Lindstedt [15], using the reaction:

L-carnitine + acetyl-CoA ↔ acetyl-L-carnitine + CoA

Briefly, the analytical method comprised four steps: sample preparation, incubation, separation and quantification of the product. Samples were prepared by mixing 10 µL of plasma, 90 µL of water and 20 µL of carnitine acetyltransferase (1.25 mg/mL, F Hoffman La Roche Ltd, Basel Switzerland). To this, 70 µL of a reagent solution was added, which consisted of ^{14}C-acetyl-CoA (0.25 mCi/2.5 mL, NEN Life Science Products Inc, Boston, MS, USA) and acetyl-CoA (Sigma-Aldrich Pty Ltd, St Louis, MO, USA) in a combined concentration of 43 µmol/L and 0.025 mCi/3.5 mL, in a potassium phosphate buffer (0.3 mmol/L, pH 7.6) containing 1.4 mmol/L *N*-ethyl maleimide (Sigma-Aldrich Pty Ltd).

Each sample was incubated for 60 minutes at room temperature and then separated using an ion exchange resin (BioRad AG2-X8, 20-50 mesh chloride form, BioRad Laboratories, Hercules, CA, USA). Radiolabelled product was quantified by scintillation counting. Standard curve samples were prepared using an L-carnitine chloride solution (Sigma-Aldrich Pty Ltd) over the range

0 - 200 µmol/L and were processed according to the same procedure as the subject plasma samples.

2.3.3 High Performance Liquid Chromatography

Plasma samples were quantified for L-carnitine, acetyl-L-carnitine and total carnitine levels by the Centre for Pharmaceutical Research, University of South Australia, Adelaide, South Australia. Sample analysis was carried out using the fluorometric detection method described by Longo et al. [16].

In short, samples were purified by solid-phase extraction (Bond Elut SAX-Isolute cartridges 100 mg/mL, Varian, Palo Alto, CA, USA) followed by derivatisation with 1-aminoanthracene (Sigma-Aldrich Chemie GmbH) with 1-3-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich Chemical Company Inc, Milwaukee, WI, USA) present as a catalyst. Chromatography was carried out using a Shimadzu pump (LC-10AD), automatic injector (SIL-10AD), system controller (SCL-10A) and spectrofluorometric detector (RF-10AXL; Shimadzu Corporation, Kyoto, Japan) together with a Varian Res Elut-BD C18 analytical column (4.6 mm x 250 mm, with spherical particles of 5 µmol/L; Varian) and an Alltech column heater (Model 530, 220V; Alltech Associates Inc, Deerfield, IL, USA). The mobile phase was run at a flow rate of 1 mL/min and consisted of 35% acetonitrile (HiPerSolv for HPLC, BDH Laboratory Supplies, Leicestershire, UK) and 65% ammonium acetate buffer (0.1 mol/L, pH 3.5). The detector wavelengths were 248 nm (excitation) and 418 nm (emission). The sample injection volume was 20 µL and run time was 20 minutes. Integration was carried out using CLASS-VP software (Version 6.10, Shimadzu Corporation).

The standard curve was constructed using samples of dialysed human plasma spiked with aqueous solutions of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine. Calibration ranges for each of the analytes were: 2.5 - 160 µmol/L for L-carnitine, 0.5 - 32 µmol/L for acetyl-L-carnitine and 3.25 - 208 µmol/L for total carnitine (L-carnitine + acetyl-L-carnitine + propionyl-L-carnitine).

2.3.4 Electrospray Tandem Mass Spectrometry

Subject plasma samples were analysed for L-carnitine, total carnitine and an array of 31 acylcarnitines (including acetyl-L-carnitine) by Pediatrix Screening, Bridgeville, Pennsylvania, USA, based on the method described by Chace et al. [17] (with modifications for plasma). Four 25 µL aliquots of each

plasma sample were pipetted onto the designated target areas on purpose-prepared filter paper (S&S grade 903, Schleicher & Schuell Bioscience Inc, Keene, NH, USA) and allowed to dry for 2 - 3 hours at room temperature. Once dry, the filter papers were sealed inside individual glassine envelopes (Westvaco number 10 stamp envelopes, MeadWestvaco Corporation, Glen Allen, VA, USA) and then placed in plastic-covered foil zipper pouches containing desiccant pillows. The filter papers were then shipped to Pediatrix Screening for analysis.

The analysis involved extraction of plasma from a $^{3}/_{16}$ inch punch of filter paper using 300 µL methanol containing stable isotopically-labelled internal standards over a period of 30 minutes. Samples were then derivatised with 50 µL acidified butanol for 15 minutes at 65°C to form butyl-esters of the carnitines. The derivatised carnitines were then analysed by flow injection electrospray tandem mass spectrometry (API-Sciex 3000, Foster City, CA, USA) with a throughput of 2 minutes per sample. The modified method utilised multiple deuterated internal standards, allowing for correction for any free carnitine produced during the derivatisation with acidified butanol.

Calibration ranges for each of the analytes were: 0 - 80 µmol/L for L-carnitine, 0 - 50 µmol/L for acetyl-L-carnitine and 0 - 10 µmol/L for non-acetyl acylcarnitines.

Total acylcarnitine concentrations were determined as the sum of all 31 of the quantified acylcarnitines. Total carnitine levels were calculated as the sum of L-carnitine and total acylcarnitines.

2.3.5 Data Analysis

Statistical comparisons were performed on Ln-transformed data using an analysis of variance (ANOVA) with posthoc analysis (Contrasts) if significance was detected. Statistical examination of the relationship between carnitine concentrations and age was conducted using linear regression. 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.

Reference interval was defined as the range of values which included 95% of the healthy population. As the distribution of carnitine concentrations within the population is slightly skewed, the upper and lower limits of the reference

intervals for each analyte were determined as the 2.5 and 97.5 percentiles of the data.

2.4 Results

Sixty healthy adult subjects participated in the study and were partitioned into groups according to gender and age. The demographic details of each study group are outlined in Table 2.2.

The mean precision and accuracy values for L-carnitine, acetyl-L-carnitine and total carnitine were within 9% for all of the analyses; the accuracy for total acylcarnitines was slightly higher for QCA (23.0%). Precision and accuracy results for each of the analytes by each analytical method are outlined in Table 2.3. It should be noted that the concentrations of each analyte quantified by electrospray tandem mass spectrometry for the high quality control samples (QCC) were outside the standard range and consequently these results were excluded in the calculation of accuracy values.

Mean ± standard deviation (SD) and 95% confidence interval for L-carnitine, acetyl-L-carnitine, total acylcarnitine (defined as the sum of all acylcarnitines, including acetyl-L-carnitine) and total carnitine concentrations for each analytical method are detailed in Table 2.4. L-carnitine concentrations quantified by both high performance liquid chromatography and electrospray tandem mass spectrometry were significantly higher than those measured by radioenzymatic assay. Both the L-carnitine and acetyl-L-carnitine concentrations measured by high performance liquid chromatography were slightly, but significantly, higher than the concentrations quantified by electrospray tandem mass spectrometry. No significant differences were found between total carnitine concentrations quantified by high performance liquid chromatography and electrospray tandem mass spectrometry.

The effects of gender on carnitine concentrations were examined using the three analytical methods (Table 2.5). L-carnitine concentrations for the male subjects were shown to be significantly higher than those for females by all three analyses. Similarly, total carnitine concentrations were significantly higher in the males when compared to female subjects for both the high performance liquid chromatography and electrospray tandem mass spectrometry analyses, whereas no differences in acetyl-L-carnitine and acylcarnitine levels were found between the two genders. Average endogenous concentrations of L-carnitine and total carnitine were in the range of 35 - 40 µmol/L and 45 - 47 µmol/L for females and 41 - 47 µmol/L and 52 - 53 µmol/L for males.

Differences in carnitine concentrations between the two different age groups (18 - 40 and >40 years) were examined (Table 2.6) and age was found to have no impact on carnitine concentrations quantified by any of the analytical methods. No significant differences between the age groups were found within each gender group. Similarly, statistical correlations between age and carnitine concentrations resulted in no significant results (data not presented).

The reference intervals for L-carnitine (stratified into females and males), acetyl-L-carnitine, total acylcarnitine and total carnitine (stratified into females and males) for each analytical method are presented in Figure 2.1. The reference intervals show significant overlap between the analytical methods and are comparable in width for each analyte.

2.5 Discussion

Numerous studies have reported plasma carnitine concentrations in healthy subjects with varying results (Table 2.1) [4, 5, 18 - 39]. Mean ± SD values for L-carnitine in healthy adults have been reported as low as 20.7 ± 0.97 µmol/L [28] and as high as 49.8 ± 2.0 µmol/L [25]. Similar variations have been observed in the reporting of total carnitine (26.6 ± 1.18 µmol/L to 57 ± 2.3 µmol/L), acetyl-L-carnitine (0.84 ± 0.20 µmol/L to 10.2 ± 0.368 µmol/L) and other carnitine concentrations [19, 22, 28]. The variability appears to be independent of the assay used, with the majority of studies reporting values obtained from radioenzymatic analysis. These inconsistencies present complexities in defining normal endogenous concentrations for carnitines in healthy adults and consequently the diagnosis of carnitine deficiency disorders becomes difficult. This study has therefore been designed to determine normal concentrations and reference intervals for endogenous L-carnitine, total carnitine and acetyl-L-carnitine in healthy adults using three different analytical methods.

The analytical methods employed in this study are validated techniques and are commonly used for diagnostic and/or research purposes. The precision and accuracy results obtained in this study for each of the assays were well within acceptable limits.

Comparison of carnitine concentrations obtained from each analytical method indicated that there were significant differences in the concentrations of L-carnitine and acetyl-L-carnitine quantified. However, examination of the data reveals that the average plasma concentrations are similar and in the range of 38 - 44 µmol/L. Analysis of samples by high performance liquid chromatography and electrospray tandem mass spectrometry resulted in average plasma concentrations which differed by only 4.8%; whereas

analysis by radioenzymatic assay produced lower average concentrations than the other analytical methods (7.8% lower than electrospray tandem mass spectrometry and 12.2% lower than high performance liquid chromatography). Endogenous acetyl-L-carnitine concentrations quantified by high performance liquid chromatography and electrospray tandem mass spectrometry were approximately 6 - 7 µmol/L, with 3.3% difference in average concentrations between the assays. There were no significant differences in total carnitine concentrations between the methods (49 - 50 µmol/L, 1.4% difference). The concentrations of carnitines obtained in the present study are comparable with those previously reported in healthy adult subjects in several studies [5, 18, 23, 26, 29 - 31, 35, 36].

Examination of the effect of gender on plasma carnitine concentrations indicated that concentrations of L-carnitine and total carnitine are significantly higher in adult males than females in each of the analytical methods utilised. In this study, average L-carnitine concentrations were in the range of 35 - 40 µmol/L for females and 41 - 47 µmol/L for males, whereas total carnitine concentrations were in the range of 45 - 47 µmol/L for females and 52 - 54 µmol/L for males. Similarly, Lombard et al. [27] has previously demonstrated significantly higher plasma L-carnitine and total carnitine concentrations in males than in females with L-carnitine concentrations of 39.9 ± 4.9 µmol/L and 32.5 ± 5.3 µmol/L and total carnitine concentrations of 49.4 ± 7.3 µmol/L and 43.3 ± 5.5 µmol/L in males and females, respectively. Niu et al. [30] also reported significantly higher concentrations of L-carnitine and total carnitine in plasma of healthy male subjects than females; however, it was also noted that acetyl-L-carnitine levels were significantly different between genders, a result which was not demonstrated in the present study. Differences between the genders in plasma total carnitine concentrations were also noted by Borum et al. [20]. Harper et al. [23] also examined the effect of gender on plasma carnitine concentrations and although a significance difference was observed in L-carnitine concentrations, no differences between males and females were noted for total carnitine. The average plasma carnitine concentrations for males and females calculated in the present study are similar to those reported in the above studies.

A limited number of studies have examined the effect of age on plasma carnitine concentrations in healthy subjects. Age was found to have no impact on any of the analytes quantified in the present study, neither with significant differences between the two age groups examined, nor differences between the age groups within the genders. Similarly, no significant correlations between age and carnitine levels were observed. Borum et al. [20] previously demonstrated a significant correlation between age and endogenous plasma total carnitine concentrations in healthy females (but not males), a result which could not be replicated in the present study.

The reference intervals calculated from the three analytical methods in this study are very similar, with significant overlap in the intervals for each of the analytes and similar interval widths (Figure 2.1). However, there were slight differences in the upper and lower limits of the reference intervals obtained in this study, illustrating the importance of the development of laboratory-specific reference intervals for use in the diagnosis of primary and secondary carnitine deficiencies. In doing so, gender should be taken into consideration in the determination of reference intervals for L-carnitine and total carnitine; however, age is shown to have no impact. Laboratory-specific reference intervals are expected to be similar to those obtained from our laboratories.

2.6 Acknowledgements

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Table 2.1: Previously reported endogenous plasma carnitine concentrations in healthy subjects

Summary of previously reported healthy subject endogenous carnitine concentrations. Data expressed as mean ± standard deviation. HPLC = high performance liquid chromatography; MS/MS = mass spectrometry; REA = radioenzymatic assay; ? = data not reported.

Reference	Population	Analytical Method	Analyte Concentration (µmol/L)			
			L-carnitine	Acetyl-L-carnitine	Total Acylcarnitine	Total Carnitine
[4]	29 Adults (19 Males, 10 Females)	REA	49 ± 11		8.3 ± 4.6	53 ± 13
[5, 18]	14 Adults (10 Males, 4 Females)	REA	46.82 ± 1.72	5.43 ± 0.42		
[19]	35 Adults (22 Males, 13 Females)	MS/MS	30.029 ± 1.902	10.227 ± 0.368	12.166 ± 0.978	
[20]	446 Males	REA				51.7 ± 10.8
	444 Females					43.8 ± 11.3
[21]	4 Adults	REA	23.9 ± 5.0			30.9 ± 6.4
[22]	? Adults	REA	48 ± 2.5			57 ± 2.3
[23]	29 Adults	REA	38.8 ± 7.3		5.9 ± 4.1	44.6 ± 8.6
	13 Males		41.3 ± 4.9		5.7 ± 4.1	47.0 ± 5.4
	16 Females		36.7 ± 8.3		6.0 ± 4.2	42.7 ± 10.3
[24]	? Adults	REA	36.6 ± 9.5		6.5 ± 3.7	43.1 ± 9.8
[25]	43 Adults	REA	49.8 ± 2.0			
[26]	12 Males	?	38.7 ± 8.7			49.3 ± 2.3
[27]	15 Males	?	39.9 ± 4.9			49.4 ± 7.3
	15 Females		32.5 ± 5.3			43.3 ± 5.5
[28]	14 Adults	REA	20.70 ± 0.97	0.84 ± 0.20		26.55 ± 1.18
[29]	18 Adults	REA	38 ± 8.7			50 ± 12
[30]	? Males	HPLC	41.2 ± 6.1	6.2 ± 0.6		53.1 ± 8.5
	? Females		35.2 ± 4.9	5.7 ± 0.7		45.4 ± 5.6
[31]	6 Adults	REA	40.1 ± 8.5		8.4 ± 4.0	48.5 ± 9.1
[32, 33]	49 Adults	REA	47.1 ± 7.7		6.2 ± 2.4	53.3 ± 8.4
	19 Males		51.4 ± 7.0		5.9 ± 3.0	57.3 ± 7.4
	30 Females		44.3 ± 6.0		6.5 ± 2.0	50.8 ± 8.1
[34]	15 Males	REA	51.0 ± 13.6			
[35]	40 Adults	REA	40.2 ± 7.7			50.9 ± 9.7
[36]	30 Adults (15 Males, 15 Females)	REA	41.9 ± 8.4			55.1 ± 9.7
[37, 38]	20 Adults (10 Males, 10 Females)	REA	37.4 ± 1.3			46.0 ± 2.5
[39]	20 Adults	REA	39.8 ± 2.1			49.7 ± 2.4

Table 2.2: Study population demographic information

Demographic data for each subject group. SD = standard deviation.

Parameter	Subject Group			
	F 18 - 40	F >40	M 18 - 40	M >40
No. of Subjects	15	15	15	15
Gender	Female	Female	Male	Male
Age: Group (years)	18 - 40	>40	18 - 40	>40
Age: Mean ± SD (years)	25.9 ± 3.87	50.8 ± 8.13	28.2 ± 7.08	52.0 ± 6.71

Table 2.3: Precision and accuracy of plasma carnitine analytical methods

Precision (data expressed as %CV) and accuracy (data expressed as mean ± standard deviation) results for L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations quantified by radioenzymatic assay (REA), high performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry (ESI MS/MS). CV = coefficient of variation; NA = not analysed.

$$\text{PRECISION} = \frac{100 \text{ X STANDARD DEVIATION OF } 10 \text{ MEASURED CONCENTRATIONS OF QCP}}{\text{MEAN OF } 10 \text{ MEASURED CONCENTRATIONS OF QCP}}$$

$$\text{ACCURACY} = \frac{100 \text{ X (MEASURED CONCENTRATION - ACTUAL CONCENTRATION)}}{\text{ACTUAL CONCENTRATION}}$$

Parameter		Analytical Method		
		REA	HPLC	ESI MS/MS
Precision				
L-carnitine		2.90	5.00	8.58
Acetyl-L-carnitine		NA	3.97	7.56
Total Acylcarnitine		NA	NA	6.59
Total Carnitine		NA	3.19	8.12
Accuracy				
L-carnitine	QCA	-1.33 ± 7.30	7.21 ± 3.77	-6.39 ± 6.32
	QCB	3.33 ± 4.71	8.15 ± 5.18	-8.47 ± 4.34
	QCC	0.133 ± 7.47	4.44 ± 4.99	NA
Acetyl-L-carnitine	QCA	NA	7.10 ± 8.33	6.91 ± 9.18
	QCB	NA	4.21 ± 4.47	3.54 ± 5.11
	QCC	NA	2.46 ± 4.89	NA
Total Acylcarnitine	QCA	NA	NA	23.0 ± 8.07
	QCB	NA	NA	5.75 ± 3.75
	QCC	NA	NA	NA
Total Carnitine	QCA	NA	3.19 ± 12.2	0.390 ± 6.57
	QCB	NA	3.96 ± 5.18	-5.19 ± 4.04
	QCC	NA	9.00 ± 7.37	NA

Table 2.4: Influence of analytical method on plasma carnitine concentrations

Comparison of analytical methods: Endogenous plasma L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations (μmol/L) obtained by radioenzymatic assay (REA), high performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry (ESI MS/MS) (n = 60). Data expressed as mean ± standard deviation [95% confidence interval]. NA = not analysed; † $p<0.05$ compared to REA; ‡ $p<0.05$ compared to HPLC.

Parameter	Analytical Method		
	REA	HPLC	ESI MS/MS
L-carnitine	38 ± 8.0 [36 - 40]	43.3 ± 8.57 [41.1 - 45.5] †	41.2 ± 10.1 [38.5 - 43.8] †‡
Acetyl-L-carnitine	NA	6.65 ± 2.11 [6.10 - 7.19]	6.43 ± 2.01 [5.92 - 6.95] ‡
Total Acylcarnitine	NA	NA	8.67 ± 2.38 [8.07 - 9.31]
Total Carnitine	NA	49.2 ± 9.33 [46.8 - 51.6]	49.9 ± 11.0 [47.0 - 52.7]

Table 2.5: Influence of gender on plasma carnitine concentrations

Effect of gender: Endogenous plasma L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations (μmol/L) for females (n = 30) and males (n = 30) obtained by radioenzymatic assay, high performance liquid chromatography and electrospray tandem mass spectrometry. Data expressed as mean ± standard deviation [95% confidence interval]. † $p<0.05$ compared to female group.

Parameter	Gender	
	Female	Male
Radioenzymatic Assay		
L-carnitine	35 ± 7.0 [32.6 - 37.8]	41 ± 7.9 [38.4 - 44.3] †
High Performance Liquid Chromatography		
L-carnitine	40.0 ± 7.70 [37.2 - 42.9]	46.6 ± 8.22 [43.5 - 49.7] †
Acetyl-L-carnitine	6.29 ± 2.21 [5.47 - 7.12]	7.00 ± 1.97 [6.27 - 7.74]
Total Carnitine	45.9 ± 8.81 [42.6 - 49.2]	52.5 ± 8.79 [49.2 - 55.7] †
Electrospray Tandem Mass Spectrometry		
L-carnitine	38.2 ± 8.01 [35.2 - 41.2]	44.1 ± 11.3 [39.9 - 48.3] †
Acetyl-L-carnitine	6.29 ± 2.31 [5.43 - 7.16]	6.58 ± 1.67 [5.96 - 7.20]
Total Acylcarnitine	8.21 ± 2.62 [7.41 - 9.47]	9.15 ± 2.03 [8.20 - 9.68]
Total Carnitine	46.7 ± 9.45 [43.1 - 50.2]	53.1 ± 11.7 [48.7 - 57.4] †

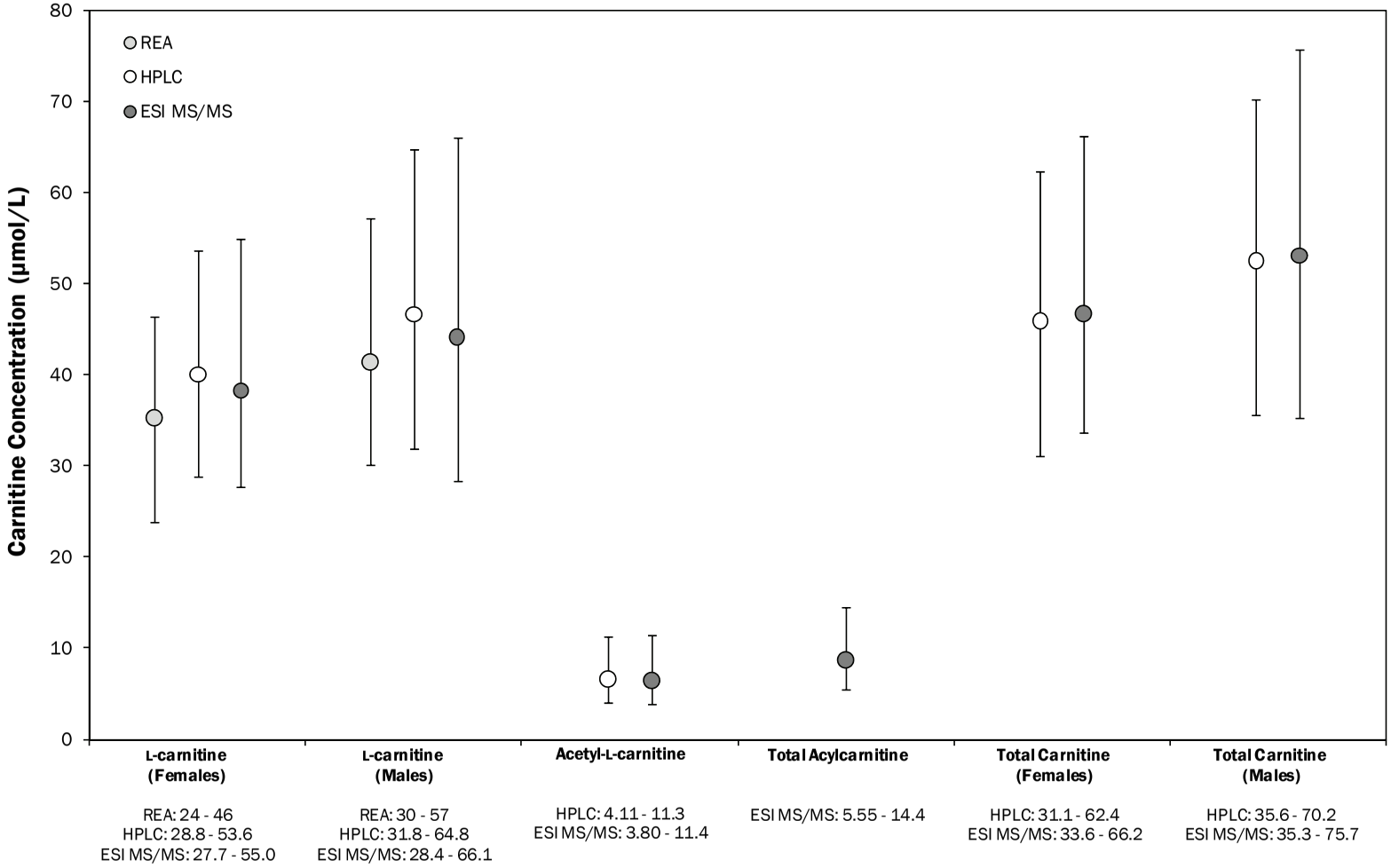
Table 2.6: Influence of age on plasma carnitine concentrations

Effect of age: Endogenous plasma L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations (µmol/L) for subjects aged 18 – 40 years (n = 30) and >40 years (n = 30) obtained by radioenzymatic assay, high performance liquid chromatography and electrospray tandem mass spectrometry. Data expressed as mean ± standard deviation [95% confidence interval].

Parameter		Age Group	
		18 - 40	>40
Radioenzymatic Assay			
L-carnitine	All Subjects	39 ± 7.3 [36 - 42]	38 ± 8.7 [34 - 41]
	Female Subjects	36 ± 6.8 [32 - 39]	35 ± 7.4 [31 - 39]
	Male Subjects	42 ± 6.2 [39 - 46]	40 ± 9.3 [35 - 45]
High Performance Liquid Chromatography			
L-carnitine	All Subjects	43.7 ± 8.29 [40.6 - 46.8]	43.0 ± 8.96 [39.6 - 46.3]
	Female Subjects	40.3 ± 8.08 [35.8 - 44.8]	39.8 ± 7.57 [35.6 - 44.0]
	Male Subjects	47.1 ± 7.24 [43.1 - 51.1]	46.1 ± 9.34 [41.0 - 51.3]
Acetyl-L-carnitine	All Subjects	6.55 ± 2.14 [5.76 - 7.35]	6.74 ± 2.11 [5.95 - 7.53]
	Female Subjects	6.22 ± 1.80 [5.23 - 7.22]	6.36 ± 2.62 [4.91 - 7.81]
	Male Subjects	6.88 ± 2.45 [5.52 - 8.23]	7.12 ± 1.42 [6.33 - 7.91]
Total Carnitine	All Subjects	48.5 ± 8.91 [45.2 - 51.8]	49.9 ± 9.83 [46.2 - 53.5]
	Female Subjects	45.7 ± 8.41 [41.0 - 50.3]	46.1 ± 9.48 [40.9 - 51.4]
	Male Subjects	51.3 ± 8.75 [46.5 - 56.2]	53.6 ± 8.98 [48.6 - 58.5]
Electrospray Tandem Mass Spectrometry			
L-carnitine	All Subjects	42.1 ± 9.91 [38.4 - 45.8]	40.2 ± 10.4 [36.3 - 44.1]
	Female Subjects	38.8 ± 8.37 [34.1 - 43.4]	37.6 ± 7.88 [33.3 - 42.0]
	Male Subjects	45.4 ± 10.5 [39.6 - 51.2]	42.8 ± 12.2 [36.1 - 49.6]
Acetyl-L-carnitine	All Subjects	6.30 ± 1.81 [5.62 - 6.98]	6.57 ± 2.21 [5.75 - 7.40]
	Female Subjects	6.11 ± 1.72 [5.15 - 7.06]	6.47 ± 2.83 [4.91 - 8.04]
	Male Subjects	6.49 ± 1.94 [5.42 - 7.56]	6.66 ± 1.42 [5.88 - 7.45]
Total Acylcarnitine	All Subjects	8.47 ± 2.16 [7.66 - 9.28]	8.90 ± 2.64 [7.92 - 9.89]
	Female Subjects	8.22 ± 2.14 [7.03 - 9.40]	8.66 ± 3.34 [6.81 - 10.5]
	Male Subjects	8.72 ± 2.21 [7.50 - 9.95]	9.16 ± 1.77 [8.18 - 10.1]
Total Carnitine	All Subjects	50.6 ± 10.4 [46.7 - 54.4]	49.1 ± 11.7 [44.8 - 53.5]
	Female Subjects	47.0 ± 9.47 [41.8 - 52.2]	46.3 ± 9.76 [40.9 - 51.7]
	Male Subjects	54.1 ± 10.4 [48.4 - 59.9]	52.0 ± 13.2 [44.7 - 59.3]

Figure 2.1 Normal reference ranges of endogenous plasma carnitine concentrations

Reference intervals for endogenous L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations obtained by radioenzymatic assay (REA), high performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry (ESI MS/MS). Data expressed as mean within reference interval.



CHAPTER 3

Endogenous Plasma Carnitine Levels in Haemodialysis Patients

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Impact of Haemodialysis on Individual Endogenous Plasma Acylcarnitine Concentrations in End-Stage Renal Disease

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	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

Previous research by our group and others has clearly established that patients with end-stage renal disease undergoing long-term haemodialysis treatment exhibit alterations in carnitine homeostasis, characterised by low endogenous L-carnitine concentrations and accumulation of acylcarnitines. The study presented in this chapter sought to examine the exact nature of these accumulated esters and to measure the efficiency of their removal by haemodialysis.

Utilising healthy adult carnitine profile data (presented in Chapter 2) as a reference, this research demonstrated that haemodialysis patients exhibit substantially altered carnitine pool composition with a significant reduction in plasma L-carnitine levels and accumulation of medium- and long-chain esters, such that the proportion of acylcarnitines within the total plasma carnitine pool is 42.8% in haemodialysis patients compared with 17.4% in healthy subjects.

The efficiency of the dialytic removal of acylcarnitines was found to decrease with increasing carbon-chain length of the acyl group and thus the accumulation of acylcarnitines, in particular medium- and long-chain acylcarnitines, in haemodialysis is considered, at least in part, a result of their limited removal by the haemodialysis procedure.

The clinical implications of these alterations in carnitine pool composition in haemodialysis patients are examined in Chapters 4 and 6.

3.1 Summary

Patients with end-stage renal disease undergoing long-term haemodialysis exhibit low L-carnitine and elevated acylcarnitine concentrations. This study evaluated endogenous concentrations of an array of acylcarnitines (carbon-chain length up to 18) in healthy individuals and end-stage renal disease patients receiving haemodialysis, and examined the impact of a single haemodialysis session on acylcarnitine concentrations. Blood samples were collected from 60 healthy subjects and 50 end-stage renal disease patients undergoing haemodialysis (pre- and post-dialysis samples). Plasma samples were analysed for individual acylcarnitine concentrations by electrospray tandem mass spectrometry. Of the 31 acylcarnitines, 29 were significantly ($p<0.05$) elevated in end-stage renal disease patients compared to healthy controls; in particular, C5 and C8:1 concentrations were substantially elevated. For acylcarnitines with a carbon-chain length less than eight, plasma acylcarnitine concentrations decreased significantly over the course of a single dialysis session; however, post-dialysis concentrations invariably remained significantly higher than those in healthy subjects. Dialytic removal of acylcarnitines diminished once the acyl-chain length exceeded 8 carbons. The accumulation of acylcarnitines during long-term haemodialysis suggests that removal by

haemodialysis is less efficient than removal from the body by the healthy kidney. Removal is significantly correlated to acyl-chain length, most likely due to the increased molecular weight and lipophilicity that accompanies increased chain length.

3.2 Introduction

L-carnitine (free carnitine) is a low molecular weight endogenous compound present in all mammalian species[1]. The primary role of L-carnitine is to facilitate the transfer of fatty acids across the inner mitochondrial membrane for β-oxidation and consequently to produce energy[2]. L-carnitine also acts to maintain inner mitochondrial levels of free CoA by accepting short-chain acyl groups from the corresponding acyl-CoA and transporting them out of the mitochondria[1, 3]. Due to this critical role is fatty acid oxidation, endogenous levels of L-carnitine and its acyl derivatives are maintained within relatively narrow limits[4, 5]. The majority of the endogenous carnitine pool is located within the skeletal and cardiac muscle (approximately 98%), which is reflective of the importance of L-carnitine in these primary sites of fatty acid oxidation[4 - 6]. Less than 1% of the body's total carnitine pool is located within the plasma.

L-carnitine levels are achieved by absorption from dietary sources, in particular red meat, and by endogenous biosynthesis from lysine[1]. Most human tissues contain the necessary enzymes to convert lysine to trimethyllysine and subsequently butyrobetaine; however, the enzymes required for the final stage of L-carnitine biosynthesis are only present in the liver, kidney and brain[1]. The plasma is therefore of critical importance for the delivery of L-carnitine from the site of synthesis to the skeletal and cardiac muscles for storage and use[4, 5].

Given that urinary excretion is the primary route of loss of L-carnitine and its esters from the body, patients with end stage renal disease not receiving dialysis treatment exhibit significantly higher plasma concentrations of L-carnitine and its acyl derivatives than the healthy population[4, 5, 7 - 10]. Bartel et al.[7] examined the composition of the endogenous serum carnitine pool in four male and four female end-stage renal disease patients not receiving dialysis treatment (age 19 - 42 years) and 64 healthy subjects (age 18 - 62; 30 men and 34 women). Serum L-carnitine concentrations were significantly higher in the end-stage renal disease patient population, with levels approximately two-fold greater than those measured in the healthy controls. However, no significant differences in total acylcarnitine concentrations were reported between the two subject groups. Rodriguez-Segade et al.[9] published a similar study in which plasma carnitine concentrations in 34 end-stage renal disease patients aged 22 - 60 years (23 men and 11 women) were compared with those in 49 healthy subjects aged 20 - 56 years

(30 men and 19 women). This study reported significantly higher L-carnitine and total carnitine concentrations in the end-stage renal disease group, with concentrations 35% and 74% greater than those measured in the healthy controls. In contrast to Bartel et al.[7], however, total acylcarnitine levels were markedly elevated, with concentrations 4.6 times greater in the end-stage renal disease patients than the healthy subjects (28.7 ± 16.5 µmol/L c.f. 6.2 ± 2.4 µmol/L). Comparable results were reported by Wanner et al.[10] in which carnitine concentrations were 1.7 and 1.9 times higher (for L-carnitine and total carnitine, respectively) in end-stage renal disease patients not undergoing haemodialysis treatment than in the healthy population. Both short- and long-chain acylcarnitine concentrations were significantly elevated, with end-stage renal disease patients exhibiting levels 2.6 and 3.2 times higher (for short- and long-chain acylcarnitines, respectively) than the healthy subjects.

Conversely, chronic haemodialysis treatment has been shown to be associated with a reduction in plasma and tissue L-carnitine concentrations and accumulation of acylcarnitines[4 - 20]. Bøhmer et al.[14] were the first to demonstrate that L-carnitine is extensively removed by the dialyser during a single dialysis session, probably due to the compound's low molecular weight and high hydrophilicity in combination with its lack of protein binding. It was later described that the dialytic extraction ratio for L-carnitine is approximately 74%[15]. Changes in endogenous carnitine pool composition in this patient group have been widely investigated. Golper et al.[16] found no significant differences in total carnitine concentrations between end-stage renal disease patients on haemodialysis and the healthy population; however, L-carnitine levels were reduced by approximately 35% and both short-chain and long-chain acylcarnitine levels were significantly elevated (3.5 and 1.5 times higher for short-chain and long-chain acylcarnitines, respectively). Rodriguez-Segade et al. reported in two papers[9, 11] that total carnitine levels were slightly elevated in end-stage renal disease patients receiving haemodialysis treatment (although not significantly); however, L-carnitine concentrations were diminished by approximately 17% and total acylcarnitine levels were almost 4-fold higher in this patient group. Similar results were reported by Wanner et al.[10, 19], with no differences in total carnitine levels between the two study populations but significantly lower L-carnitine levels and significantly higher total acylcarnitine levels in the haemodialysis patients. In these studies, both short- and long-chain acylcarnitines were found to be elevated in the end-stage renal disease patients, with a two-fold increase in both instances. A number of other research groups have investigated the effect of haemodialysis on endogenous plasma carnitine levels with comparable results[13, 15, 17, 18].

Recent research by our group has investigated the relationship between the duration of haemodialysis treatment (dialysis age) and carnitine status [21]. The study demonstrated a significant negative correlation between plasma L-carnitine concentrations and dialysis age, with the majority of the reduction in L-carnitine concentrations occurring within the first few months of haemodialysis treatment. The decline in plasma L-carnitine with increasing dialysis age was accompanied by a similar reduction in plasma acetyl-L-carnitine concentrations (acetyl-L-carnitine is the most abundant acylcarnitine in the body); however, acetyl-L-carnitine levels remained above the normal range for healthy individuals. Compared with the healthy population, acylcarnitine levels (calculated as total carnitine subtracted by L-carnitine concentrations) were shown to be significantly higher in end-stage renal disease patients, particularly in those patients who had been receiving haemodialysis treatment for more than 12 months.

Until recently, measurements of acylcarnitines have given values for the sum of all of the carnitine esters or the sum of all short-chain and/or long-chain acylcarnitines. Chace et al. [22] has developed an electrospray tandem mass spectrometry method in which individual acylcarnitines can be quantified in human blood. The present study evaluated the concentrations of L-carnitine and an array of short-, medium- and long-chain carnitine esters in healthy individuals and end-stage renal disease patients receiving haemodialysis. For the first time, the impact of a single haemodialysis session on individual plasma acylcarnitine concentrations was examined.

3.3 Methods

3.3.1 Study Design

This study was reviewed and approved by the Royal Adelaide Hospital Research Ethics Committee and the Human Research Ethics Committee of the University of South Australia. Patients were recruited from the Royal Adelaide Hospital Dialysis Unit and its associated satellite dialysis centres. Healthy subjects were recruited from the general population via advertisement, and were assessed at the Centre for Pharmaceutical Research of the University of South Australia. All subjects were enrolled into the study once it had been established that they were not receiving any carnitine supplementation. Patients and healthy subjects were fully informed of the study procedures and provided written informed consent prior to study participation.

3.3.1.1 Healthy Subjects

Sixty healthy subjects (Table 3.1) were selected for study participation. Subjects attended the study centre on one occasion for collection of a single 20 mL blood sample.

3.3.1.2 End-Stage Renal Disease Patients

Fifty end-stage renal disease patients receiving haemodialysis treatment (Table 3.1) were recruited to participate in the study. Arterial blood samples (5 mL) were collected from the arteriovenous fistula on one occasion prior to commencement of dialysis (pre-dialysis) and after completion of the dialysis session (post-dialysis).

3.3.2 Analytical Methods

Plasma samples were analysed for L-carnitine and acylcarnitine profiles using an electrospray tandem mass spectrometry (API/Sciex MDS 3000) assay, based on the method reported by Chace et al.[22] but with modifications. The target biological matrix was modified to plasma rather than blood spots. An improved quantitative analysis was achieved using dried plasma spots as haematocrit and spot diameter have less effect on variations in the actual volume of the matrix for plasma compared to blood. Dialysed plasma prepared as described[15, 23] was used for the preparation of calibration curves. Overall, from repeat analysis, the assay was shown to have a precision and accuracy within 9%.

Individual acylcarnitines analysed are detailed in Table 3.2. It should be noted that, due to the nature of the tandem mass spectrometry method, some assay results represent the sum of 2 or 3 carnitine esters; for example, C4 represents the sum of two structural isomers with a 4 carbon-chain acyl group (butyryl-L-carnitine and isobutyryl-L-carnitine) (Table 3.2).

3.3.3 Statistical Methods

Acylcarnitine concentrations obtained from healthy subject samples were statistically compared with pre- and post-dialysis concentrations in end-stage renal disease patients using an analysis of variance (ANOVA). Pre- and post-dialysis acylcarnitine levels were compared with a paired student's t-test.

Significance was set at an α-level of 0.05. WinNonlin® Professional, Version 4.0 (Pharsight Corporation, Mountain View, CA, 94040, USA) was used for all statistical analyses.

Acylcarnitines were grouped according to carbon-chain length as short- (C2 - C6), medium- (C8 - C12) and long-chain (C14 - C18) acylcarnitines.

3.4 Results

Sixty healthy subjects and 50 end-stage renal disease patients receiving chronic haemodialysis treatment on a regular basis (approximately three times per week) participated in the study. The demographic and dialysis details are presented in Table 3.1. Although there is a notable difference in the mean age for each study group, previous investigations by our group have demonstrated that there is no significant effect of age on carnitine concentrations.

Mean L-carnitine and total carnitine pre-dialysis concentrations in end-stage renal disease patients were 24.16 ± 9.95 μmol/L and 42.22 ± 16.12 μmol/L respectively. In comparison, mean concentrations in the healthy population were 41.16 ± 10.13 μmol/L and 49.85 ± 11.03 μmol/L, respectively, with the difference being statistically significant ($p<0.05$) in both cases. It should be noted that, despite the fact that plasma L-carnitine concentrations were 41.3% lower in the end-stage renal disease population than in normal volunteers, total carnitine concentrations were only 15.3% lower.

End-stage renal disease patients displayed significantly higher concentrations of total short-, medium- and long-chain acylcarnitines ($p<0.05$), with concentrations of 11.77 ± 5.78 μmol/L, 4.36 ± 1.44 μmol/L and 1.93 ± 0.79 μmol/L compared with normal concentrations of 7.47 ± 2.10 μmol/L, 0.80 ± 0.33 μmol/L and 0.43 ± 0.12 μmol/L, respectively.

Upon examination of the individual L-carnitine acyl derivatives, it can be seen that in all cases, with the exception of C3 and C16-OH, the carnitine esters were significantly elevated in the end-stage renal disease patient population compared to their healthy counterparts (Table 3.3). Most notably, carnitine esters of 18 carbon-chain length were much higher in the end-stage renal disease population with concentrations of 0.17 ± 0.11 μmol/L, 0.51 ± 0.25 μmol/L and 0.22 ± 0.12 μmol/L for C18, C18:1 and C18:2 respectively, compared with 0.05 ± 0.02 μmol/L, 0.09 ± 0.03 μmol/L and 0.03 ± 0.01 μmol/L in the healthy subjects. In addition, C5 and C8:1 concentrations were almost seven times higher in the end-stage renal disease population, with values of 0.82 ± 1.40 μmol/L and 1.00 ± 0.57 μmol/L, respectively, compared with

concentrations of 0.12 ± 0.04 µmol/L and 0.15 ± 0.08 µmol/L in healthy subjects. Despite the high variability in C5 and C8:1 levels in the end-stage renal disease population, the difference in the endogenous concentrations of these compounds is statistically significant between the two populations.

L-carnitine and total carnitine concentrations were significantly reduced over the course of a single dialysis session, with mean post-dialysis concentrations of 9.93 ± 6.04 µmol/L and 17.39 ± 9.80 µmol/L, respectively, compared to concentrations of 24.16 ± 9.95 µmol/L and 42.22 ± 16.12 µmol/L prior to dialysis. Statistical comparisons of the pre- and post-dialysis acylcarnitine concentrations revealed that all of the carnitine esters were significantly removed during the dialysis session, with the exception of the 18 carbon-chain esters (C18, C18:1 and C18:2) and C16-OH. There was a significant correlation ($p<0.05$) between the ratio of post-dialysis to pre-dialysis acylcarnitine concentrations and carbon-chain length of the acyl group (indicating decreasing dialytic removal with increasing chain length); with a notable reduction in the amount of acylcarnitine removed once the acyl-chain length exceeded eight carbons (Figure 3.1). Despite significant removal of acylcarnitines during a single dialysis session, for the majority of the carnitine esters, post-dialysis concentrations remained significantly higher than those in the healthy population, in particular the long-chain acylcarnitines (Table 3.3).

3.5 Discussion

Disturbances in carnitine pool homeostasis during long-term haemodialysis have been implicated as a contributing factor for certain clinical problems in end-stage renal disease patients, including skeletal myopathies, poor exercise performance, cardiomyopathy and intradialytic complications such as hypotension, cramps, weakness and fatigue [12, 13, 20].

It has been widely established that long-term haemodialysis treatment results in alterations in the endogenous carnitine pool, with a large number of studies reporting significantly lower plasma endogenous L-carnitine concentrations in haemodialysis patients when compared with healthy individuals [4 - 21]. Despite significant reductions in L-carnitine concentrations in the haemodialysis population, total carnitine concentrations remain relatively unchanged. This is due to a significant accumulation of the acylated forms of L-carnitine, with acylcarnitine concentrations between 33% and 47.6% of total concentrations in haemodialysis patients, compared with 11.6 - 17.4% in healthy subjects [8 - 11, 16, 17, 19, 21]. However, the pattern of this accumulation, in terms of the composition of the accumulated acyl pool, has not been studied. To our knowledge,

this is the first study which has quantified individual acylcarnitines in end-stage renal disease patients receiving haemodialysis.

In this study, total carnitine levels in the end-stage renal disease patients were 15.3% lower than the healthy population, a relatively small reduction considering that L-carnitine levels were reduced by 41.3%. Upon examination of the data, it can be seen that the carnitine pool composition is considerably different between the two study populations. L-carnitine concentrations in the healthy population make up 82.6% of the total carnitine pool (17.4% acylcarnitine), whereas in the end-stage renal disease patients on haemodialysis, the L-carnitine and acylcarnitine proportions of the carnitine pool are 57.2% and 42.8%, respectively. Haemodialysis patients exhibited significantly higher acylcarnitine concentrations than healthy individuals for 29 out of the 31 individual acylcarnitines measured. A result of particular interest is the considerable accumulation noted for the C5 and C8:1 acylcarnitines.

Comparison of the pre- and post-dialysis plasma acylcarnitine concentrations indicated that dialytic removal of acylcarnitine is significantly correlated to carbon-chain length of the acyl group, with no significant removal of the 18 carbon-chain length esters. Removal of the longer chain esters across the dialysis membrane is obviously impaired due to the increased molecular weight and lipophilicity that accompanies increasing chain length.

Interestingly, despite the fact that the short- and medium-chain esters are removed by haemodialysis, the concentrations of the majority of carnitine esters remain significantly higher after the dialysis session than concentrations found in the healthy individuals. The accumulation of acylcarnitines during long-term haemodialysis suggests that removal by haemodialysis is less efficient than removal from the body by the healthy kidney. Alternatively, enhanced formation of acylcarnitines or diminished utilisation of fatty acids may also contribute to these alterations to the carnitine pool in this population.

The clinical and toxicological implications of the accumulation of acylcarnitines in end-stage renal disease patients receiving long-term haemodialysis are yet to be determined. However, Yamada et al.[24] have demonstrated that palmitoyl-L-carnitine and stearoyl-L-carnitine enhance calcium release from rabbit cardiac sarcoplasmic reticulum-enriched membrane vesicles, an observation that was found to be both concentration-dependent and carbon-chain length-dependent, a finding which suggests that these compounds could contribute to the development of ventricular arrhythmias. This, coupled with the high incidence of cardiomyopathy and other cardiac disorders in

haemodialysis patients, gives us a basis from which future research can be conducted investigating the clinical implications of this altered carnitine profile, including the impact of L-carnitine supplementation on the pattern of accumulation of acylcarnitines, in particular long-chain acylcarnitines.

3.6 Acknowledgements

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Table 3.1 Study population demographic data

Subject demographic and dialysis data. Data expressed as mean ± standard deviation (range). ESRD = end-stage renal disease; NA = not applicable.

Parameter	ESRD Patients	Healthy Subjects
Subjects (n)	50	60
Age (years)	56 ± 15 (22 - 71)	39 ± 14 (18 - 76)
Gender	30 men / 20 women	30 men / 30 women
Dialysis Age (years)	3.15 ± 3.07 (0.13 - 18.43)	NA
Dialysis Session Duration (hours)	4.00 ± 0.59 (2.50 - 5.15)	NA

Table 3.2 Quantified endogenous acylcarnitines

Acylcarnitines measured as part of the electrospray tandem mass spectrometry method.

Abbreviation	Carnitine Name
LC	L-carnitine
TC	Total carnitine
C2	Acetyl-L-carnitine
C3	Propionyl-L-carnitine
C4	Butyryl-L-carnitine / Isobutyryl-L-carnitine
C4-OH	3-OH-Butyryl-L-carnitine
C4DC	2-Methylmalonyl-L-carnitine
C5	Valeryl-L-carnitine / Isovaleryl-L-carnitine / 2-Methylbutyryl-L-carnitine
C5-OH	3-OH-Valeryl-L-carnitine
C5:1	Pentenoyl-L-carnitine
C5DC (C10-OH)	Glutaryl-L-carnitine (minor contribution of 3-OH-Decanoyl-L-carnitine)
C6	Hexanoyl-L-carnitine
C6-OH	3-OH-Hexanoyl-L-carnitine
C6DC	Adipoyl-L-carnitine
C8	Octanoyl-L-carnitine
C8:1	Octenoyl-L-carnitine (?)
C8-OH (C3DC)	3-OH-Octanoyl-L-carnitine (minor contribution of Malonyl-L-carnitine)
C10	Decanoyl-L-carnitine
C10:1	Decenoyl-L-carnitine
C10:2	Decandienoyl-L-carnitine
C12	Lauroyl-L-carnitine
C12:1	Dodecenoyl-L-carnitine
C12-OH	3-OH-Dodecanoyl-L-carnitine
C14	Myristoyl-L-carnitine
C14:1	Tetradecenoyl-L-carnitine
C14:2	Tetradidecenoyl-L-carnitine
C14-OH	3-OH-Myristoyl-L-carnitine
C16	Palmitoyl-L-carnitine
C16:1	Palmitoeoyl-L-carnitine
C16-OH	3-OH-Palmitoyl-L-carnitine
C18	Stearoyl-L-carnitine
C18:1	Oleoyl-L-carnitine
C18:2	Linoleoyl-L-carnitine

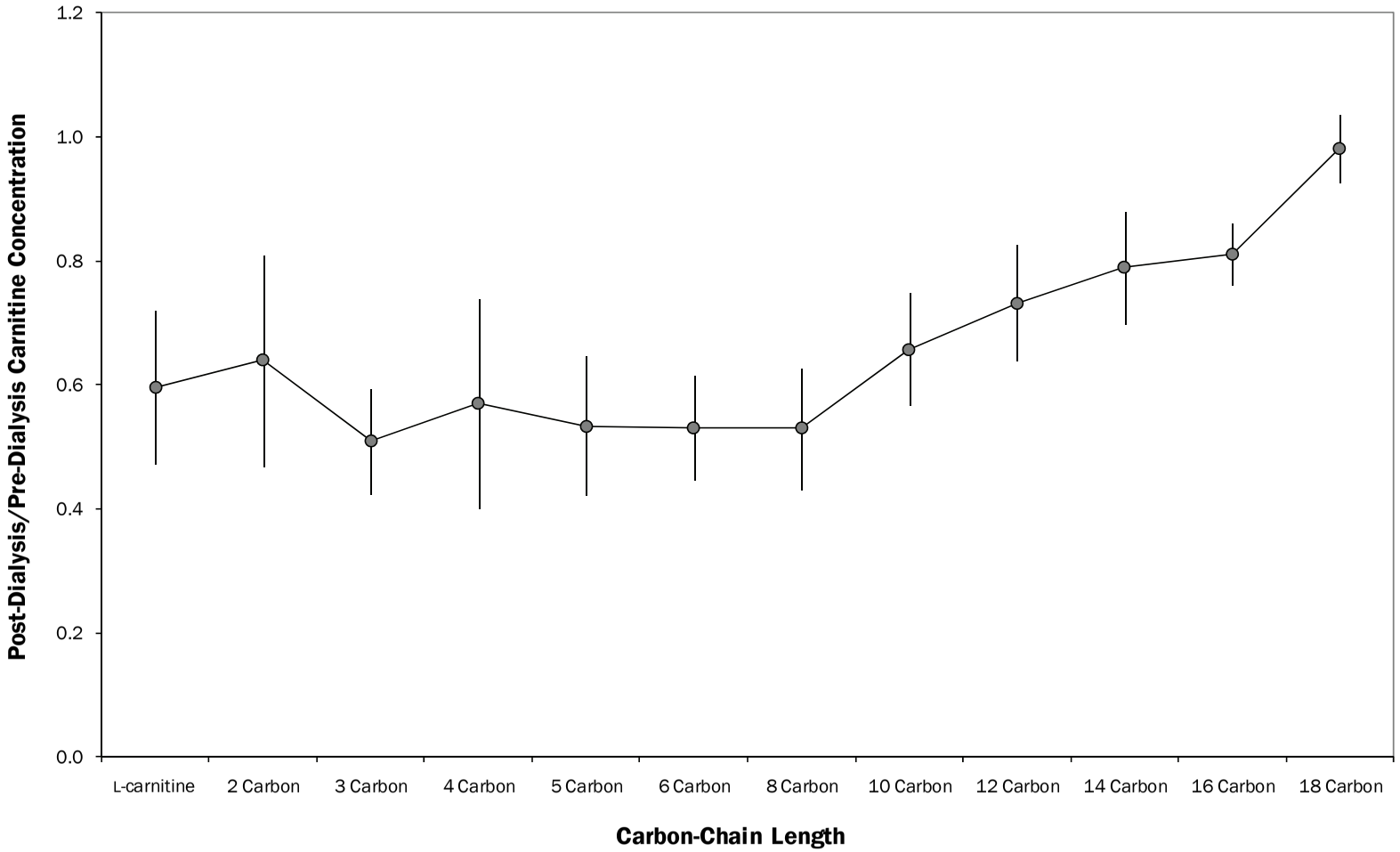
Table 3.3 Impact of haemodialysis on endogenous plasma carnitine concentrations

Plasma acylcarnitine concentrations (μmol/L) for healthy subjects and end-stage renal disease (ESRD) patients receiving chronic haemodialysis treatment. Data expressed as mean ± standard deviation. † $p<0.05$ compared to healthy subjects; ‡ $p<0.05$ compared to ESRD pre-dialysis.

Abbreviation	Healthy Subjects	ESRD Patients	
		Pre-Dialysis	Post-Dialysis
LC	41.16 ± 10.13	24.16 ± 9.95 †	9.93 ± 6.04 †‡
TC	49.85 ± 11.03	42.22 ± 16.12 †	17.39 ± 9.80 †‡
C2	6.43 ± 2.01	9.23 ± 4.76 †	3.32 ± 2.35 †‡
C3	0.52 ± 0.17	0.57 ± 0.37	0.21 ± 0.14 †‡
C4	0.24 ± 0.09	0.68 ± 0.51 †	0.19 ± 0.15 ‡
C4-OH	0.02 ± 0.01	0.08 ± 0.04 †	0.03 ± 0.02 ‡
C4DC	0.03 ± 0.01	0.31 ± 0.14 †	0.10 ± 0.07 †‡
C5	0.12 ± 0.04	0.82 ± 1.40 †	0.32 ± 0.74 ‡
C5:1	0.03 ± 0.01	0.08 ± 0.04 †	0.03 ± 0.02 ‡
C5-OH	0.03 ± 0.01	0.11 ± 0.05 †	0.04 ± 0.02 ‡
C5DC (C10-OH)	0.04 ± 0.02	0.65 ± 0.29 †	0.18 ± 0.15 †‡
C6	0.05 ± 0.02	0.12 ± 0.09 †	0.05 ± 0.03 ‡
C6-OH	0.02 ± 0.01	0.08 ± 0.04 †	0.03 ± 0.02 †‡
C6DC	0.02 ± 0.01	0.63 ± 0.43 †	0.16 ± 0.18 †‡
C8	0.10 ± 0.07	0.29 ± 0.16 †	0.11 ± 0.06 ‡
C8:1	0.15 ± 0.08	1.00 ± 0.57 †	0.41 ± 0.36 †‡
C8-OH (C3DC)	0.03 ± 0.02	0.23 ± 0.11 †	0.06 ± 0.07 †‡
C10	0.15 ± 0.11	0.37 ± 0.19 †	0.18 ± 0.09 ‡
C10:1	0.09 ± 0.04	0.29 ± 0.14 †	0.15 ± 0.08 †‡
C10:2	0.05 ± 0.02	0.16 ± 0.08 †	0.10 ± 0.04 †‡
C12	0.06 ± 0.03	0.18 ± 0.07 †	0.10 ± 0.05 †‡
C12:1	0.06 ± 0.03	0.11 ± 0.05 †	0.07 ± 0.04 ‡
C12-OH	0.01 ± 0.005	0.14 ± 0.14 †	0.05 ± 0.05 †‡
C14	0.03 ± 0.01	0.16 ± 0.07 †	0.09 ± 0.05 †‡
C14:1	0.05 ± 0.03	0.12 ± 0.06 †	0.09 ± 0.06 †‡
C14:2	0.02 ± 0.01	0.05 ± 0.02 †	0.03 ± 0.02 †‡
C14-OH	0.01 ± 0.005	0.22 ± 0.30 †	0.10 ± 0.20 †‡
C16	0.09 ± 0.03	0.35 ± 0.13 †	0.27 ± 0.12 †‡
C16:1	0.02 ± 0.01	0.07 ± 0.04 †	0.05 ± 0.03 †‡
C16-OH	0.03 ± 0.01	0.06 ± 0.18	0.05 ± 0.19
C18	0.05 ± 0.02	0.17 ± 0.11 †	0.14 ± 0.07 †
C18:1	0.09 ± 0.03	0.51 ± 0.25 †	0.53 ± 0.32 †
C18:2	0.03 ± 0.01	0.22 ± 0.12 †	0.20 ± 0.13 †

Figure 3.1 Dialytic removal and acylcarnitine carbon-chain length

Relationship between the ratio of post-dialysis/pre-dialysis acylcarnitine concentrations and carbon-chain length of the acyl group. Data expressed as mean ± standard error of the mean.



CHAPTER 4

Carnitine and rHuEPO Requirements in Haemodialysis Patients

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Endogenous Plasma Carnitine Pool Composition and Response to Erythropoietin Treatment in Chronic Haemodialysis Patients

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	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

In light of the pattern of accumulation of acylcarnitines established in Chapter 3, this study examined the clinical impact of the alterations in carnitine pool composition observed in haemodialysis patients with respect to erythropoietin-resistant anaemia. This study was able to demonstrate a highly significant negative correlation between the proportion of medium- and long-chain acylcarnitines (i.e. non-acetyl acylcarnitines) within the total carnitine pool and the effectiveness of erythropoietin treatment. Whilst the specific mechanism by which carnitine levels affect erythropoietin requirements is not fully understood, it is proposed that carnitine contributes to the stabilisation of the erythrocyte membrane via the modulation of red blood cell carnitine palmitoyltransferase activity. As carnitine palmitoyltransferase has been shown to have a critical role in red blood cell membrane acyl trafficking, decreased in carnitine palmitoyltransferase activity would lead to a reduction in red blood cell membrane repair and turnover thereby resulting in reduced membrane stabilisation and decreased lifespan of the erythrocyte.

4.1 Summary

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. Pre-dialysis blood samples were collected from 87 patients and analysed for plasma L-carnitine and individual acylcarnitine levels by electrospray tandem mass spectrometry. As an indication of rHuEPO responsiveness, erythropoietin resistance index (ERI) was calculated as rHuEPO dose/kg/week normalised for haemoglobin levels. A significant negative correlation between L-carnitine levels and ERI was found ($p = 0.0421$). All patients categorised as high ERI (>0.02 µg/kg/week/gHb) exhibited subnormal L-carnitine levels (<30 µmol/L); conversely, patients with normal L-carnitine levels (>30 µmol/L) displayed low ERI values (<0.02 µg/kg/week/gHb). More importantly, the ratio of non-acetyl acylcarnitines/total carnitine was significantly positively correlated with ERI ($p = 0.0062$). 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.

4.2 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 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 suboptimal [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, characterised 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 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.

4.3 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 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:

$$\mathbf{ERI} = \frac{\mathbf{rHuEPO / kg / week}}{\mathbf{g\ Haemoglobin}}$$

Study patients were prospectively divided into pre-defined Low ERI and High ERI groups based on a cut-off ERI value of 0.02 µg/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 µg/kg/week) are recommended

for L-carnitine therapy[28]. An endogenous L-carnitine concentration <30 µmol/L was defined as subnormal based on the lower limit of the normal reference range in healthy adults[29].

4.3.1 Analytical Methods

Plasma samples were analysed for L-carnitine and individual acylcarnitine concentrations using a 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 (synthesised in-house). After a 15 minute 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 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 4.1 were calculated using quantified plasma carnitine concentrations.

4.3.2 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, statistically 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.

4.4 Results

A total of 87 end-stage renal disease 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 4.2. 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 indictors of carnitine pool composition are outlined in Table 4.1. Mean ± standard deviation (SD) values for each carnitine parameter for all of the study patients are summarised in Table 4.3, along with mean ± 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 µmol/L). Similarly, all patients with normal L-carnitine levels had ERI values <0.02 µg/kg/week/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 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 ± 5.13 µmol/L c.f. 15.0 ± 4.28 µmol/L, $p = 0.0116$) 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 ± 9.50 µmol/L and 0.200 ± 0.0534, respectively, compared with values of 22.9 ± 4.62 µmol/L 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 4.1). 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 4.2); this relationship was found to be independent of dialysis age.

4.5 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 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 approximately 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 which 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, Kletzmayr 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 stabilisation of the erythrocyte membrane with studies demonstrating improvements in membrane fluidity, osmotic fragility and red blood cell deformability with L-carnitine supplementation[23, 39, 41]. Based on the findings of previous studies, we have hypothesised that alterations in the AcylLC-C2/TC ratio may have an effect on erythrocyte membrane by regulation of red blood cell carnitine palmitoyltransferase. Acylcarnitines, in particular, long-chain acylcarnitines, have been shown to inhibit carnitine palmitoyltransferase activity[42] and conversely L-carnitine increases carnitine palmitoyltransferase activity[43 - 45]; accordingly accumulation of medium- and long-chain acylcarnitines and a deficiency in L-carnitine levels (characterised by a high AcylLC-C2/TC ratio) would result in a decrease in red blood cell carnitine palmitoyltransferase activity. As carnitine palmitoyltransferase has been shown to have a critical role in red blood cell membrane acyl trafficking, decreases in carnitine palmitoyltransferase activity would lead to a reduction in red blood cell membrane repair and turnover thereby resulting in reduced membrane stabilisation 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) red blood cell carnitine palmitoyltransferase 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 red blood cell [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 red blood cell, a finding which 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 which 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.

This 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. ≥0.02 µg/kg/week/gHb), and hence have 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 randomised, double-blind, placebo-controlled study is now being conducted to investigate this hypothesis.

4.6 Acknowledgements

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Table 4.1 Indices of carnitine pool composition

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

Table 4.2 Study population demographic data

Patient demographic and dialysis data. Data expressed as mean ± standard deviation. ERI = erythropoietin resistance index.

Parameter	All Patients	Low ERI Patients	High ERI Patients
Subjects (n)	87	75	12
Age (years)	59.1 ± 13.8	58.6 ± 13.8	61.8 ± 14.2
Gender	56 Males / 31 Females	53 Males / 22 Females	3 Males / 9 Females
Dialysis Age (years)	2.78 ± 2.72	2.47 ± 2.04	4.70 ± 5.02
Dialysis Session Duration (hours)	4.10 ± 0.461	4.09 ± 0.452	4.17 ± 0.531

Table 4.3 Endogenous plasma carnitine pool composition and erythropoietin requirements

Endogenous plasma carnitine levels for all subjects and each erythropoietin resistance index (ERI) subgroup. Data expressed as mean ± standard deviation. † $p<0.05$ compared to the Low ERI group.

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

Figure 4.1 Endogenous plasma L-carnitine concentrations and erythropoietin requirements

Relationship between endogenous plasma L-carnitine concentrations and erythropoietin resistance index (ERI). ○ Low ERI patients; ● High ERI patients; p = 0.0421; R = -0.2184.

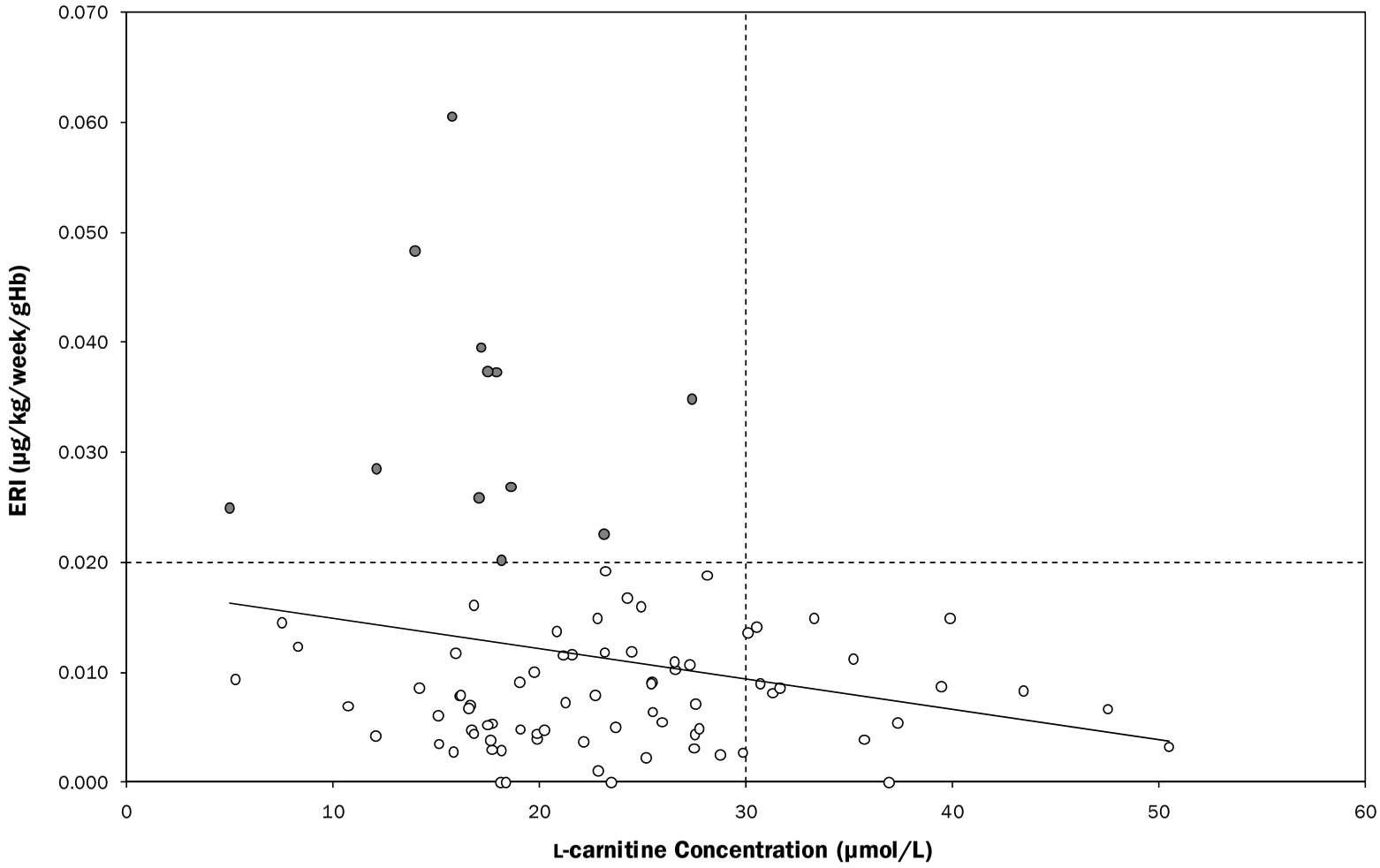
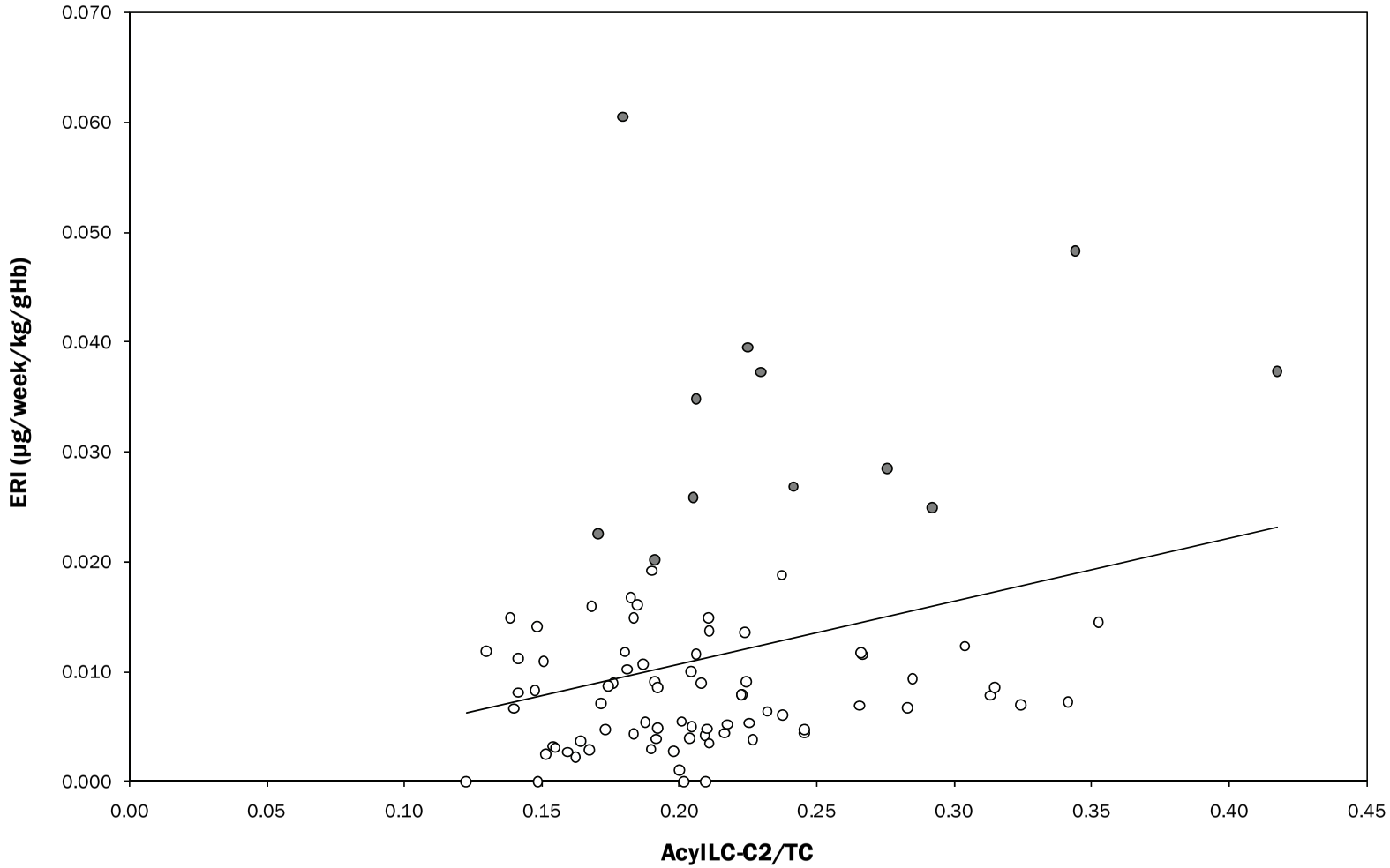


Figure 4.2 Endogenous plasma carnitine pool composition and erythropoietin requirements

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; p = 0.0062; R = +0.2911.



CHAPTER 5

Assessment of Muscle Fatigue

Stephanie E Reuter, Nicola Massy-Westropp, Allan M Evans

Reliability and Validity of Indices of Hand-Grip Strength and Endurance

Australian Occupational Therapy Journal 2011; Volume 58(2): Pages 82 - 87

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

In light of carnitine's critical role in muscle energy utilisation, an association between carnitine pool composition and muscle functionality is proposed. Given the practical limitations in conducting traditional treadmill or bicycle-based tests in haemodialysis patients, alternate assessments of muscle function are needed. The intention of this study was to develop an assessment tool which could be utilised in haemodialysis patients for the purposes of examining the relationship between carnitine status and muscle functionality; these results are presented in Chapter 6.

The study presented in this chapter was able to demonstrate excellent test-retest reliability of the developed hand-grip dynamometry test protocol and its validity in relation to distance walked during the six-minute walk test, a standardised exercise capacity test. The developed hand-grip fatigue test was therefore considered suitable for use in a clinical setting.

5.1 Summary

Grip strength is useful in clinical practice for the assessment of disease and/or rehabilitation progression. Brief maximal gripping is seldom required in everyday occupations, with repeated or sustained gripping at sub-maximal power more commonly involved. It has been proposed that assessment of both maximal hand-grip force and endurance is utilised. While the suitability of maximal contraction measures has been clearly established, the reliability and validity of other hand-grip indices have not been investigated. This study examined the reliability of various hand-grip indices and their validity in relation to distance walked during the six-minute walk test, a standardised exercise capacity test. Subjects undertook static sub-maximal (50%) and maximal force contraction hand-grip testing from which various indices were derived, and six-minute walk testing from which distance walked was determined. Testing was repeated on three separate occasions for determination of test-retest reliability. Pre- and post-fatigue maximal contraction measurements demonstrated excellent test-retest reliability and validity. Conversely, other hand-grip indices were shown to be unreliable and exhibited no relationship with distance walked hence concurrent validity could not be established. Based on the results of this study, it is recommended that pre- and post-fatigue maximal contraction may be utilised for the assessment of client ability and progression due to their established validity and test-retest reliability. However, previously proposed measures of fatigue such as endurance (duration of sustained contraction), strength decrement index and work performed (function of endurance and force of contraction) are unreliable and invalid and may have limited use in clinical practice.

5.2 Introduction

There is an abundance of daily activities for which some degree of hand-grip strength is necessary for a successful outcome. Hand strength is often affected by injury or disease and therefore measurement of grip strength is critical for occupational therapists and other health professionals for assessment of disease progress and progression in rehabilitation [1, 2]. Hand-grip dynamometry is a useful assessment tool for multiple purposes in clinical practice, including 'the assessment of upper limb impairment; in evaluating work capacity for those with hand injuries and other work related injuries; the evaluation of people with other impairments and disabilities such as rheumatoid arthritis, chronic fatigue syndrome, developmental disabilities, muscular dystrophy and stroke; determining the efficacy of different treatments for people with a range of disabilities; part of an overall fitness assessment; and in determining the level of effort exerted' [2]. In addition, assessments of grip strength have been shown to be strongly related to functional status and other health conditions such as osteoporosis in a range of patient groups [3 - 7].

In light of the importance of grip ability for the monitoring of patient outcomes in practice, it is critical that the reliability and validity of these test protocols are established. Research has clearly established that the assessment of maximal contraction by hand-grip dynamometry is appropriate for use in healthy subjects [1, 8] and in those with neurological conditions [1, 9, 10]. The assessment of the fatigue of maximal hand-grip has also proven reliable in healthy study participants [11]. However, the majority of occupations require the use of sustained or repeated gripping at less than maximal forces and thus it has been proposed that both maximal hand-grip force and endurance are utilised in clinical practice for assessment of patient progress [12]. This therefore raises the need for reliable measurement protocols and normative data. Past studies have assessed hand-grip endurance utilising a range of protocols and indices. A majority of sub-maximal (20 - 80%) static hand-grip endurance tests previously conducted have relied on the duration of contraction as an indicator of hand-grip fatigability [13 - 16]. Similarly, duration has been used in sub-maximal tests of other muscle groups [16, 17], as well as in maximal hand-grip tests [12, 14]. Previous studies have also suggested that strength decrement index (SDI), calculated as the deterioration of strength as a proportion of initial values, is a useful assessment of muscle fatigue [18]. Despite the wide use of the duration of contraction and SDI as indicators of fatigue, the reliability of these measures has not been reported. The aim of this study was to examine the test-retest reliability of indices of sub-maximal, static hand-grip testing in healthy adult subjects.

Given that grip strength is not only a direct measure of hand skeletal muscle strength but also an index of overall muscle strength, endurance and ability, an association between peripheral strength and exercise capacity has been proposed [3, 4, 19]. The six-minute walk test (6MWT) is a standardised test for the assessment of exercise capacity. It was primarily developed for the assessment of functionality in patients with cardiac or respiratory disease; however, its use has extended to other patient groups [19]. It has been established as superior to subjective patient self-reporting tools and safer, easier to administer and better tolerated than other exercise capacity tests [19, 20]. Distance walked during the 6MWT has been shown to strongly correlate with exercise capacity determined by maximal cardiopulmonary exercise testing and is strongly related to patient mortality in patients with primary pulmonary hypertension [21]. Studies have also demonstrated that peripheral muscle strength, including maximal hand-grip strength, is an important predictor of distance walked during the 6MWT. Enright et al. [19] have demonstrated a significant ($p<0.003$) correlation between maximal grip strength and distance walked during the 6MWT in 2281 adults aged ≥68 years. Similar associations between peripheral strength and results of the 6MWT have been reported in patients with chronic obstructive pulmonary disease [3, 4]. Although the concurrent validity of maximal grip strength and distance walked during the 6MWT has been previously established, this has not been examined for other indices of hand-grip strength and endurance. The secondary aim of this study was to determine the concurrent validity of sub-maximal, static hand-grip testing in healthy adult subjects in relation to results of the 6MWT.

5.3 Methods

This study was reviewed and approved by the Human Research Ethics Committee of the University of South Australia. Participants were fully informed of the study procedures and provided written informed consent prior to study initiation. The study was conducted in accordance with the study protocol, the Declaration of Helsinki and the National Statement of Ethical Conduct in Human Research issued by the National Health and Medical Research Council (Australia).

Healthy adult subjects underwent hand-grip testing and the 6MWT according to the protocols outlined below. Testing was performed on three separate occasions (separated by at least 24 hours) at a similar time of day, by the same assessor for determination of test-retest reliability. In order to reduce bias and possible effects on motivation, subjects were not informed of their progress during any of the testing procedures.

5.3.1 Hand-Grip Test

Hand-grip was assessed using a Smedley Hand Dynamometer (Stoelting Co., Wood Dale, IL, USA) modified with grip cushioning. Subjects were seated in an upright position with their shoulder adducted and elbow flexed at 90°, the forearm allowed to rest lightly on the arm of the chair or on the subject's thigh. For each subject, maximal voluntary contraction was determined as the highest of three contractions (INITIAL MAX)[22]. After a 1 minute rest period[23], subjects were instructed to squeeze the dynamometer in a static contraction at a level of 50% maximal for as long as possible, the duration of which was recorded (DURATION). The level of static contraction was monitored by the assessor who provided verbal feedback to ensure a level of 50% maximal was maintained. Subjects were then required to perform an additional post-fatigue maximal contraction within 10 seconds of completion of the 50% maximal static contraction (FINAL MAX). Hand-grip testing was performed on both the dominant and non-dominant hands for each subject.

5.3.2 Six-Minute Walk Test

The 6MWT was conducted according to the American Thoracic Society Guidelines for the Six-Minute Walk Test[24]. Briefly, subjects were instructed to walk as far as possible for 6 minutes on a flat, non-undulating surface. Participants were directed to walk in a circuit between two markers spaced 30 metres apart. The total distance walked during the six minute testing period was recorded (DISTANCE).

5.3.3 Statistical Methods

Indices of hand-grip testing were calculated for each subject, on each testing day, for both the dominant and non-dominant hands. Indices included:

- Pre-fatigue maximal contraction (INITIAL MAX)
- Post-fatigue maximal contraction (FINAL MAX)
- Duration of 50% maximal static contraction (DURATION)
- Work performed (WORK = DURATION x 50% INITIAL MAX)
- Strength decrement index (SDI = 100% x (INITIAL MAX – FINAL MAX)/INITIAL MAX)

Reliability of indices was calculated as the single measures intraclass correlation coefficient (ICC) of Day 1 – 3 data for which an ICC value of ≥0.9 is classified as 'excellent'; ≥0.7 is 'acceptable'; and ≤0.7 indicates that the measurement system is inadequate. In order to determine the presence of a training effect, an analysis of variance (ANOVA) was used to compare test results on each of the assessment days. Concurrent validity was investigated as the relationship between hand-grip indices and distance walked during the 6MWT using linear regression.

WinNonlin® Professional Version 5.2 (Pharsight Corporation, Mountain View, CA, USA) and SPSS Version 16.0 (SPSS Inc., Chicago, IL, USA) were used for the conduct of statistical analyses. Significance was set at an α-level of 0.05.

5.4 Results

Twenty-one healthy adult subjects (12 men, nine women) were recruited for study participation, of whom 16 underwent both testing protocols on three separate occasions for determination of test-retest reliability. The mean ± standard deviation (SD) age of the subjects was 29.8 ± 6.9 years. All subjects were free of significant illnesses, which would impact on the study results.

Mean ± SD INITIAL MAX values were 34 ± 8.2 kg and 33 ± 10 kg for the dominant and non-dominant hands respectively. Comparably, FINAL MAX values were 26 ± 7.2 kg after sub-maximal static contraction for 93 ± 32 seconds using the dominant hand and 24 ± 8.6 kg after contraction for 85 ± 25 seconds using the non-dominant hand. Mean ± SD DISTANCE values were 651 ± 77.5 m.

Test-retest reliability values for each parameter are presented in Table 5.1. Values for INITIAL MAX and FINAL MAX data were excellent (ICC >0.9), and were comparable to that obtained for the distance walked during the 6MWT (ICC = 0.819). Test-retest reliability values for the other indices measured were poorer, in particular for SDI and DURATION (Table 5.1).

Test parameters obtained on each study day were statistically compared in order to determine the presence of a training effect. No significant between-day differences were observed in any of the hand-grip indices or in the results of the 6MWT. Results of the ANOVA are presented in Table 5.1.

In the statistical correlation of calculated indices of hand-grip and results of the 6MWT, there was a highly significant correlation between DISTANCE and INITIAL MAX for both the

dominant and non-dominant hands ($p = 0.017$ and $p = 0.016$, respectively), as well as DISTANCE and FINAL MAX for the dominant and non-dominant hands ($p = 0.003$ and $p = 0.007$, respectively) (Figure 5.1). No significant correlations were found for the other hand-grip indices. Linear regression results for each index are presented in Table 5.1.

5.5 Discussion

The use of hand-grip dynamometry has extended beyond the assessment of maximal voluntary contraction alone and has also been used for the assessment of grip endurance to gain a better overall picture of patient functionality [12 - 16]. While the suitability of maximal grip strength measurements has been clearly established [1, 8 - 11], the reliability and validity of these additional hand-grip assessments have not been reported. Consequently, this study aimed to establish the reliability of various indices of hand-grip strength and endurance and their concurrent validity in relation to distance walked during the 6MWT, a standardised exercise capacity test.

Maximal hand-grip contraction (INITIAL MAX) values and results of the 6MWT obtained in this study were comparable with age- and gender-specific normative values previously reported for healthy adults [8, 20, 21]. These parameters showed excellent test-retest reliability, which is in keeping with the results of previous research in healthy volunteers [1, 8, 11] and patients with neurological conditions [1, 9, 10]. A highly statistically significant correlation between the distance walked during the 6MWT (DISTANCE) and maximal force contraction (INITIAL MAX) was observed for both the dominant and non-dominant hands, consistent with the findings of previous studies in chronic obstructive pulmonary disease patients [3, 4] and in elderly adults [19]. This is also consistent with previous studies, which have demonstrated that maximal hand-grip strength is strongly related, even predictive of other physical characteristics of concern to occupational therapists and health professionals, for example, disability and frailty in clients aged above 65 years [6], bone mineral density [5], the risk of falls [7] and nutritional status [25]. These findings support the use of maximal hand-grip force in clinical practice and research as an indicator of patient status and progression.

For the first time, this study has also investigated the reliability and validity of other measures of hand-grip strength and endurance. Static contraction at a sub-maximal level (50%) until fatigue was found to result in an approximate 25% reduction in maximal grip force (FINAL MAX). Similar to pre-fatigue maximal contraction, these post-fatigue maximal contraction values were shown to have excellent test-retest reliability (ICC >0.9) and correlated well with results of the 6MWT ($p<0.01$) indicating its suitability for monitoring patient outcomes in practice and a research setting. In addition, statistical

comparisons of FINAL MAX data on each of the assessment days indicated the lack of a learning effect, suggesting that this parameter may be used for both single point evaluations as well as in longitudinal assessments.

Conversely, other previously proposed indices of hand-grip fatigability such as DURATION, WORK and SDI demonstrated poor test-retest reliability and concurrent validity of these measures against the 6MWT was unable to be established. As a result, these parameters cannot be recommended for clinical use. Furthermore, previous research has commonly utilised these measures as indicators of fatigability[12-17], particularly duration of contraction, and thus results of these studies may need to be interpreted with caution.

Based on the results of this study, it is recommended that pre- and post-fatigue maximal contraction may be utilised individually or in combination for the assessment of client ability and progression because of their established validity and test-retest reliability. However, previously proposed measures of fatigue such as endurance (duration of sustained contraction), strength decrement index and work performed (function of endurance and force of contraction) have been shown to be unreliable and invalid and therefore may have limited use in clinical practice and research.

In addition, the highly significant correlations between pre- and post-fatigue maximal contraction and distance walked during the 6MWT observed in this study suggest that these indices of grip strength are predictive of exercise capacity. The use of these hand-grip measurements as an indicator of exercise capacity may be particularly useful for assessment of patient groups for whom performing the 6MWT or traditional treadmill or bicycle-based tests is not feasible due to limited mobility or lack of a suitable test environment; however, this warrants further investigation.

5.6 Acknowledgements

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5.7 References

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Table 5.1 Reliability and validity of indices of hand-grip strength and endurance

Results of the test-retest reliability (intraclass correlation coefficient (ICC) of Day 1 - 3 data) and the determination of a training effect (ANOVA of Day 1 - 3 data) for each parameter measured, as well as the examination of the relationship between hand-grip indices and distance walked during the six-minute walk test (linear regression).

Index	Reliability	Training Effect	Regression (DISTANCE vs Index)	
	ICC	p Value	p Value	R^2
Hand-Grip Test (Dominant Hand)				
INITIAL MAX	0.924	0.928	0.017	0.265
FINAL MAX	0.924	0.942	0.003	0.374
DURATION	0.582	0.992	0.455	0.030
WORK	0.593	0.823	0.117	0.124
SDI	0.465	0.839	0.114	0.126
Hand-Grip Test (Non-Dominant Hand)				
INITIAL MAX	0.967	0.983	0.016	0.271
FINAL MAX	0.947	0.919	0.007	0.328
DURATION	0.572	0.473	0.972	0.0001
WORK	0.753	0.644	0.143	0.110
SDI	0.408	0.683	0.196	0.087
Six-Minute Walk Test				
DISTANCE	0.819	0.567	-	-

Figure 5.1 Post-fatigue maximal hand-grip contraction and distance walked during the six-minute walk test

Relationship between post-fatigue maximal hand-grip contraction (FINAL MAX) for the dominant (a) and non-dominant (b) hands and distance walked during the six-minute walk test (DISTANCE).

Figure 5.1a: p = 0.003; R^2 = 0.374

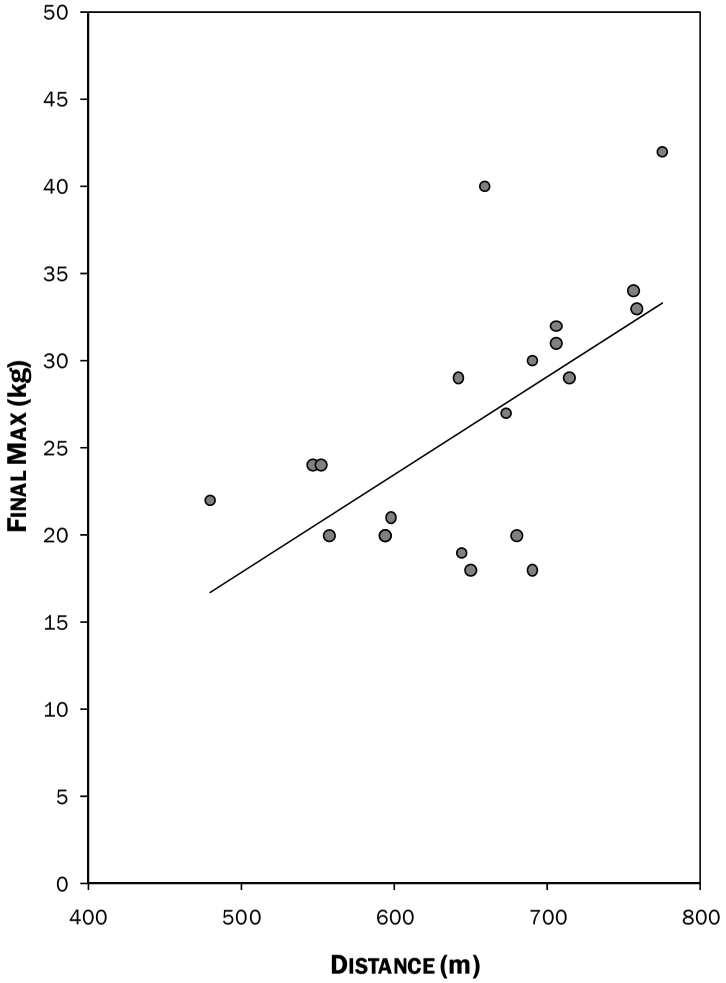
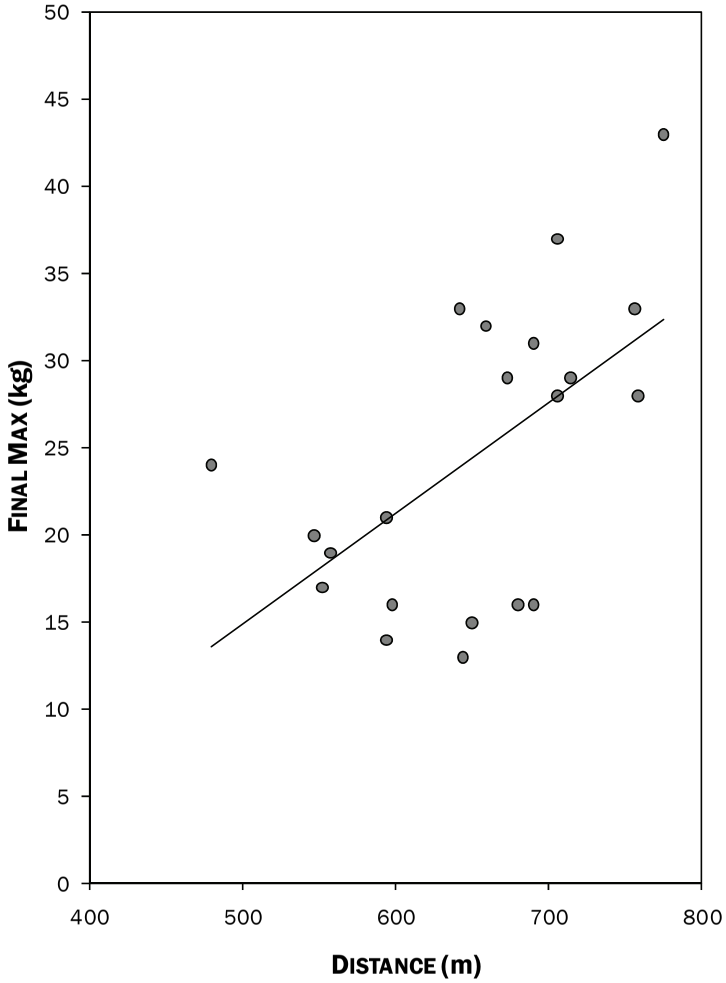


Figure 5.1b: p = 0.007; R^2 = 0.328



CHAPTER 6

Carnitine and Functionality in Haemodialysis Patients

Stephanie E Reuter, Randall J Faull, Allan M Evans

Patient Functionality in Chronic Haemodialysis Patients: The Importance of Carnitine Pool Composition

Nephrology Dialysis Transplantation; Submitted

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87..
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

The relationship between disturbances in the composition of the endogenous plasma carnitine pool in haemodialysis patients (as established in Chapter 3) and skeletal and cardiac muscle functionality in this population is examined in this chapter. For this, skeletal muscle fatigue was assessed utilising the developed methodology presented in Chapter 5.

This study demonstrated that patients with muscle dysfunctionality (i.e. muscle fatigue, muscle cramps, cardiac dysfunction and/or intradialytic hypotension) exhibit a significantly more disturbed carnitine profile than non-symptomatic patients, with a greater proportion of acylcarnitines, in particular medium- and long-chain acylcarnitines, as part of the total plasma carnitine pool. It is conceivable that this disruption in carnitine pool composition may alter carnitine palmitoyltransferase activity that could lead to an impairment in fatty acid metabolism, which in-turn would contribute to dialysis-related muscle symptomology.

6.1 Summary

Chronic haemodialysis patients exhibit an altered endogenous carnitine profile with a significant reduction in L-carnitine levels and accumulation of acylcarnitines. This cross-sectional study was conducted to investigate the relationship between the composition of the endogenous plasma carnitine pool and skeletal and cardiac muscle functionality in chronic haemodialysis patients not receiving L-carnitine supplementation. For the first time, the association between the incidence of dialysis-related disorders and a full complement of indices of carnitine pool composition has been investigated. Forty-nine patients undergoing stable, long-term haemodialysis treatment were recruited from the Department of Renal Medicine, Royal Adelaide Hospital and its associated satellite dialysis centres. Patients were assessed for objective and subjective measures of skeletal muscle functionality, cardiac dysfunction, incidence of intradialytic hypotension and plasma carnitine profiling. This study demonstrated that patients with skeletal and/or cardiac muscle dysfunctionality, specifically muscle fatigue, muscle cramps, cardiac dysfunction and intradialytic hypotension, exhibit a significantly more disturbed carnitine profile than non-symptomatic patients. In all cases, symptomology was associated with alterations in the proportion of L-carnitine and acylcarnitines, in particular medium- and long-chain acylcarnitines, as part of the total plasma carnitine pool rather than differences in the absolute carnitine levels. These findings suggest that the relative composition of the endogenous carnitine pool may be more important than the levels of carnitines alone. It is conceivable that this disruption in carnitine pool composition may alter carnitine palmitoyltransferase activity which could lead to an impairment in fatty acid metabolism which would in-turn contribute to dialysis-related muscle symptomology.

6.2 Introduction

Dialysis-related conditions, such as muscle fatigue, cramps, intradialytic hypotension, restless legs, exercise intolerance and cardiac dysfunction, are commonly experienced by chronic haemodialysis patients [1, 2]. These symptoms are life-altering, and in many cases debilitating, for the numerous haemodialysis patients who experience them regularly and can have a significant impact on quality-of-life [3]. A reduction in the perceived quality-of-life has been shown to be associated with poor compliance with dialysis treatment and medication use which can in-turn result in a further decline in health [3]. Studies have established that a better perceived quality-of-life is associated with a significant reduction in morbidity and mortality [4].

L-carnitine is an endogenous compound critical for the transfer of fatty acids across the inner mitochondrial membrane for fatty acid oxidation via the reversible binding of acyl groups from coenzyme A (CoA) (Figure 6.1) [5, 6]. It has also been established that carnitine has a role in regulating the cellular to mitochondrial ratio of free CoA to acyl-CoA, in the transport of short and medium acyl groups from the peroxisome to the mitochondria and in the removal of excess acyl groups from the body [5, 6]. In healthy individuals, endogenous plasma and tissue levels of L-carnitine and acylcarnitines remain relatively constant due to homeostatic control mechanisms, predominantly mediated by the kidney. Haemodialysis, whilst being an invaluable substitute for the excretory role of the human kidney, lacks the control mechanisms necessary for carnitine homeostasis and consequently, chronic haemodialysis patients exhibit an altered endogenous carnitine profile with a significant reduction in L-carnitine levels and accumulation of acylcarnitines [7 - 12].

Carnitine deficiency has been implicated as a contributing factor in the incidence and/or severity of common dialysis-related conditions. A number of studies have investigated the effect of L-carnitine supplementation on dialytic symptoms; however, in most cases these studies are poorly designed and inadequately controlled. Of the studies that have been conducted, some have demonstrated a significant improvement in dialytic symptoms and associated quality-of-life with L-carnitine supplementation; whereas others have shown no effect (reviewed by Miller & Ahmad [13] and Reuter et al. [14]). In one study, although no overall improvement in the severity of dialytic symptoms was observed, the investigators noted that 4 of the 7 patients treated with L-carnitine improved substantially [15]. Based on this observation, the authors proposed that the haemodialysis population is divided into two subgroups of patients: 'responders' and 'non-responders' to L-carnitine supplementation; however, no distinction between these two groups has been proposed. Previous research has reported that patients

experiencing dialysis-related conditions, including muscle fatigue, cramps and intradialytic hypotensive events, exhibit significantly lower endogenous L-carnitine and/or higher acylcarnitines levels than their non-symptomatic counterparts[16, 17]. Consequently, it is suggested that responsiveness to L-carnitine supplementation may be related to a person's carnitine profile, in particular, disturbances in endogenous carnitine esters.

In order to investigate this further, this study was conducted to investigate the relationship between the composition of the endogenous plasma carnitine pool and skeletal and cardiac muscle functionality in chronic haemodialysis patients not receiving L-carnitine supplementation. For the first time, the association between the incidence of dialysis-related conditions and a full complement of indices of carnitine pool composition has been investigated.

6.3 Methods

This study was reviewed and approved by the institutional human research ethics committees. Participants 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 patients undergoing chronic haemodialysis were recruited for study participation from the Department of Renal Medicine, Royal Adelaide Hospital and its associated satellite dialysis centres. Patients were required to be stable on long-term haemodialysis treatment and to have not received carnitine supplementation within the 2 months prior to the study.

Patients were assessed for skeletal muscle functionality, cardiac dysfunction, incidence of intradialytic hypotension and plasma carnitine profiling according to the protocols outlined below.

6.3.1 Skeletal Muscle Function

Skeletal muscle functionality was assessed using an objective measure of skeletal muscle fatigue as well as subjective measures of the incidence of muscle cramps, asthenia and restless legs, as well as exercise tolerance.

Skeletal muscle fatigue was objectively assessed using a hand-grip dynamometer method which was developed and validated in-house [18]. The level of the maximum contraction after fatigue (50% maximum static contraction until exhaustion) was taken as an indicator of skeletal muscle fatigue. In preliminary studies, post-fatigue maximum contraction has been shown to be a reliable indicator of hand-grip fatigue (intraclass correlation coefficient ≥0.9), and was show to correlate well with results of standardised exercise capacity testing ($p<0.01$) [18]. A cut-off value of 20 kg corresponds to the 25th percentile of results observed in a healthy population [18] and thus patients with a post-fatigue recording of <20kg were considered to have 'high fatigability' whereas patients with values ≥20kg were classified as having 'normal fatigability'. Skeletal muscle fatigue testing was conducted prior to the commencement of the haemodialysis session.

Patients were asked to subjectively assess the incidence of muscle cramps, restless legs and asthenia. Incidence was recorded as the average frequency of symptoms over a 1 month period. Patients who reported ≥1 episode were considered to be 'symptomatic'; patients who reported no symptoms were considered to be 'non-symptomatic'.

Exercise tolerance was assessed using a self-reporting scale questionnaire in which patients were asked to assess their ability to walk on a level surface and an incline. Results of the questionnaire have previously been shown to correlate well with exercise tolerability determined using treadmill assessment methods [19]. Patients who had a rating less than normal (<11, i.e. <6 for level and/or <5 for incline) were considered to have a compromised exercise tolerance ('poor exercise tolerance'); patients with a normal rating were considered to have 'normal exercise tolerance' [19].

6.3.2 Cardiac Function

Cardiac function was assessed by a physician using the New York Heart Association (NYHA) classification. Patients assessed as Class II - IV were considered to be 'symptomatic'; patients classified as Class 0 - I were considered 'non-symptomatic'.

6.3.3 Intradialytic Hypotension

Intradialytic hypotension was defined as 'a decrease in systolic blood pressure by ≥ 20 mmHg or a decrease in mean arterial pressure by 10 mmHg

associated with symptoms that include: abdominal discomfort; yawning; sighing; nausea; vomiting; muscle cramps; restlessness; dizziness or fainting; and anxiety' [20]. Intradialytic hypotension was subjectively assessed as the average incidence over a 1 month period, whether or not the event was prevented by intravenous infusion of normal saline or colloidal solution. Patients who reported ≥1 episode were considered to be 'symptomatic'; patients who reported no symptoms were considered to be 'non-symptomatic'.

6.3.4 Plasma Carnitine Profile

An arterial blood sample was collected from the arteriovenous fistula of each patient immediately prior to commencement of a single haemodialysis session.

Plasma samples were analysed for L-carnitine and individual acylcarnitine concentrations by the Department of Genetic Medicine, Women's and Children's Hospital (Adelaide, SA, Australia). Analysis was conducted using a 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 (synthesised in-house). After a 15 minute 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 precursor scan of 85.1 amu. The levels of acylcarnitines were determined against the respective deuterated stable isotope using Analyst® software (Applied Biosystems Inc, Foster City, CA, USA).

As indicators of carnitine pool composition, the parameters outlined in Table 6.1 were calculated using quantified plasma carnitine concentrations.

6.3.5 Statistical Methods

Data are expressed as mean ± standard deviation, unless otherwise indicated. Statistical comparisons of carnitine parameters between groups were

conducted using analysis of variance (ANOVA). Significance was set at an α-level of 0.05. WinNonlin® Professional Version 5.2 (Pharsight Corporation, Mountain View, CA, USA) was used for ANOVA analysis.

6.4 Results

A total of 49 patients (34 male, 15 female) undergoing chronic haemodialysis treatment participated in the study. Patients were aged 61.0 ± 13.1 years and had been undergoing haemodialysis treatment for 2.42 ± 2.11 years. Dialysis treatment was administered 3 - 4 times per week, for a duration of 4.1 ± 0.34 hours. In all cases, dialysis treatment was considered to be adequate (Kt/V Urea 1.5 ± 0.19).

In the entire study population, endogenous plasma L-carnitine and total carnitine concentrations were 21.3 ± 6.50 μmol/L and 34.5 ± 7.97 μmol/L, respectively. Total acylcarnitine and non-acetyl acylcarnitine concentrations were 13.2 ± 2.13 μmol/L and 6.71 ± 1.14 μmol/L, respectively.

6.4.1 Skeletal Muscle Function

Carnitine pool composition data for each skeletal muscle function index subgroup are presented in Tables 6.2a – 6.2e. Statistical comparisons of carnitine data between the skeletal muscle fatigue test subgroups indicated that patients classified as having a 'high fatigability' had significantly lower L-carnitine levels and a lower proportion of L-carnitine and a higher proportion of acylcarnitines and non-acetyl acylcarnitines within the total plasma carnitine pool than patients with 'normal fatigability' (Table 6.2a). In addition, patients who reported experiencing muscle cramps had significantly higher levels of non-acetyl acylcarnitines and a higher proportion of non-acetyl acylcarnitines within the total plasma carnitine pool than non-symptomatic patients (Table 6.2c). There were no significant differences in carnitine parameters between the patient subgroups for the subjective assessments of asthenia, restless legs and exercise tolerance (Tables 6.2b, 6.2d and 6.2e, respectively). There were no significant differences in the duration of dialysis treatment (dialysis age) between the subgroups.

6.4.2 Cardiac Function

Patients who were considered to be symptomatic according to the NYHA classification had a significantly lower proportion of L-carnitine and a higher

proportion of acylcarnitines within the total plasma carnitine pool than asymptomatic patients (Table 6.3). Comparison of AcylLC-C2/TC values between the subgroups was of borderline significance ($p = 0.0521$). There was no significant difference in dialysis age between the symptomatic and asymptomatic patient groups.

6.4.3 Intradialytic Hypotension

Statistical comparisons of carnitine data indicated that patients who reported experiencing intradialytic hypotension had a significantly lower proportion of L-carnitine and a higher proportion of acylcarnitines within the total plasma carnitine pool than non-symptomatic patients (Table 6.4). There was no significant difference in dialysis age between patients who experienced intradialytic hypotension and those who did not.

6.5 Discussion

Skeletal and cardiac muscle metabolism is predominantly dependent on fatty acid oxidation and therefore requires delivery of acyl moieties into the mitochondria; a step which is dependent on the presence of L-carnitine [21]. Chronic haemodialysis is associated with alterations in the composition of the endogenous carnitine pool, with a reduction in L-carnitine levels and accumulation of non-acetyl acylcarnitines [7 - 12]. It has therefore been proposed that these alterations in the endogenous carnitine pool may contribute to skeletal muscle and cardiac dysfunction commonly experienced in this patient population. Consequently, supplementation with L-carnitine could conceivably result in an improvement in certain dialysis-related conditions by increasing the transport of free fatty acids into the mitochondria, thereby increasing the availability of energy substrates.

A number of studies have investigated the efficacy of L-carnitine supplementation for the treatment of these dialysis-related disorders; however, there is a lack of consistency in the findings and no clear conclusions have been made [13, 14]. Studies have reported that the patient population appears to be divided into groups of 'responders' and 'non-responders' to L-carnitine treatment [15], although there appears to be no clear distinction between these two groups. Recent research has suggested that the extent of effectiveness of L-carnitine supplementation for long-term haemodialysis patients may be related to their endogenous plasma carnitine profile which may explain the disparity in the findings of previous studies [22]. The current study has therefore been conducted to examine the relationship between carnitine pool composition and various indices of

patient functionality in order to determine if patients with a more severe symptomology exhibit a more disturbed carnitine protocol than non-symptomatic patients.

In this study, patients exhibited considerable alterations in carnitine homeostasis compared to levels previously reported for healthy subjects using the same analytical method [11, 23], with lower endogenous plasma levels of L-carnitine (21.3 ± 6.50 µmol/L c.f. 41.2 ± 10.1 µmol/L) and total carnitine (34.5 ± 7.97 µmol/L c.f. 49.9 ± 11.0 µmol/L) and accumulation of acylcarnitines (13.2 ± 2.13 µmol/L c.f. 8.67 ± 2.38 µmol/L). Consequently, the total endogenous plasma carnitine pool for these patients comprises of a lower proportion of L-carnitine and a higher proportion of acylcarnitines, in particular medium- and long-chain acylcarnitines; in fact, the proportion of non-acetyl acylcarnitines within the total carnitine pool for these patients is approximately 20%, much higher than that observed in the healthy population (~5%). These findings are consistent with those previously reported for patients undergoing long-term haemodialysis treatment [9, 11, 12, 24 - 28].

6.5.1 Skeletal Muscle Function

It is well recognised that haemodialysis patients commonly experience muscular symptoms, such as fatigue, muscle cramps, restless legs, asthenia and reduced exercise tolerance. Whilst, in some cases, these symptoms can be attributed to an inadequate dialysis prescription, thyroid function abnormalities and/or depression, for some patients the underlying cause of their muscular symptoms cannot be established. In light of L-carnitine's critical role in muscle fatty acid metabolism, it has been suggested that carnitine deficiency may also contribute to dialysis-related muscle symptomology.

Numerous studies have investigated the impact of L-carnitine supplementation on skeletal muscle function, using both objective and subjective measures, with varying results. Bellinghieri et al. [29] reported that L-carnitine supplementation (2 g/day p.o. for 60 days) resulted in significant improvements in muscle fatigue assessed using knee flexor exercise and stair climbing measurements. Similarly, using self-reporting methods, studies have also demonstrated a significant improvement in asthenia with L-carnitine treatment [17, 30 - 34]. Conversely, Fagher et al. [35] described no effect on maximum dynamic strength and dynamic endurance of knee extensor muscles with carnitine supplementation compared with placebo treatment. A similar lack of effect of L-carnitine supplementation on objective measurements of muscle fatigue was reported by Rogerson et al. [36]. In the present study, patients

classified as having high skeletal muscle fatigability using the hand-grip fatigue test were shown to have a significantly more disturbed carnitine profile, with reduced L-carnitine levels and higher acylcarnitine levels (in particular non-acetyl acylcarnitines) as a proportion of the total plasma carnitine pool, than patients who were classified as having normal fatigability. This finding is supported by those of Sakurauchi et al.[17], who reported that haemodialysis patients experiencing fatigue had significantly lower levels of L-carnitine and a higher proportion of acylcarnitines relative to L-carnitine than non-symptomatic patients.

Whilst the current study has demonstrated that, using objective measurements, high muscle fatigability is associated with a compromised carnitine profile, the same is not true for subjective assessment of fatigue. In the present study, there were no significant differences in endogenous carnitine pool composition between patients who classified themselves as experiencing general fatigue and those who did not. The lack of significance in the current study may be explained by the highly subjective nature of the fatigue assessment and that some patients may not considered themselves to suffer from fatigue when in fact they would be classified as such by other methods. In fact, examination of the data from the present study reveals that only half of the patients who were classified as having high fatigability using the objective hand-grip test considered themselves to suffer from fatigue. Consequently, it is difficult to draw any firm conclusions from these subjective results.

The incidence of muscle cramps has also been shown to be reduced with L-carnitine supplementation. Ahmad et al.[37] reported a drop in incidence from 36% of patients to only 13% after 2 months of L-carnitine supplementation, with no significant changes in the incidence of cramps in placebo-treated patients. Similar improvements in the incidence of muscle cramps with L-carnitine supplementation have been reported by others[17, 29, 31 - 33]; however, the finding has not been consistently found across all studies[35]. A recent meta-analysis was unable to confirm the benefits of L-carnitine supplementation for the treatment of muscle cramps and has indicated the need for further well-designed studies[38]. The present study demonstrated that patients who experience muscle cramps have significantly higher non-acetyl acylcarnitine levels and a higher proportion of non-acetyl acylcarnitines within the total carnitine pool than non-symptomatic patients. This finding is supported by the results of Sakurauchi et al.[17], in which it was demonstrated

that patient who experience muscle cramps have a significantly more disturbed carnitine profile (lower L-carnitine levels and higher AcylLC/LC ratio) than non-symptomatic patients. The incidence of restless legs was not associated with differences in carnitine pool composition in the present study, a finding which has not been previously demonstrated.

Studies have also examined the impact of L-carnitine administration on exercise tolerance in chronic haemodialysis studies. Ahmad et al.[37] noted a 10% improvement in VO_{2max} in L-carnitine treated haemodialysis patients, compared to only a 1% change (not significant) in placebo treated patients. On the other hand, Siami et al.[15] reported no significant improvement in subjective assessment of exercise tolerance in patients supplemented with L-carnitine compared to placebo treated patients. Although in the latter study the authors noted that there was a clear cluster of 4/7 patients who improved substantially with L-carnitine treatment, and therefore suggested that the population is divided into 'responders' and 'non-responders'. A positive correlation between exercise tolerance and endogenous L-carnitine levels in female (but not male) haemodialysis patients has also been shown[19]. In the current study there were no significant differences in carnitine levels between patients who had compromised exercise tolerance and those who did not.

6.5.2 Cardiac Function

Cardiac disease is a significant problem amongst haemodialysis patients, accounting for approximately half of all deaths in this patient population[39]. Due to its important role in fatty acid oxidation, L-carnitine has been explored as a potential treatment for cardiovascular disease in haemodialysis patients.

The beneficial role of L-carnitine supplementation for the treatment of cardiac dysfunction has been previously reported. Studies examining the impact of long-term L-carnitine supplementation on left ventricular ejection fraction in chronic haemodialysis patients have described a 6 - 10% improvement in left ventricular ejection fraction with treatment[40 - 44]. Similarly, administration of L-carnitine to chronic haemodialysis patients for 4 - 8 weeks resulted in a significant reduction in the incidence and severity of cardiac arrhythmias[45]. On the other hand, studies have also reported no significant improvement in left ventricular ejection fraction[46, 47] or the frequency of cardiac arrhythmias[37] with L-carnitine supplementation.

It has previously been described that there is an association between left ventricular ejection fraction as an indication of cardiac function and endogenous plasma carnitine levels, with haemodialysis patients who have a reduced left ventricular ejection fraction exhibiting lower L-carnitine concentrations than those patients who have high left ventricular ejection fraction values [44]. Although an association between cardiac functionality and absolute L-carnitine levels was not demonstrated in this study, it was shown that patients who experience cardiac dysfunction have a more disturbed carnitine profile, with a lower proportion of L-carnitine and a higher proportion of acylcarnitines within the total carnitine pool, than asymptomatic patients. This may reflect the importance of carnitine pool composition in cardiac function in chronic haemodialysis patients.

6.5.3 Intradialytic Hypotension

Intradialytic hypotension occurs in approximately 25% of all haemodialysis sessions [48] and is associated with impaired patient wellbeing, a predisposition to coronary and/or cerebral ischaemic events and an increased risk of mortality [49, 50]. L-carnitine's involvement in intradialytic hypotension is not clear; however, it has been suggested that L-carnitine supplementation may be beneficial for intradialytic hypotension due to an improvement in vascular smooth muscle and cardiac muscle function.

Administration of L-carnitine (990 mg/day p.o. for 2 months) has been shown to be associated with a significant reduction in the incidence of intradialytic hypotensive events when compared with placebo treatment [31]. Similarly, Ahmad et al. [37] reported a reduction in the incidence of hypotensive events from 44% of patients at baseline compared to 18% after 2 months of intravenous L-carnitine supplementation; no change in the incidence was observed in the placebo-treated group. These results are also supported by the findings of Fujita et al. [51] and Sohn et al. [52]. Based on the findings of these studies the Centers for Medicare and Medicaid Services have approved coverage of intravenous L-carnitine administration for the treatment of dialysis-related hypotension in haemodialysis patients with low endogenous plasma L-carnitine levels. Despite this, the benefit of L-carnitine supplementation for intradialytic hypotension remains a contentious subject; a recent meta-analysis failed to confirm a significant beneficial effect of L-carnitine supplementation on dialysis-related hypotension and has suggested that further research is needed [38].

It has previously been noted that long-term haemodialysis patients who experience hypotensive episodes have lower endogenous levels of L-carnitine than their normotensive counterparts[16]. The present study was unable to confirm this finding, with no significant differences in endogenous L-carnitine levels between symptomatic and non-symptomatic patients observed. However, it was demonstrated that patients who experienced intradialytic hypotension had a significantly lower proportion of L-carnitine and a higher proportion of acylcarnitines as part of the total plasma carnitine pool than asymptomatic patients, suggesting that carnitine pool composition has a role in intradialytic hypotension.

6.5.4 Conclusions

Previous studies, investigating all aspects of skeletal and cardiac muscle functionality, have commonly reported that some patients 'respond' to L-carnitine supplementation, whilst others do not. Similarly, some studies report an overall improvement in symptoms in their patient population with L-carnitine therapy, whilst others fail to not any significant change. The basis for this lack of a consistent response between patients has not been established and treatment is still largely empirical.

This study has demonstrated an association between endogenous carnitine pool composition and indicators of skeletal and cardiac muscle functionality in haemodialysis patients, specifically muscle fatigue, muscle cramps, cardiac dysfunction and intradialytic hypotension. Interestingly, in all cases the incidence in these dialytic symptoms was associated with alterations in the proportion of L-carnitine and acylcarnitines, in particular medium- and long-chain acylcarnitines, as part of the total plasma carnitine pool rather than differences in the absolute levels of these carnitines, thereby suggesting that the relative composition of the endogenous carnitine pool is more important than the levels of carnitines alone. These more complex indicators of carnitine pool composition have not been previously investigated.

Whilst the involvement of carnitine pool composition in the symptomology of these dialysis-related conditions is yet to be elucidated, it is feasible that this impairment of muscle functionality may be a result of alterations in carnitine palmitoyltransferase activity (Figure 6.1). Acylcarnitines, in particular long-chain acylcarnitines, have been shown to inhibit carnitine palmitoyltransferase activity[53], whereas L-carnitine enhances carnitine palmitoyltransferase

activity[54, 55]; accordingly, the accumulation of acylcarnitines and deficiency of L-carnitine (characterised by a low LC/TC ratio and a high AcylLC/TC ratio) commonly observed in this patient population would result in reduced carnitine palmitoyltransferase activity. This reduction in carnitine palmitoyltransferase activity could conceivably lead to an impairment in fatty acid metabolism which would in-turn contribute to dialysis-related muscle symptomology. Conversely, patients who have a lower proportion of acylcarnitines and a higher proportion of L-carnitine within the total plasma carnitine pool would exhibit better carnitine palmitoyltransferase activity due to a better 'balance' between acylcarnitine inhibition and L-carnitine activation and consequently these patients display better muscle functionality. This also suggests that 'responsiveness' to L-carnitine supplementation may be related to a normalisation of carnitine pool composition rather than simply an increase in absolute L-carnitine levels; this may explain the disparity in the findings of previous studies. A randomised, double-blind, placebo-controlled trial investigating the responsiveness to L-carnitine supplementation in long-term haemodialysis patients with altered carnitine pool composition is currently being conducted.

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Table 6.1 Indices of carnitine pool composition

Carnitine profile 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

Table 6.2a Endogenous plasma carnitine pool composition and skeletal muscle fatigue

Skeletal muscle fatigue: Endogenous plasma carnitine levels for patients classified as having normal fatigability (n = 23) and poor fatigability (n = 20) according to the skeletal muscle fatigue hand-grip test. Data expressed as mean ± standard deviation. † $p<0.05$ compared to normal fatigability patients.

Parameter	Normal Fatigability Patients	Poor Fatigability Patients	
LC (µmol/L)	22.7 ± 6.61	18.6 ± 4.62 †	p = 0.0487
TC (µmol/L)	36.2 ± 8.67	31.5 ± 5.92	
AcylLC (µmol/L)	13.5 ± 2.49	12.9 ± 1.84	
AcylLC-C2 (µmol/L)	6.67 ± 1.25	6.62 ± 0.927	
LC/TC	0.620 ± 0.0489	0.587 ± 0.0471 †	p = 0.0309
AcylLC/TC	0.380 ± 0.0489	0.413 ± 0.0471 †	p = 0.0234
AcylLC-C2/TC	0.190 ± 0.0374	0.214 ± 0.0340 †	p = 0.0193

Table 6.2b Endogenous plasma carnitine pool composition and asthenia

Asthenia: Endogenous plasma carnitine levels for patients classified as asymptomatic (n = 30) and symptomatic (n = 14) according to the subjective assessment of asthenia. Data expressed as mean ± standard deviation.

Parameter	Asymptomatic Patients	Symptomatic Patients
LC (μmol/L)	20.9 ± 6.78	20.5 ± 4.15
TC (μmol/L)	34.3 ± 8.74	33.3 ± 5.15
AcylLC (μmol/L)	13.4 ± 2.47	12.8 ± 1.59
AcylLC-C2 (μmol/L)	6.83 ± 1.12	6.32 ± 0.955
LC/TC	0.599 ± 0.0547	0.612 ± 0.0427
AcylLC/TC	0.401 ± 0.0547	0.388 ± 0.0427
AcylLC-C2/TC	0.207 ± 0.0396	0.192 ± 0.0309

Table 6.2c Endogenous plasma carnitine pool composition and muscle cramps

Muscle cramps: Endogenous plasma carnitine levels for patients classified as asymptomatic (n = 18) and symptomatic (n = 26) according to the subjective assessment of muscle cramps. Data expressed as mean ± standard deviation. † $p<0.05$ compared to asymptomatic patients.

Parameter	Asymptomatic Patients	Symptomatic Patients	
LC (µmol/L)	20.9 ± 6.17	20.9 ± 5.72	
TC (µmol/L)	34.1 ± 8.43	34.2 ± 6.94	
AcylLC (µmol/L)	13.2 ± 2.64	13.3 ± 1.85	
AcylLC-C2 (µmol/L)	6.22 ± 1.14	6.99 ± 0.936 †	p = 0.0136
LC/TC	0.607 ± 0.0498	0.604 ± 0.0496	
AcylLC/TC	0.393 ± 0.0498	0.396 ± 0.0496	
AcylLC-C2/TC	0.189 ± 0.0415	0.208 ± 0.0301 †	p = 0.0447

Table 6.2d Endogenous plasma carnitine pool composition and restless legs

Restless legs: Endogenous plasma carnitine levels for patients classified as asymptomatic (n = 23) and symptomatic (n = 21) according to the subjective assessment of restless legs. Data expressed as mean ± standard deviation.

Parameter	Asymptomatic Patients	Symptomatic Patients
LC (µmol/L)	20.4 ± 6.95	21.1 ± 4.96
TC (µmol/L)	33.8 ± 9.18	34.1 ± 5.99
AcylLC (µmol/L)	13.4 ± 2.62	13.0 ± 1.74
AcylLC-C2 (µmol/L)	6.72 ± 1.10	6.61 ± 1.10
LC/TC	0.593 ± 0.0521	0.614 ± 0.0489
AcylLC/TC	0.407 ± 0.0521	0.386 ± 0.0489
AcylLC-C2/TC	0.208 ± 0.0431	0.196 ± 0.0296

Table 6.2e Endogenous plasma carnitine pool composition and exercise tolerance

Exercise tolerance: Endogenous plasma carnitine levels for patients classified as having normal exercise tolerance (n = 22) and poor exercise tolerance (n = 23) according to the subjective assessment of exercise tolerance. Data expressed as mean ± standard deviation.

Parameter	Normal Exercise Tolerance Patients	Poor Exercise Tolerance Patients
LC (μmol/L)	21.5 ± 6.88	19.9 ± 4.96
TC (μmol/L)	35.3 ± 8.91	32.5 ± 6.13
AcylLC (μmol/L)	13.8 ± 2.42	12.6 ± 1.85
AcylLC-C2 (μmol/L)	6.71 ± 1.18	6.60 ± 1.01
LC/TC	0.600 ± 0.0522	0.606 ± 0.0497
AcylLC/TC	0.400 ± 0.0522	0.394 ± 0.0497
AcylLC-C2/TC	0.197 ± 0.0437	0.206 ± 0.0295

Table 6.3: Endogenous plasma carnitine pool composition and cardiac function

Cardiac function: Endogenous plasma carnitine levels for patients classified as asymptomatic (n = 22) and symptomatic (n = 23) according to the New York Heart Association (NYHA) assessment of cardiac function. Data expressed as mean ± standard deviation. † $p<0.05$ compared to asymptomatic patients.

Parameter	Asymptomatic Patients	Symptomatic Patients	
LC (µmol/L)	21.9 ± 6.90	19.4 ± 4.86	
TC (µmol/L)	35.0 ± 8.51	33.1 ± 6.07	
AcylLC (µmol/L)	13.1 ± 2.22	13.6 ± 1.83	
AcylLC-C2 (µmol/L)	6.61 ± 0.998	7.02 ± 1.50	
LC/TC	0.615 ± 0.0575	0.569 ± 0.0698 †	p = 0.0244
AcylLC/TC	0.385 ± 0.0575	0.431 ± 0.0698 †	p = 0.0291
AcylLC-C2/TC	0.196 ± 0.0403	0.233 ± 0.0775	

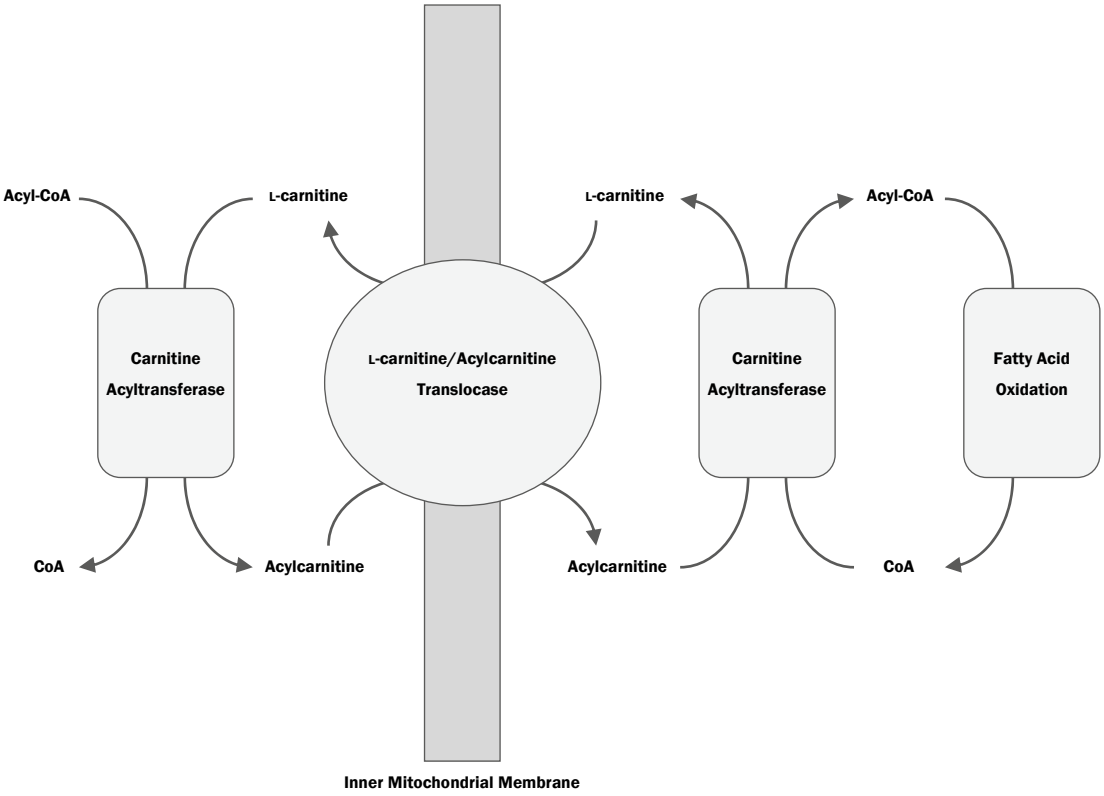
Table 6.4: Endogenous plasma carnitine pool composition and intradialytic hypotension

Intradialytic hypotension: Endogenous plasma carnitine levels for patients classified as asymptomatic (n = 22) and symptomatic (n = 23) according to the subjective assessment of intradialytic hypotension. Data expressed as mean ± standard deviation. † $p<0.05$ compared to asymptomatic patients.

Parameter	Asymptomatic Patients	Symptomatic Patients	
LC (µmol/L)	21.5 ± 5.79	19.8 ± 6.17	
TC (µmol/L)	34.5 ± 7.63	33.2 ± 7.83	
AcylLC (µmol/L)	13.0 ± 2.30	13.4 ± 2.13	
AcylLC-C2 (µmol/L)	6.55 ± 0.972	6.77 ± 1.22	
LC/TC	0.617 ± 0.0444	0.586 ± 0.0530 †	p = 0.0382
AcylLC/TC	0.383 ± 0.0444	0.414 ± 0.0530 †	p = 0.0431
AcylLC-C2/TC	0.195 ± 0.0318	0.210 ± 0.0413	

Figure 6.1 Role of L-carnitine in fatty acid metabolism

Schematic diagram of the function of L-carnitine in the transport of fatty acids across the inner mitochondrial membrane. CoA = coenzyme A.



CHAPTER 7

Carnitine Therapy in Haemodialysis

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L-carnitine Supplementation in the Dialysis Population: Are Australian Patients Missing Out?

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	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

The results of the studies presented within this thesis have clearly demonstrated that chronic haemodialysis treatment is associated with substantial alterations in the composition of the endogenous plasma carnitine pool (Chapter 3), which has been shown to be related to erythropoietin-resistant anaemia (Chapter 4) and skeletal and carnitine muscle dysfunction (Chapter 6) experienced within this patient group.

Despite widespread use of L-carnitine supplementation for the treatment of these dialysis-related disorders in the USA and other countries, prescription within Australia is virtually non-existent.

This chapter presents an invited review that examines the previous research associated with the potential benefit of L-carnitine administration for the treatment of renal anaemia, cardiac dysfunction, dyslipidaemia and dialytic symptoms in chronic haemodialysis patients.

Whilst there are a considerable number of studies in the literature which have examined the contribution of carnitine to these conditions, for the most part these studies are poorly designed and thus limited conclusions can be made. Nonetheless, the collective data suggests that L-carnitine supplementation is beneficial for the treatment of erythropoietin-resistant anaemia, cardiac dysfunction, intradialytic hypotension and muscle fatigue; however, it appears that some patients benefit from treatment more than others. It is proposed that further, well-designed studies evaluating the relationship between these conditions and indices of carnitine pool composition are needed, only then will we have measures to better predict those patients for whom L-carnitine supplementation will be most beneficial.

7.1 Summary

It has been widely established that patients with end-stage renal disease undergoing chronic haemodialysis therapy exhibit low endogenous levels of L-carnitine and elevated acylcarnitine levels; however, the clinical implication of this altered carnitine profile is not as clear. It has been suggested that these disturbances in carnitine homeostasis may be associated with a number of clinical problems common in this patient population, including erythropoietin-resistant anaemia, cardiac dysfunction and dialytic complications such as hypotension, cramps and fatigue. In January 2003, the Centers for Medicare and Medicaid Services (USA) implemented coverage of intravenous L-carnitine for the treatment of erythropoietin-resistant anaemia and/or intradialytic hypotension in patients with low endogenous L-carnitine concentrations. It has been estimated that in the period of 1998 - 2003, 3.8 - 7.2% of all haemodialysis patients in the USA received at least one dose of L-carnitine, with 2.7 - 5.2% of patients receiving at least 3 months of supplementation for one or both of these conditions. The use of

L-carnitine within Australia is virtually non-existent, which leads us to the question: Are Australian haemodialysis patients missing out? This review examines the previous research associated with L-carnitine administration to chronic dialysis patients for the treatment of anaemia, cardiac dysfunction, dyslipidaemia and/or dialytic symptoms, and discusses whether supplementation is warranted within the Australian setting.

7.2 Introduction

L-carnitine is an endogenous compound critical for the transfer of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation[1, 2]. Plasma L-carnitine concentrations are regulated by the healthy kidney, with active tubular reabsorption and preferential excretion of acylcarnitines[3 - 5]. It has been widely established that patients with end-stage renal disease undergoing chronic haemodialysis treatment exhibit altered carnitine levels[6, 7].

In light of this alteration in carnitine homeostasis and evidence for its impact on a number of clinical conditions, the Centers for Medicare and Medicaid Services (USA) implemented coverage of intravenous L-carnitine administration for the treatment of erythropoietin-resistant anaemia and/or intradialytic hypotension in patients who exhibit low endogenous L-carnitine levels (<40 μmol/L)[8]. It has been estimated that in the period of 1998 - 2003, 3.8 - 7.2% of all haemodialysis patients in the USA received at least one dose of L-carnitine, with 2.7 - 5.2% of patients receiving at least 3 months of supplementation[9]. In a recent survey conducted by our group, it was established that carnitine administration within the Australian nephrology community is virtually non-existent (unpublished data).

Until now, the debate over carnitine use in dialysis patients has not been brought to Australia. The aim of this review is to evaluate the existing findings associated with carnitine administration and discuss whether the use of L-carnitine supplementation is warranted within the Australian setting.

7.3 Carnitine Homeostasis

L-carnitine is a low molecular weight, polar molecule found in all mammalian species (Figure 7.1)[1]. The principal biological role of carnitine is in the transport of fatty acids across the inner mitochondrial membrane for fatty acid oxidation (Figure 7.2)[2]. It has also been established that carnitine has a role in regulating the cellular to mitochondrial ratio of free CoA to acyl-CoA, in the transport of short and medium acyl groups from the

peroxisome to the mitochondria, and in the removal of unwanted acyl groups from the body [1, 10].

Adequate levels of L-carnitine are largely achieved from dietary sources, in particular from red meat; however, it can also be biosynthesised from lysine and methionine [1]. The enzymes required for the first four stages of the synthesis of L-carnitine are located within most tissues throughout the body; however, γ-butyrobetaine 2-oxoglutarate dioxygenase (required for the conversion of γ-butyrobetaine to L-carnitine) is present only in the kidneys, liver and brain [1]. Consequently, these organs are important in the biosynthesis of L-carnitine. Plasma plays a critical role in the transport of L-carnitine from these sites of production to the muscles for storage and mediation of mitochondrial fatty acid uptake [11, 12].

The endogenous plasma levels of L-carnitine in the healthy human are 40 - 50 μmol/L with plasma total carnitine levels (L-carnitine + acylcarnitines) 50 - 60 μmol/L; acetyl-L-carnitine (the most abundant acylcarnitine) levels are usually 3 - 6 μmol/L [5, 13 - 16]. Nonetheless, plasma carnitines only account for approximately 1% of the total body carnitine pool, with over 98% of carnitines present in the skeletal and cardiac muscles. A small amount is also present in the kidney, liver and brain [11, 12, 17].

L-carnitine is not protein bound and is therefore extensively filtered at the glomerulus; however, extensive tubular reabsorption maintains adequate endogenous levels [3, 5]. Loss of endogenous carnitine occurs primarily via renal excretion, with preferential excretion of the unwanted acylcarnitines, such that the ratio of acylcarnitine to L-carnitine in urine exceeds that in plasma [4, 5].

7.4 Carnitine Deficiency

It has been well established that alterations in carnitine homeostasis can have a detrimental effect on human health [18, 19]. Disorders of carnitine deficiency may be categorised as either primary or secondary. These disorders have been reviewed extensively elsewhere [18 - 20]; in short, primary carnitine deficiency primarily results from an impairment in the OCTN2 carnitine transporter which restricts tissue uptake and potentiates renal loss; primary carnitine deficiency is not associated with any other illness which may result in the depletion of carnitine. Primary carnitine deficiency may be further categorised as either systemic carnitine deficiency or myopathic carnitine deficiency [19]. In the systemic form, there is a significant reduction in carnitine levels within all the carnitine compartments; whereas in the myopathic form, carnitine deficiency is restricted to the muscular compartment only. Patients with primary

carnitine deficiency develop progressive cardiomyopathy, encephalopathy and muscle weakness, resulting in death from heart failure [19, 20]. For these patients, L-carnitine supplementation is a life-saving treatment.

Secondary carnitine deficiency is less severe with respect to its short-term clinical impact; however, it is much more common. As opposed to primary carnitine deficiency, secondary carnitine deficiency is associated with, or caused by, another disorder, such as liver or kidney disease, a defect in fatty acid metabolism or administration of certain pharmacologic agents (such as pivampicillin or valproic acid) [18, 19, 21].

7.4.1 End-Stage Renal Disease

Considering that the kidney has such a critical role in carnitine homeostasis, it is hardly surprising that alterations in carnitine pool composition occur in patients with end-stage renal disease. Numerous studies investigating these changes have established that as kidney function deteriorates, plasma concentrations of endogenous L-carnitine and its acyl derivatives increase significantly [11, 12, 22 - 25]. Plasma L-carnitine concentrations have been shown to be up to two-fold higher in the end-stage renal disease population than in healthy controls, while total acylcarnitine levels in this patient population have been observed at levels up to 4.6 times greater than in healthy subjects [11, 12, 22 - 25].

Conversely, chronic haemodialysis treatment has been shown to be associated with a reduction in plasma and tissue L-carnitine concentrations and an accumulation of acylcarnitines [11, 12, 17, 22 - 35]. It has been demonstrated that L-carnitine is extensively removed by the dialyser during a single dialysis session, due to the compound's low molecular weight and high hydrophilicity in combination with its lack of protein binding [29, 30]. As a consequence, plasma L-carnitine levels in end-stage renal disease patients receiving haemodialysis treatment have been shown to be substantially lower than endogenous levels observed in healthy subjects [6, 24 - 26, 31, 34], with levels of 22.0 ± 5.4 µmol/L reported in long-term dialysis patients compared with an average plasma concentration of 43.3 ± 8.6 µmol/L in healthy adults [6]. Recent research has demonstrated a significant negative correlation between plasma endogenous L-carnitine levels and the duration of dialysis treatment (dialysis age), with the majority of the reduction occurring within the first few months of haemodialysis treatment [6] (Figure 7.3). Long-term haemodialysis treatment (>12 months) has also been shown to be associated with a significant decrease in muscle

carnitine stores, with a 38% reduction in L-carnitine levels compared with levels prior to the initiation of dialysis treatment[6]. Similar decreases in muscle total carnitine and acetyl-L-carnitine levels were also observed.

On the other hand, compared with the healthy population, acylcarnitine levels are significantly higher in end-stage renal disease patients on chronic haemodialysis therapy, particularly those who have been receiving haemodialysis treatment for at least 12 months[6]. In fact, acylcarnitine levels make up approximately 50% of the total plasma carnitine pool in these patients (of which only half is accounted for by acetyl-L-carnitine), compared with 15% in healthy subjects (consisting primarily of acetyl-L-carnitine and negligible levels of the non-acetyl acylcarnitines) (Figure 7.4)[6]. The dialytic removal of acylcarnitines during a single dialysis session is significantly correlated to carbon-chain length of the acyl group, with no significant removal of the 18 carbon-chain length esters (Figure 7.5)[7]. Removal of these acylcarnitines is obviously impaired due to the increased molecular weight and lipophilicity that accompanies increasing chain length, in addition to protein binding which has been demonstrated for the longer-chain acylcarnitines[36].

7.5 Carnitine Supplementation in Chronic Haemodialysis Patients

Disturbances in carnitine homeostasis during long-term haemodialysis have been implicated as a contributing factor for certain clinical problems in end-stage renal disease patients, including skeletal myopathies, poor exercise performance, cardiomyopathy, and dialytic complications such as hypotension, cramps, weakness and fatigue[37]. Recent research has demonstrated that supplementation with L-carnitine is associated with a reduction in hospital use in the USA[38, 39].

A large number of studies have been conducted over the past 20 years to assess the efficacy of supplemental L-carnitine in treating certain dialysis-related clinical disorders. However, many of these studies have been small, retrospective trials, and few blinded, placebo-controlled, large-scale trials have been conducted. In addition, the prescription of L-carnitine (e.g. dose, route, duration of treatment) and patient population (e.g. dialysis age) differ greatly from one study to the next, making between-study comparisons difficult. However, perhaps the most important factor is in the definition of carnitine deficiency itself in this patient population. Thus, in long term haemodialysis patients it may be more important to examine relationships between the clinical response to L-carnitine treatment and pre-treatment L-carnitine (or acylcarnitine) levels as a proportion of the carnitine pool, rather than focussing on the pre-treatment plasma

L-carnitine levels (or change in L-carnitine levels after treatment) alone; no studies have investigated these more complex relationships.

Despite this, there appears to be an evidential basis for the use of L-carnitine in chronic haemodialysis patients; however, more controlled comprehensive trials are needed before any definitive conclusions can be made.

7.5.1 Pharmacokinetics and Safety

L-carnitine has been administered to chronic haemodialysis patients according to a variety of different prescriptions for the treatment of dialysis-related carnitine deficiency. In most cases L-carnitine is given as an i.v. or oral (tablet or solution) dose; however, the pharmacokinetics of these two dose forms are vastly different, and therefore must be considered separately.

The i.v. form of L-carnitine is 100% bioavailable and undergoes negligible metabolism (with the exception of reversible acylation). A typical 1 - 2 g i.v. dose of L-carnitine to a healthy adult results in initial plasma carnitine concentrations up to 10-fold greater than the threshold for tubular reabsorption, and as a consequence 70 - 90% is excreted unchanged in urine within 12 - 24 hours post-dose. Thus, in healthy people, a single dose of L-carnitine is not present in the body long enough for any significant amount to equilibrate into the body's carnitine pool (i.e. skeletal and cardiac muscle). On the other hand, as end-stage renal disease patients have no efficient removal process during the interdialysis period, the i.v. dose of L-carnitine remains in the bloodstream for sufficient time for it to be incorporated into the extravascular tissue compartments. In fact, in a study performed by our group, it was demonstrated that only 10% of a single i.v. dose of L-carnitine was recovered in dialysate during the next dialysis session, suggesting that approximately 90% of the dosed L-carnitine had moved into the tissues [30]. Chronic i.v. L-carnitine administration has been shown to result in a 60 - 200% increase in muscle L-carnitine levels [17, 31, 40, 41].

The usual, prescribed i.v. dose of L-carnitine is 20 mg/kg during the initiation of therapy, which represents approximately 5 - 10% of total body carnitine levels. It is thought that a dose of this magnitude may drive L-carnitine from the plasma into muscle stores; in any case, this dose level results in a steady increase in muscle carnitine levels over a period of weeks to months.

Administration of a more physiologically relevant dose (e.g. 2 mg/kg) would obviously result in a much longer equilibration time.

Studies in healthy subjects have shown that oral L-carnitine is poorly bioavailable, with only 15% of a usual dose of 2 g being absorbed into the bloodstream, reducing to 5% bioavailability after a 6 g dose [3, 42, 43]. The bioavailability of oral L-carnitine in renal disease patients is unknown. The unabsorbed portion of the oral dose of L-carnitine undergoes bacterial degradation in the gastrointestinal tract to form trimethylamine and γ-butyrobetaine. Trimethylamine is absorbed into the bloodstream and converted to trimethylamine-*N*-oxide. In healthy subjects, trimethylamine (which has the odour of rotting fish) is rapidly converted to trimethylamine-*N*-oxide and is excreted efficiently by the kidneys, and consequently these individuals experience no toxic effects. On the other hand, patients with kidney failure are unable to excrete trimethylamine-*N*-oxide effectively and these patients experience accumulation of trimethylamine-*N*-oxide and trimethylamine. A high level of these compounds might contribute to neurotoxicity and 'uraemic breath' in these patients [44].

Previous studies have also demonstrated that orally administered L-carnitine undergoes a high degree of acetylation during gastrointestinal absorption in animals [45, 46]. Although it is still unknown whether this is also true for humans, this deserves careful consideration as to whether high acetyl-L-carnitine levels are desirable in haemodialysis patients who already have high endogenous acylcarnitine levels.

L-carnitine administration has shown to have an excellent safety profile. Early studies investigated the effects of D,L-carnitine administration and noted in a number of reports that this was associated with the development of myasthenia-like symptoms. It was later established that this was due to toxicity related to the D-isomer only. Transient nausea and vomiting have been associated with L-carnitine administration, and less frequently body odour, nausea, gastritis and seizures [47].

7.5.2 Anaemia

Anaemia is a common complication associated with chronic haemodialysis treatment, characterised by both a decrease in erythrocyte precursor production and reduced survival of red blood cells; however, the most

important cause of renal anaemia is a decrease in erythropoietin secretion by the kidney[48]. Recombinant human erythropoietin (rHuEPO) is the commonly used treatment for anaemia of end-stage renal disease patients; however, the rHuEPO dose required to maintain a target haemoglobin level varies dramatically within the patient population[49, 50]. In addition, the cost of this treatment is extremely high, with rHuEPO therapy accounting for up to 10% of the total treatment costs for chronic haemodialysis patients[51]. It is estimated that approximately AU$100 - 150 million is spent on erythropoietin or darbepoetin treatment in Australia per year, with more than AU$20 million under the Pharmaceutical Benefits Scheme[52]. However, it is important to note that the rationale for improving rHuEPO resistance in not merely economic; recent research by Zhang et al.[50] investigating the relationship between rHuEPO requirements and mortality has indicated that the highest mortality rate is observed in patients receiving high doses of rHuEPO but with low haematocrit levels (i.e. rHuEPO-resistant). Interestingly, patients with haematocrit levels in the desirable range (33 - 36%) receiving high doses of rHuEPO had a mortality rate twice that of those receiving a low dose of rHuEPO with the haematocrit levels in the same range, thereby suggesting that, wherever possible, the minimum rHuEPO dose necessary to achieve acceptable haematocrit levels should be prescribed.

L-carnitine has been proposed as a potential adjunct treatment to rHuEPO in the treatment of anaemia. From January 2003, the Centers for Medicare and Medicaid Services implemented coverage of intravenous L-carnitine administration for the treatment of erythropoietin-resistant anaemia in haemodialysis patients who have low endogenous levels of L-carnitine[8]. Numerous studies have investigated the potential use of L-carnitine for anaemia, with promising results[53 - 68]. These studies have been summarised in Table 7.1.

Studies have demonstrated that L-carnitine administration improved the response to rHuEPO in the haemodialysis population, with either an increase in haematocrit levels[53 - 59], a decrease in the rHuEPO dose required[60 - 65], or a decrease in erythropoietin resistance index (ERI; rHuEPO dose/week normalised for haematocrit or haemoglobin)[66 - 68]. In a randomised, placebo-controlled trial, Trovato et al.[59] observed a significant increase in haematocrit levels in patients treated with 825 mg L-carnitine p.o. b.i.d. for 12 months; in contrast, haematocrit levels decreased by almost 10% in placebo-treated patients during the 12 month treatment period. Similarly, Labonia[62] noted a

significant decrease in rHuEPO dose in patients treated with L-carnitine (1 g i.v. after dialysis for 6 months), compared with no change in placebo-treated subjects. Hurot et al.[69] performed a meta-analysis of 18 clinical trials investigating the effects of L-carnitine administration on anaemia, and noted that co-therapy with L-carnitine resulted in a significant improvement in haematocrit and/or rHuEPO requirements.

The underlying mechanism by which L-carnitine exerts its favourable effects on these anaemic indices in dialysis patients is still not yet fully understood. It has been suggested that L-carnitine may act via stabilisation of the red blood cell membrane, thereby increasing the lifespan of the erythrocyte[59]; this is in direct contrast to the described effects of long-chain acylcarnitines on red blood cells[70-73]. In one such study it was demonstrated that co-incubation of erythrocytes with L-carnitine can reverse the damaging detergent effects produced by palmitoyl-L-carnitine (a long-chain acylcarnitine)[73]. Relationships between L-carnitine and erythrocyte osmotic fragility, deformability and membrane fluidity have been demonstrated[58, 73-75]. Alternatively, the beneficial effects of L-carnitine on anaemia may be a result of a reduction in lipid peroxidation. Gallucci et al.[76] have demonstrated that patients with rHuEPO-resistant anaemia have significantly higher levels of malondialdehyde than responders to rHuEPO therapy and patients not receiving rHuEPO treatment. Following on from this, Veselá et al.[65] showed that L-carnitine administration for 6 months results in a significant reduction in malondialdehyde levels.

It should, however, be noted that, despite the fact that there is an overall improvement in anaemic parameters with L-carnitine supplementation, a number of studies have shown that the patient population appears to be separated into 'responders' and 'non-responders'[62, 66, 67]. Although in all of these studies the division of patients into these subgroups was retrospective, the authors of one paper reported 'a greater benefit was found in those patients with the higher increase in carnitine plasma levels'[66]. Kletzmayr et al.[67] noted that responders had higher baseline levels of all carnitines compared to non-responders, and therefore suggested that 'severely carnitine-depleted patients might need a higher dosage or longer supplementation of carnitine in order to reduce the rHuEPO requirement'. The apparent subgrouping of patients as responders and non-responders needs to be investigated further.

Our group has recently demonstrated that there is a significant relationship between a patient's endogenous carnitine profile and ERI; specifically, the proportion of non-acetyl acylcarnitines within the total carnitine pool is positively correlated with ERI, independent of dialysis age (Figure 7.6). In other words, a higher rHuEPO dose is required in patients exhibiting a higher proportion of medium- to long-chain carnitine esters in plasma. It is hypothesised that L-carnitine supplementation may be most beneficial in these patients. Only one study has investigated the effect of supplementation in a specific group of patients who were considered to be rHuEPO-resistant; however, this study was not controlled and utilised oral supplementation [57]. Further studies are needed to investigate the effect of L-carnitine administration in this specific population.

It is of our opinion that L-carnitine shows great promise as a potential adjunct treatment to rHuEPO for the treatment of anaemia in haemodialysis patients, at least in a subgroup of patients. However, other known factors, such as iron deficiency, should be first ruled out as a possible cause of hyporesponsiveness to rHuEPO in these patients. Further studies are needed to determine whether the effectiveness of L-carnitine for anaemia is constant in all haemodialysis patients or only a select population.

7.5.3 Cardiac Function

Cardiac disease is a significant problem among dialysis patients, accounting for approximately half of all deaths in this patient population [77]. In addition, approximately three-quarters of end-stage renal disease patients commencing haemodialysis treatment suffer from some form of left ventricular dysfunction [78] as indicated by an impaired left ventricular ejection fraction which has been shown to be an independent predictor of congestive heart failure [79]. Intradialytic hypotension has been associated with a significant increased risk of mortality [80] and impaired myocardial contractile reserve [81].

Several studies have investigated the effect of L-carnitine administration on cardiac function [40, 63, 64, 82 - 90], these are summarised in Table 7.2.

The relationship between L-carnitine and the incidence of hypotensive episodes in haemodialysis patients has been widely investigated. It has been noted that patients who experience hypotensive episodes have lower endogenous L-carnitine levels than their normotensive counterparts [91]. Administration of

L-carnitine (990 mg/day p.o. for 2 months) has been shown to be associated with a significant reduction in the incidence of intradialytic hypotensive events when compared to placebo treatment [82]. The results of these studies are supported by the findings of Ahmad et al. [40] and Fujita et al. [83]. Based on these studies, the Centers for Medicare and Medicaid Services have approved coverage of intravenous L-carnitine supplementation for the treatment of dialysis-related hypotension in haemodialysis patients with low endogenous levels of L-carnitine [8].

The effect of L-carnitine on left ventricular ejection fraction has also been described. A significant correlation between left ventricular ejection fraction and endogenous L-carnitine levels has been reported, with these patients exhibiting considerable improvement in left ventricular ejection fraction after administration of L-carnitine (1 g i.v. after dialysis) for 3 months, a result which was particularly pronounced in patients experiencing recurrent hypotensive episodes [84]. These symptomatic patients had a significantly lower ejection fraction than their asymptomatic counterparts, suggesting a significantly higher risk of mortality [81]. Additional studies have provided similar findings [63, 64, 85, 86]. Although mounting evidence exists in favour of the role of L-carnitine in the treatment of left ventricular dysfunction, other studies have been performed with negative results [87, 88]; however, the results of these studies should be interpreted with caution, as the patients in these studies had normal baseline ejection fractions.

The use of L-carnitine therapy for the treatment of cardiac arrhythmias remains a contentious subject. Suzuki et al. [90] reported that the administration of L-carnitine (2 g p.o. prior to dialysis) for 4 - 8 weeks resulted in a significant reduction in the incidence and severity of arrhythmias in the patient population. Conversely, no change in the frequency of arrhythmias was reported after administration of 20 mg/kg i.v. L-carnitine after dialysis for 6 months when compared to a placebo-treated group, although the authors of this study did note that very few dialysis-related arrhythmias were reported in both the carnitine- and placebo-treated groups [40].

The findings of these studies indicate that L-carnitine shows a great deal of potential for use in the treatment of cardiac dysfunction, in particular in the treatment of dialysis-related hypotension. However, the relationship between L-carnitine and cardiac arrhythmias is less compelling, and additional well-designed trials are needed to investigate this further.

7.5.4 Plasma Lipids

Approximately 40% of all chronic haemodialysis patients experience some form of dyslipidaemia [92]. The majority of patients present with Type IV hyperlipidaemia, with a reduction in plasma high density lipoprotein (HDL) cholesterol levels [92]. As a consequence, these dyslipidaemic haemodialysis patients may be at a high risk of developing atherosclerotic cardiovascular disease [93, 94]. In addition, recent research has found that traditional treatment of dyslipidaemia with statins is associated with no clinical benefit in cardiovascular end-points and an increase in the incidence of fatal strokes in some haemodialysis patients [95]. Consequently, treatment with statins is not recommended in these patients, and the investigation of alternative agents is warranted [96].

Although the aetiology of dyslipidaemia in these patients is complex, carnitine deficiency has been implicated as a possible contributing factor in uraemic hyperlipidaemia. It has been hypothesised that carnitine supplementation may improve dyslipidaemia in these patients by stimulating oxidation of long-chain fatty acids, thereby decreasing their availability for esterification to triglycerides [92]. Based on this theory, numerous studies have investigated the effects of L-carnitine administration on plasma lipids in chronic haemodialysis patients, with varying results [31, 34, 89, 92, 97 - 109]. The findings of these studies are presented in Table 7.3.

A number of studies have demonstrated that long-term L-carnitine administration results in a significant reduction in triglyceride levels [97 - 99, 101], whereas others report that supplementation is associated with a significant increase in HDL cholesterol [92, 102, 103]. In a study by Vacha et al. [104], administration of L-carnitine for at least 12 months resulted in both a decrease in triglycerides and an increase in HDL cholesterol, with levels returning to baseline after cessation of L-carnitine treatment. Reintroduction of L-carnitine treatment produced the same improvement in plasma lipid levels. Trivelli et al. [105] reported comparable findings. Although numerous studies have demonstrated the beneficial effects of L-carnitine supplementation on dyslipidaemia in chronic haemodialysis patients, many studies have failed to show any significant changes in plasma lipid profiles [31, 89, 106 - 108], with some studies reporting a negative effect on levels [34, 109]. A meta-analysis of these studies indicated that L-carnitine supplementation was not associated with any

significant effect on lipid parameters [69]. The authors suggested that additional adequately sized trials should be performed.

The results of these studies indicate that the potential use of L-carnitine for the treatment of dyslipidaemia is a complex issue. Additional studies have suggested that the haemodialysis population can be divided into groups of 'responders' and 'non-responders', although there appears to be no clear distinction between these two groups. Elisaf et al. [98] noted that patients with hypertriglyceridaemia (triglycerides >200 mg/dL) exhibited significant reductions in triglyceride levels with L-carnitine treatment; however, normotriglyceridaemic patients showed no significant changes. A significant correlation between response to carnitine supplementation and baseline triglyceride levels was described. Similarly, Vacha et al. [110] observed that patients with low HDL cholesterol (<400 mg/L) responded significantly to L-carnitine treatment (20 mg/kg/dialysis session i.v.) with a decrease in triglyceride levels and an increase in HDL cholesterol; whereas patients with high HDL cholesterol (>400 mg/L) had no changes in plasma lipid profiles. However, administration of a higher dose of L-carnitine (60 mg/kg/dialysis session i.v.) to hyporesponsive patients resulted in a significant reduction in cholesterol and triglyceride levels. Other studies have noted that responders exhibited significantly higher baseline short- and long-chain acylcarnitine levels than non-responders, and responders had significantly higher L-carnitine levels with administration than non-responders [111, 112].

The potential use of L-carnitine for the treatment of dyslipidaemia in chronic haemodialysis patients is not a clear-cut case, as evidenced by the discrepancy in the findings in the above-mentioned studies. Limited research in this area has been conducted over the past few years investigating this, which is surprising considering the recent significant increases in the incidence of dyslipidaemia in our society. Additional, appropriately designed studies are needed to examine this potential role for L-carnitine, in particular in specific dyslipidaemic populations, such as patients with high triglyceride levels and low HDL cholesterol levels.

7.5.5 Dialytic Symptoms

Muscle weakness is a common complaint experienced by haemodialysis patients, as are other symptoms of altered muscle function, such as cramps.

Carnitine deficiency has been implicated as a contributing factor to these conditions.

In a study investigating the effect of L-carnitine supplementation on haemodialysis patients, both with and without muscular symptoms such as cramps and asthenia, it was noted that significantly lower endogenous plasma carnitine levels were observed in the symptomatic patients that their non-symptomatic counterparts[113]. Of the 30 symptomatic patients in this study, approximately two-thirds had some improvement in their symptoms with L-carnitine treatment, which in most cases was classified as a mild or moderate improvement. Comparable results were described by Bellinghieri et al.[28], in which L-carnitine treatment resulted in a significant improvement in symptom scores for muscle cramps, post-dialysis asthenia and interdialysis asthenia in haemodialysis patients. These symptoms worsened during the washout phase of the trial when L-carnitine treatment was ceased. Conflicting results were reported in another study, in which no significant changes in muscle cramps or fatigue were observed after L-carnitine treatment[41]. However, it is worth noting that these patients were only administered L-carnitine treatment for 6 weeks and these patients exhibited normal muscle carnitine levels at baseline.

The benefit of L-carnitine therapy on measurements of muscle function does, however, appear to be limited, as evidenced by the findings of Rogerson et al.[107] and Siami et al.[114]. It was noted in the latter study that, although no overall significant effects on muscle activity were found, four out of the seven patients who received carnitine treatment showed an improvement of 2 or more grades in the assessment of muscle activity. The authors postulated that there is a subset of patients in whom L-carnitine administration appears to improve muscle function.

The benefit of L-carnitine treatment on muscle function and weakness is not established. These dialytic symptoms are life-altering and, in many cases, debilitating for the numerous haemodialysis patients who experience them regularly. Consequently, L-carnitine treatment may be justified, in some cases, for the potential improvement of these symptoms.

7.6 Conclusions

Although large clinical trials demonstrating the positive effect of L-carnitine on rHuEPO-resistant anaemia in haemodialysis patients are limited, the collective data suggest that

L-carnitine treatment is beneficial in the treatment of this condition. This is supported by a formal meta-analysis which demonstrated that L-carnitine co-therapy was associated with a significant improvement in haematocrit and/or rHuEPO requirements. Although the direct link between an improvement in anaemic parameters as a result of L-carnitine supplementation and an improvement in a patient's clinical status has not been established, studies have demonstrated that L-carnitine supplementation is associated with a reduction in hospital utilisation in the USA [38, 39].

There is by no means definitive evidence for the role of L-carnitine supplementation in the treatment of cardiac function, dyslipidaemia or dialytic symptoms in long-term haemodialysis patients; however, there are mounting data which indicate that L-carnitine may be a useful adjunct to traditional therapy in the treatment of these conditions. Interestingly, Steiber et al. [68, 115] recently reported a significant correlation between the acylcarnitine/L-carnitine ratio and results of the physical component of the Short Form-36 in males, and a significant improvement in these scores from baseline with L-carnitine treatment. This is in keeping with the findings of Sloan et al. [116] in which it was demonstrated that L-carnitine supplementation had a positive effect on measures of quality-of-life.

Although numerous studies have demonstrated that L-carnitine is beneficial for the treatment of dialysis-related disorders, little is known about the specific patient groups for which L-carnitine supplementation provides the most benefit. Studies have suggested that response to L-carnitine treatment may be related to endogenous L-carnitine levels or changes in levels from baseline; however, this has not been investigated fully. Additional studies investigating the response to treatment and the relationship to more complex indices, such as the proportion of L-carnitine within the total carnitine pool or the accumulation of specific acylcarnitines, are needed.

Although it should never be a case of 'monkey see, monkey do', that L-carnitine treatment is covered within the US system is a fact that we should not ignore. Due to the excellent safety profile associated with this treatment, it is of our opinion that supplementation with L-carnitine may be warranted for the treatment of dialysis-related carnitine deficiency, particularly in those patients with rHuEPO-resistant anaemia, cardiac dysfunction, intradialytic hypotension and dialytic symptoms. Nevertheless, further well-designed studies evaluating the relationship between these conditions and L-carnitine levels are needed, in particular investigations into the phenomenon of 'responders' and 'non-responders', only then will we have measures to predict those patients for whom L-carnitine administration will be beneficial.

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Table 7.1: Previously reported impact of L-carnitine supplementation for the treatment of anaemia in haemodialysis patients

Summary of anaemia studies. Dx = dialysis session; rHuEPO = recombinant human erythropoietin dose; ERI = erythropoietin resistance index; HCT = haematocrit; HD = haemodialysis.

Reference	Study Design	Population	L-carnitine Treatment	Findings	
[53]	Two-way, parallel, open-label	12 HD Patients	1 g/day p.o. for 6 months	↑ HCT	T_0 23.1 ± 5.2%; T_6 26.5 ± 4.4%
		11 HD Patients	100 µmol/L dialysate for 6 months		
[54]	Case report	2 HD Patients	2 mg/kg/Dx i.v. for 9 months	↑ HCT	T_0 22.3 & 20.9%; T_9 24.3 & 28.6%
[55]	One-way, open-label	10 HD Patients	1 g b.i.d. p.o. for 2 months	↑ HCT	T_0 25.6 ± 2.4%; T_2 29.0 ± 2.6%
[56]	One-way, open-label	8 HD Patients	1 g/Dx i.v. + 1 g/day p.o. for 2 months	↑ HCT	T_0 22.3 ± 3.6%; T_2 24.9 ± 2.7%
[57]	One-way, open-label	14 HD Patients (rHuEPO-Resistant)	500 mg/day p.o. for 3 months	↑ HCT	T_0 24.0 ± 2.0%; T_3 26.1 ± 2.0%
[58]	One-way, open-label	15 HD Patients	30 mg/kg/Dx i.v. for 3 months	↑ HCT	T_0 30.8 ± 1.9%; T_3 34.2 ± 2.4%
[59]	Two-way, parallel, double-blind	13 HD Patients	825 mg b.i.d. p.o. for 12 months	↑ HCT	T_0 25.5 ± 1.4%; T_{12} 37.4 ± 2.2%
		11 HD Patients	Placebo for 12 months		T_0 24.0 ± 3.6%; T_{12} 21.8 ± 3.2%
[60]	One-way, open-label	14 HD Patients	40 - 60 mg/kg/wk i.v. for 3 months	↓ rHuEPO	T_0 151 ± 21 U/kg/wk; T_3 87 ± 32 U/kg/wk
[61]	One-way, open-label	8 HD Patients	2 g/Dx i.v. for 5 months	↓ rHuEPO	T_0 50 - 60 U/kg/Dx; T_5 20 - 25 U/kg/Dx
[62]	Two-way, parallel, double-blind	13 HD Patients	1 g/Dx i.v. for 6 months	↓ rHuEPO	T_0 102.2 ± 52.6 U/kg/wk; T_6 63.3 ± 37.8 U/kg/wk
		11 HD Patients	Placebo i.v. for 6 months		T_0 78.6 ± 32.1 U/kg/wk; T_6 79.9 ± 46.7 U/kg/wk
[63]	One-way, open-label	23 HD Patients	1 g/Dx i.v. for 8 months	↓ rHuEPO	T_0 7000 ± 3000 U; T_8 6000 ± 2000 U
[64]	Two-way, parallel, open-label	25 HD Patients	1 g/Dx i.v. + 1 g/Non-Dx p.o. for 36 months	↓ rHuEPO	T_0 5976 ± 1732 U/wk; T_{36} 3391 ± 659 U/wk
		35 HD Patients	No treatment for 36 months		T_0 6100 ± 1587 U/wk; T_{36} 5519 ± 1360 U/wk
[65]	One-way, open-label	12 HD Patients	15 mg/kg/Dx i.v. for 6 months	↓ rHuEPO	T_0 5500 U/wk; T_{36} 3500 U/wk
[66]	Two-way, parallel, double-blind	10 HD Patients (>65 years)	1 g/Dx i.v. for 6 months	↓ ERI	T_0 135 ± 79 IU/kg/wk/%; T_6 118 ± 108 IU/kg/wk/%
		11 HD Patients (>65 years)	Placebo i.v. for 6 months		T_0 136 ± 66 IU/kg/wk/%; T_6 217 ± 204 IU/kg/wk/%
[67]	Two-way, parallel, double-blind	14 HD Patients	5 mg/kg/Dx i.v. for 4 months	↓ ERI	T_0 16.0 ± 11.0 U/kg/gHb; T_4 13.6 ± 10.5 U/kg/gHb
		5 HD Patients	25 mg/kg/Dx i.v. for 4 months		
[68]	Two-way, parallel, double-blind	13 HD Patients	20 mg/kg/Dx i.v. for 6 months	↓ ERI	Values not reported
		14 HD Patients	Placebo for 6 months		

Table 7.2: Previously reported impact of L-carnitine supplementation for the treatment of cardiac dysfunction in haemodialysis patients

Summary of cardiac studies. ARR = arrhythmias; Dx = dialysis session; ECG = echocardiogram; HD = haemodialysis; HT = hypotensive episodes; LVEF = left ventricular ejection fraction.

Reference	Study Design	Population	L-carnitine Treatment	Findings	
[82]	Two-way, crossover, double-blind	9 HD Patients 9 HD Patients	990 mg/day p.o. then placebo for 2 months each Placebo then 990 mg/day p.o. for 2 months each	↓HT	Values not reported
[40]	Two-way, parallel, double-blind	38 HD Patients 44 HD Patients	20 mg/kg/Dx i.v. for 6 months Placebo for 6 months	↓HT	T_0: 17 patients with HT; T_6: 7 patients with HT T_0: 9 patients with HT; T_6: 11 patients with HT
[89]	Two-way, parallel, double-blind	15 HD Patients 15 HD Patients	1 - 1.5 g/Dx i.v. for 2 months Placebo for 2 months	↓HT	Values not reported
[83]	One-way, open-label	18 HD Patients (>4 HT/wk)	1200 mg/day p.o. for 3 months	↓HT	Values not reported
[84]	One-way, open-label	13 HD Patients	1 g/Dx i.v. for 3 months	↑LVEF	T_0: 42.4 ± 19.4%; T_3: 48.6 ± 17.6%
[85]	One-way, open-label	6 HD Patients (Children)	15 - 30 mg/kg/day p.o. for 18 months	↑LVEF	Values not reported
[86]	One-way, open-label	9 HD Patients (Impaired LVEF)	500 mg/day p.o. for 6 months	↑LVEF	T_0: 44.9 ± 12.2%; T_6: 53.8 ± 13.8%
[63]	One-way, open-label	11 HD Patients	1 g/Dx i.v. for 8 months	↑LVEF	T_0: 32.0%; T_8: 41.8%
[64]	Two-way, parallel, open-label	25 HD Patients 35 HD Patients	1 g/Dx i.v. + 1 g/Non-Dx p.o. for 36 months No treatment for 36 months	↑LVEF	Values not reported
[87]	Two-way, parallel, double-blind	14 HD Patients 14 HD Patients	2 g/Dx i.v. for 6 weeks Placebo for 6 weeks	↔ECG	No difference in echocardiogram measurements
[88]	One-way, open-label	11 HD Patients	1 g/day p.o. then 0.5 g/day p.o. for 1 month each	↔ECG	No difference in echocardiogram measurements
[90]	One-way, open-label	17 HD Patients	2 g/Dx p.o. for 1 - 2 months	↓ARR	Values not reported
[40]	Two-way, parallel, double-blind	38 HD Patients 44 HD Patients	20 mg/kg/Dx i.v. for 6 months Placebo for 6 months	↔ARR	No difference in incidence of arrhythmias

Table 7.3: Previously reported impact of L-carnitine supplementation for the treatment of dyslipidaemia in haemodialysis patients

Summary of lipid studies. Dx = dialysis session; HD = haemodialysis; HDL = high density lipoprotein; HL = hyperlipidaemia; LDL = low density lipoprotein; TRIG = triglycerides.

Reference	Study Design	Population	L-carnitine Treatment	Findings	
97	One-way, open-label	10 HD Patients	50 mg/kg/Dx i.v. for 2 months (D,L-carnitine)	↓TRIG	T_0: 550 ± 115 mg/mL; T_2: 357 ± 75 mg/mL
98	One-way, open-label	28 HD Patients	5 mg/kg/Dx i.v. for 6 months	↓TRIG	T_0: 225 ± 76 mg/dL; T_6: 201 ± 79 mg/dL
99	Two-way, parallel, open-label	8 HD Patients	0.5 g/Dx i.v. for 2 months then 1.0 g/Dx i.v. for 1.5 months	↓TRIG	T_0: 336 ± 56 mg/dL; $T_{3.5}$: 244 ± 82 mg/dL
		8 HD Patients	Placebo for 3.5 months		T_0: 329 ± 72 mg/dL; $T_{3.5}$: 444 ± 269 mg/dL
100	One-way, open-label	8 HD Patients	900 mg/day p.o. for 2 months	↓TRIG	T_0: 287 ± 74 mg/dL; T_2: 188 ± 22 mg/dL
101	One-way, open-label	25 HD Patients	5 mg/kg b.i.d. p.o. for 3 weeks	↓TRIG	Values not reported
102	Two-way, crossover, double-blind	13 HD Patients	1 g/Dx i.v. then placebo for 40 days each	↑HDL	Values not reported
		14 HD Patients	Placebo then 1 g/Dx i.v. for 40 days each		
92	Two-way, parallel, single-blind	9 HD Patients	660 mg/day p.o. for 1 month	↑HDL	T_0: 42.4 ± 5.9 mg/100mL; T_1: 52.7 ± 5.4 mg/100mL
		6 HD Patients	Placebo for 6 months		Values not reported
103	One-way, open-label	20 HD Patients	2 g/Dx i.v. + 2 g/Non-Dx p.o. for 3 months	↑HDL/LDL	Values not reported
104	Two-way, parallel, open-label	11 HD Patients	1 g/Dx i.v. for 1 month then 2 g/Dx dialysate for 3 months	↓TRIG & ↑HDL	Values not reported
		11 HD Patients	1 g/Dx i.v. for 1 month then 4 g/Dx dialysate for 3 months		
105	Two-way, parallel, open-label	6 HD Patients (Type IV HL)	2 g/Dx i.v. for 3 months	↓TRIG & ↑HDL	Values not reported
		6 HD Patients (Type IV HL)	Placebo for 3 months		
106	Two-way, crossover, double-blind	9 HD Patients	1 g t.i.d. p.o. then placebo for 5 weeks each	↔LIPID	No difference in plasma lipid measurements
		9 HD Patients	Placebo then 1 g t.i.d. p.o. for 5 weeks each		
31	Two-way, parallel, double-blind	38 HD Patients	20 mg/kg/Dx i.v. for 6 months	↔LIPID	No difference in plasma lipid measurements
		44 HD Patients	Placebo for 6 months		
107	One-way, open-label	6 HD Patients (Asthenia)	2 g/day p.o. for 6 weeks	↔LIPID	No difference in plasma lipid measurements
89	Two-way, parallel, double-blind	15 HD Patients	1 - 1.5 g/Dx i.v. for 2 months	↔LIPID	No difference in plasma lipid measurements
		15 HD Patients	Placebo for 2 months		
108	Two-way, parallel, double-blind	21 HD Patients	100 µmol/L dialysate for 6 months	↔LIPID	No difference in plasma lipid measurements
			Placebo for 6 months		
109	Two-way, parallel, open-label	6 HD Patients	990 mg t.i.d. p.o. for 1 month	↑TRIG	T_0: 180 ± 66 mg%; T_1: 219 ± 88 mg%
		4 HD Patients	Placebo for 1 month		T_0: 222 ± 35 mg%; T_1: 222 ± 35 mg%
34	One-way, open-label	23 HD Patients	1 g/Dx i.v. for 1 month	↑TRIG	T_0: 185 ± 22 mg/dL; T_1: 273 ± 23 mg/dL

Figure 7.1 Chemical structure of L-carnitine

Structure of carnitine, with the site of esterification illustrated in bold.

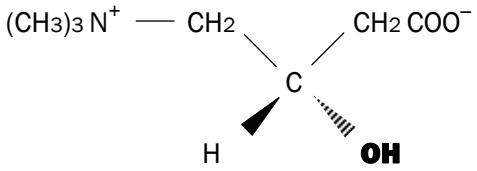


Figure 7.2 Role of L-carnitine in fatty acid metabolism

Schematic diagram of the function of L-carnitine in the transport of fatty acids across the inner mitochondrial membrane. CoA = coenzyme A.

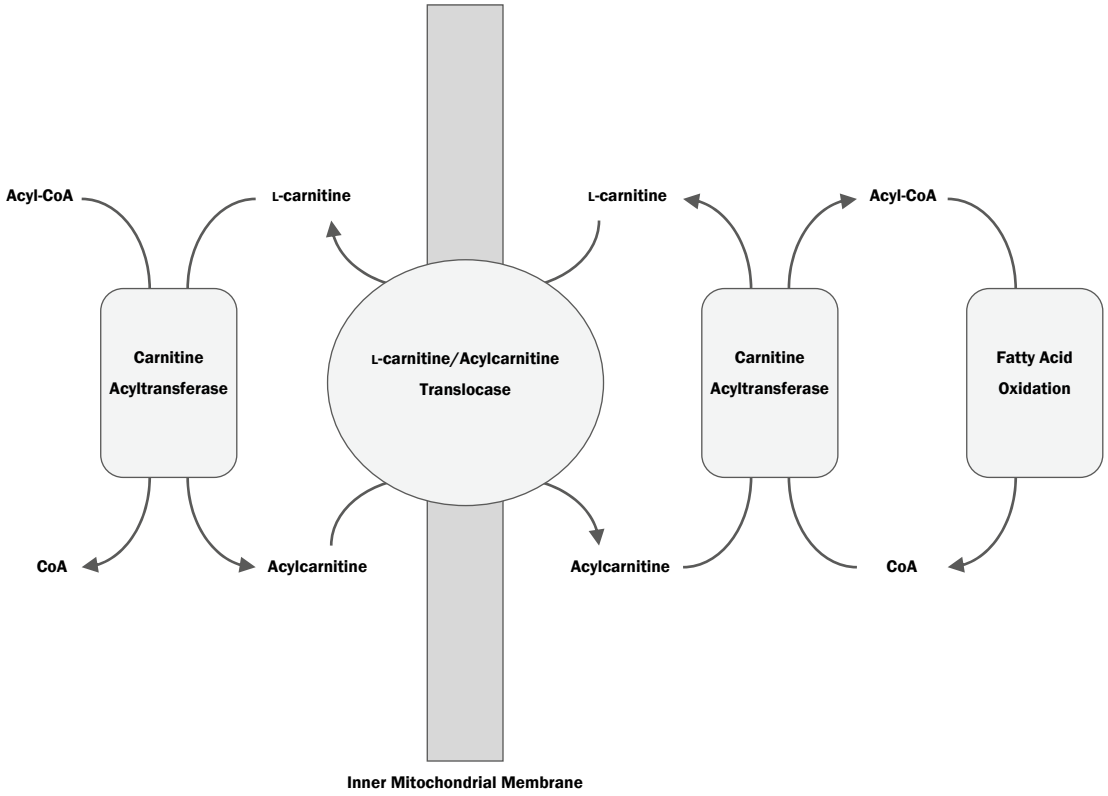


Figure 7.3 Dialysis age and endogenous plasma L-carnitine concentrations

Endogenous plasma L-carnitine levels during the first 12 months of haemodialysis. Endogenous pre-dialysis plasma L-carnitine concentrations were examined in 21 end-stage renal disease patients throughout the first 12 months of dialysis treatment. Plasma L-carnitine concentrations significantly decreased within the first week of dialysis and continued to progressively decline during the first 12 months of treatment.

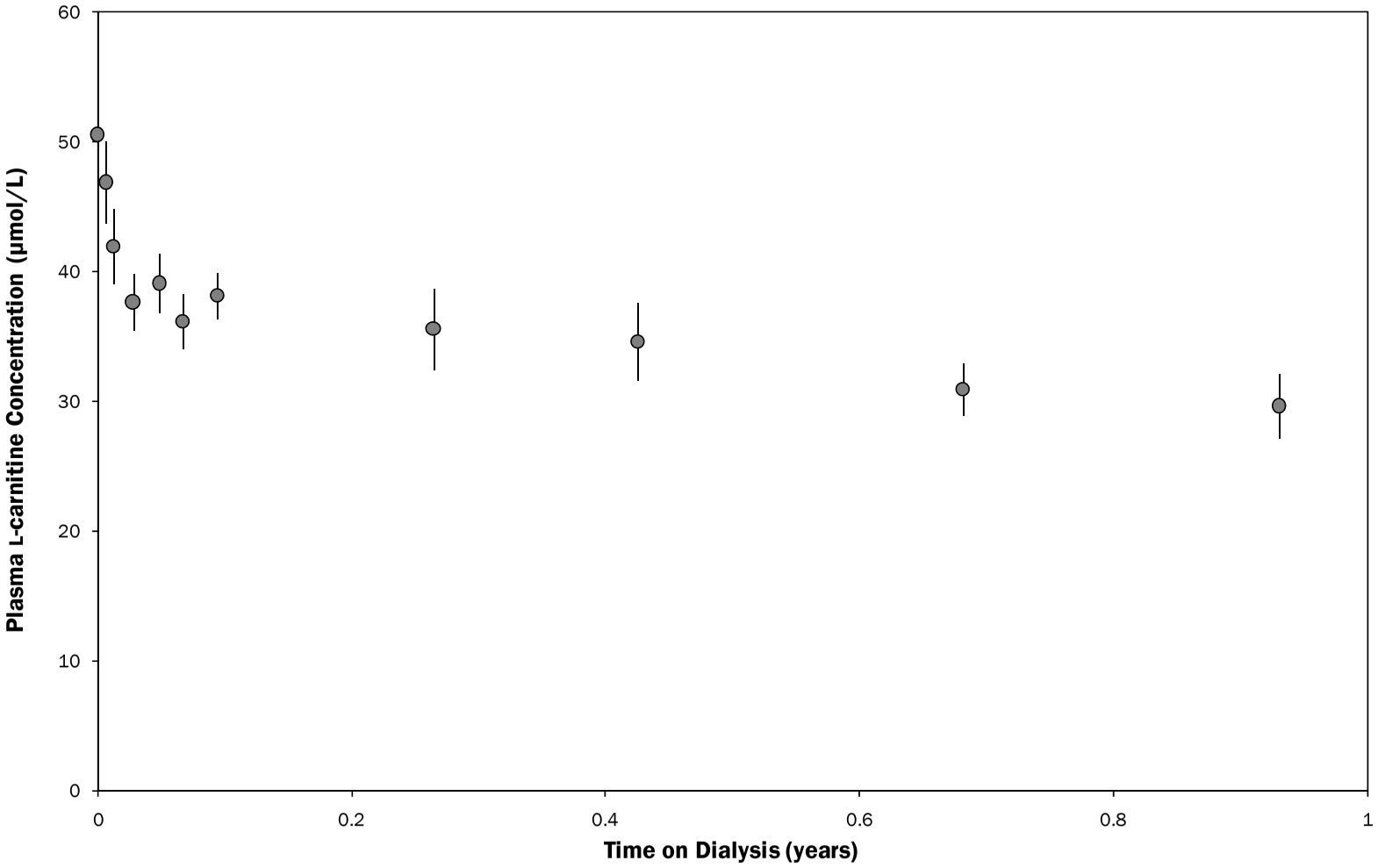


Figure 7.4 Dialysis age and endogenous plasma carnitine pool composition

Impact of haemodialysis on the composition of the plasma carnitine pool. The proportion of endogenous L-carnitine (□), acetyl-L-carnitine (▒) and non-acetyl acylcarnitines (■) within the total carnitine pool was examined in healthy subjects, end-stage renal disease (ESRD) patients not receiving haemodialysis treatment, and ESRD patients undergoing haemodialysis treatment for various lengths of time. An increase in dialysis age is associated with a decrease in the proportion of L-carnitine and an increase in the proportion of non-acetyl acylcarnitines within the total carnitine pool. HD = haemodialysis.

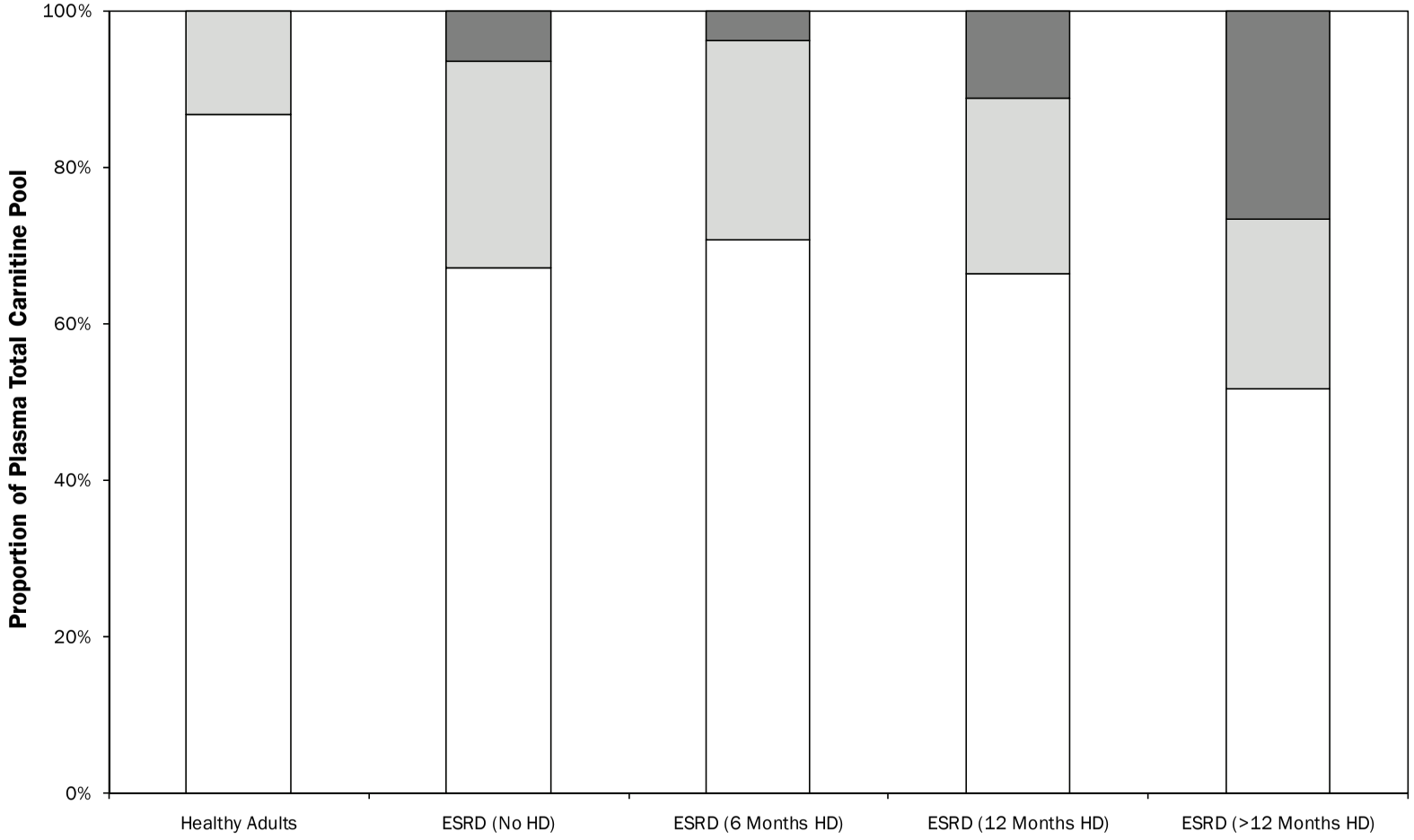


Figure 7.5 Dialytic removal and acylcarnitine carbon-chain length

Relationship between dialytic removal of carnitines and acyl group carbon-chain length. A significant correlation between the ratio of post-dialysis to pre-dialysis acylcarnitine concentrations (as an indicator of dialytic removal) and carbon-chain length of the acyl group has been demonstrated, indicating decreased dialytic removal with increasing chain length. Data expressed as mean ± standard error of the mean.

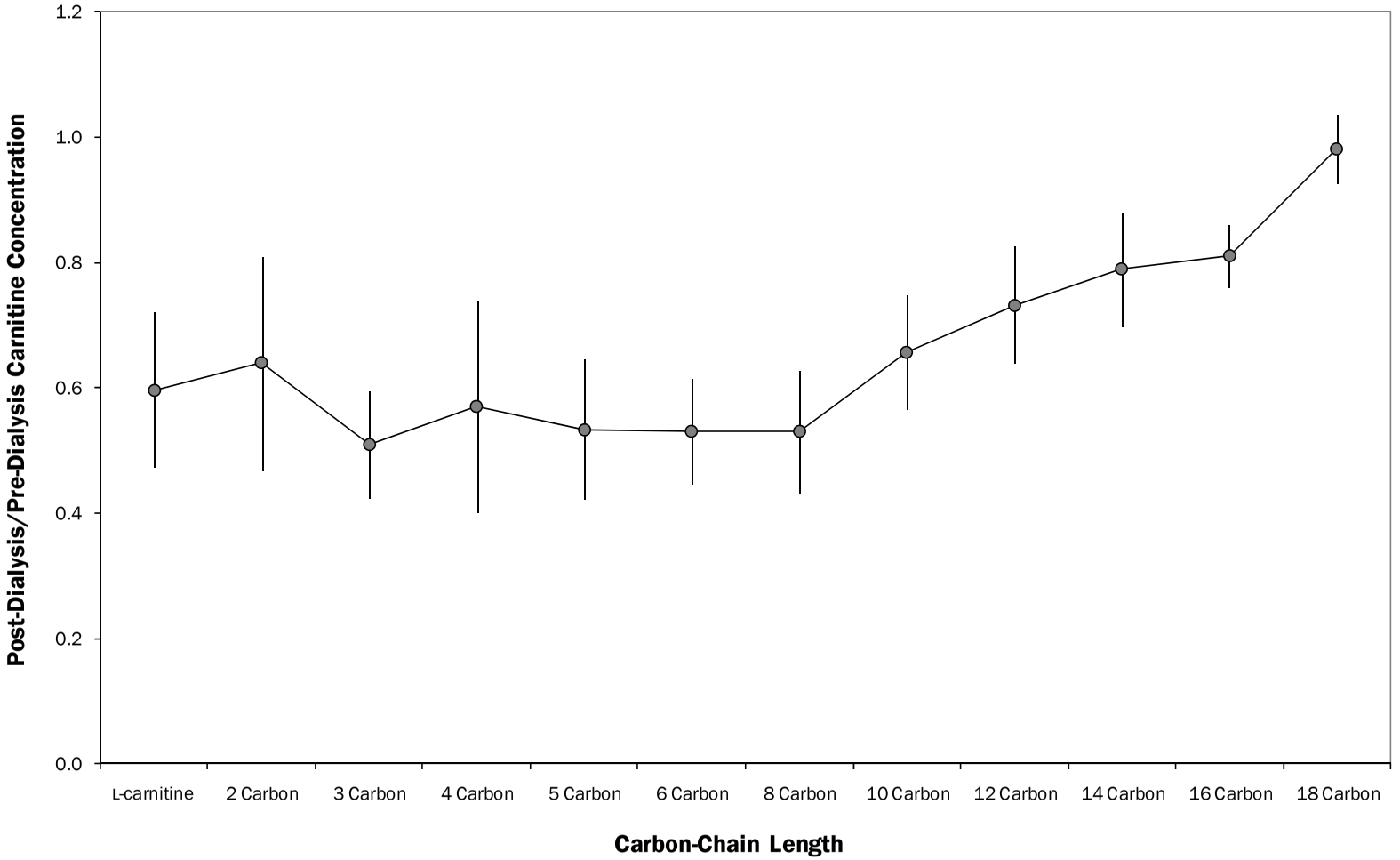
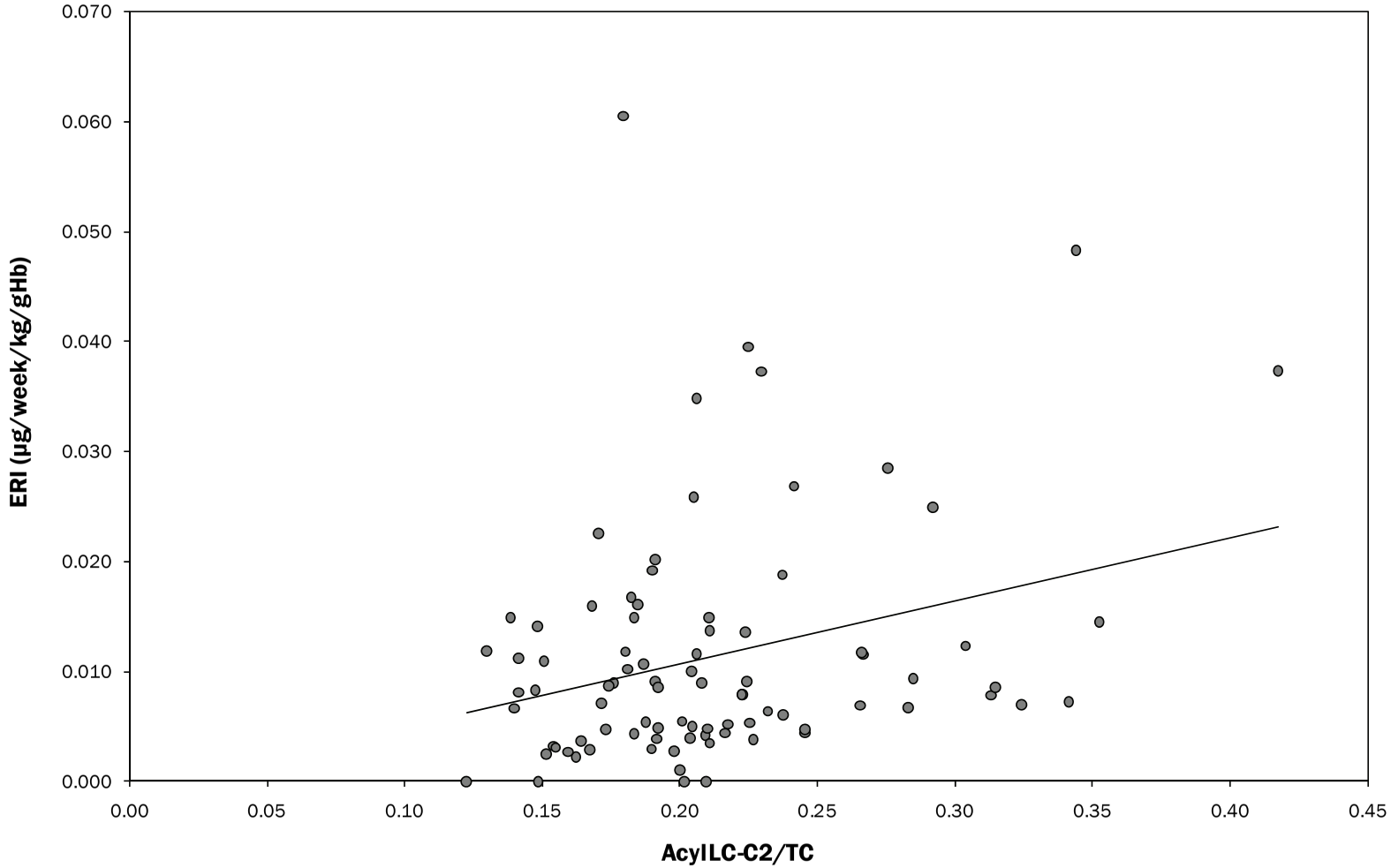


Figure 7.6 Endogenous plasma carnitine pool composition and erythropoietin requirements

Relationship between proportion of non-acetyl acylcarnitines with respect to the total carnitine pool (AcylLC-C2/TC) and erythropoietin resistance index (ERI). A significant relationship ($p = 0.006$) between AcylLC-C2/TC and ERI was demonstrated in 87 end-stage renal disease patients undergoing chronic haemodialysis treatment.



CHAPTER 8

Carnitine Supplementation Guidelines for Haemodialysis Patients

Stephanie E Reuter, Allan M Evans

What is the Value of L-carnitine Level Determination for Carnitine Supplementation in Haemodialysis Patients?

Kidney International; Prepared for Submission

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

Based on previous literature and clinical experience the National Kidney Foundation has recommended the use of L-carnitine supplementation for the treatment of dialysis-related disorders associated with carnitine deficiency; however, treatment is still largely empirical. In January 2003, the Centers for Medicare and Medicaid Services (USA) issued coverage of intravenous L-carnitine for end-stage renal disease patients who have been on dialysis for a minimum of 3 months. Patients must have 'documented carnitine deficiency, defined as a plasma free carnitine level <40μmol/L (determined by a professionally accepted method as recognized in current literature),along with signs and symptoms of: (i) erythropoietin-resistant anaemia (persistent haematocrit <30% with treatment) that has not responded to standard erythropoietin dosage (that which is considered clinically appropriate to treat the particular patient) with iron replacement, and for which other causes have been investigated and adequately treated; or (ii) hypotension on haemodialysis that interferes with delivery of the intended dialysis despite application of usual measures deemed appropriate (e.g., fluid management). Such episodes of hypotension must have occurred during at least 2 dialysis treatments in a 30-day period.

Taking into consideration the more complex pattern of carnitine deficiency which has been established in haemodialysis patients and the lack of a clear association between absolute L-carnitine levels and the severity and/or incidence of dialysis-related conditions, it is proposed that the requirement for patients to document plasma L-carnitine levels <40 μmol/L in order to be eligible for L-carnitine reimbursement is unjustified. Given the excellent safety profile of L-carnitine and the high incidence of carnitine deficiency in haemodialysis patients, we suggest that coverage of treatment based on the presence of symptomology in conjunction with patient dialysis age can have significant economic and clinical implications.

8.1 Commentary

Long-term haemodialysis therapy is a life-saving medical intervention for patients with end-stage renal disease. Despite the medical advancements in the treatment of renal disease, the incidence of conditions such as renal anaemia, cardiac dysfunction and dyslipidaemia is high and the vast majority of patients experience dialytic symptoms such as muscle cramps, fatigue, intradialytic hypotension and restless legs [1, 2].

These dialysis-related conditions are associated with high financial costs, both directly and indirectly. For example, on average, erythropoietin administration for the treatment of renal anaemia can account for up to 10% of the total treatment expenditure for chronic haemodialysis patients [3]. In addition, numerous patients remain hyporesponsive to rHuEPO treatment, despite adequate iron stores. These patients receive a particularly high dose of rHuEPO to achieve clinically acceptable haemoglobin concentrations,

although levels usually remain sub-optimal [4, 5]. The economic impact of treatment of these hyporesponsive patients is considerable due to the higher cost of rHuEPO treatment and the expense associated with inadequate anaemia management. However, the rationale for improving the treatment of renal anaemia is not merely economic, with recent research demonstrating that patients receiving high doses of rHuEPO have a substantially higher mortality rate that those patients on lower doses, irrespective of haemoglobin levels [6]. Similarly, it has been established that these common dialysis-related conditions can have a significant impact on quality-of-life which in-turn has been shown to be associated with poor compliance with dialysis treatment and medication use, leading to a further decline in health [7, 8]. Ultimately it is clear that better treatment of these dialysis-related conditions can have both a significant financial and clinical impact for the treatment of haemodialysis patients.

L-carnitine deficiency in haemodialysis patients has been implicated as a contributing factor in dialysis-related disorders due to its vital role in the utilisation of fatty acids and the safe removal of excess acyl groups from the body [9, 10]. A large number of studies have been conducted which have investigated the potential benefit of L-carnitine supplementation for the treatment of these dialytic conditions; however, for the most part, this body of literature comprises of studies which are poorly designed and inadequately controlled with few blinded, placebo-controlled, large-scale trials (reviewed by Reuter et al. [11] and Miller & Ahmad [12]). Nonetheless, after careful consideration of the published literature and clinical experience, a consensus panel convened by the National Kidney Foundation established practice recommendations for the use of L-carnitine supplementations for dialysis patients with (i) erythropoietin-resistant anaemia, (ii) intradialytic hypotension, (iii) cardiomyopathy, and/or (iv) muscle weakness and fatigue which results in a reduced quality-of-life [13]. Subsequently, commencing in January 2003, the Centers for Medicare and Medicaid Services issued a national coverage determination for the use of intravenous L-carnitine for the treatment of erythropoietin-resistant anaemia and intradialytic hypotension [14]. However, reimbursement is only applicable for patients who have documented carnitine deficiency (arbitrarily) defined as a plasma L-carnitine level <40 μmol/L as determined by a professionally accepted method.

Our group and others have demonstrated that plasma levels of L-carnitine decline by approximately 60-70% during the course of a single dialysis session [15 - 17]. While mobilisation of L-carnitine from tissue stores compensates for this loss, ongoing haemodialysis treatment leads to permanent depletion of both plasma and muscle stores of L-carnitine, such that after 12 months of haemodialysis endogenous plasma and muscle L-carnitine levels are approximately half of those measured prior to the

initiation of haemodialysis treatment[18-20]. On the other hand, compared with the healthy population, acylcarnitine levels are significantly higher in chronic haemodialysis patients, particularly in patients who have been receiving dialysis treatment for at least 12 months[18]. In fact, acylcarnitine levels make up approximately 50% of the total plasma carnitine pool in these patients (of which only half is accounted for by acetyl-L-carnitine); this compares with 15% in healthy subjects (consisting primarily of acetyl-L-carnitine and negligible levels of the non-acetyl acylcarnitines) (Figure 8.1)[18]. More recently, analytical methods have been able to determine the specific pattern of accumulation of individual acylcarnitines in dialysis patients, and whilst most of the quantified acylcarnitines have been shown to be significantly higher than levels in healthy subjects, considerable accumulation of the long-chain esters has been noted[17]. A significant correlation between carbon-chain length of the acyl group and dialytic removal of the acylcarnitines has been observed, with a notable reduction in the removal of medium- and long-chain esters[17].

Given that the pattern of carnitine deficiency in chronic haemodialysis patients is not only characterised by low endogenous L-carnitine levels but also an accumulation of medium- and long-chain acylcarnitine levels such that these patients exhibit a substantially lower proportion of L-carnitine and a higher proportion of acylcarnitines within the total carnitine pool, the appropriateness of demonstrating plasma L-carnitine levels <40 μmol/L for Medicare reimbursement is questioned. Recent research has investigated the relationship between the incidence and/or severity of dialysis-related disorders and composition of the endogenous plasma carnitine pool in a carnitine-naive haemodialysis population[21, 22]. These studies have demonstrated an association between endogenous plasma carnitine pool composition and indicators of erythropoietin resistance and skeletal and cardiac muscle functionality. A highly significant relationship between a patient's endogenous plasma carnitine profile and rHuEPO requirements was demonstrated; specifically, the proportion of non-acetyl acylcarnitines within the total carnitine pool was positively correlated with erythropoietin resistance index (ERI; rHuEPO dose/hg/week normalised for haemoglobin levels), independent of dialysis age[21]. Similarly, patients who experience skeletal and/or cardiac muscle dysfunctionality, specifically muscle fatigue, muscle cramps, cardiac dysfunction and intradialytic hypotension, exhibited a significantly more disturbed carnitine profile than non-symptomatic patients[22]. In all cases, symptomology was associated with alterations in the proportion of L-carnitine and acylcarnitines within the total plasma carnitine pool rather than differences in absolute L-carnitine levels. In light of the lack of clear association between endogenous L-carnitine levels and rHuEPO requirements and incidence of intradialytic hypotension, we propose that the requirement of documented

plasma L-carnitine concentrations <40 µmol/L for Medicare reimbursement in this population is not warranted.

Even if L-carnitine supplementation is only of benefit to symptomatic patients with low endogenous plasma L-carnitine levels, we propose that very few patients who exhibit these dialysis-related conditions would fail to meet the Centers for Medicare and Medicaid Services L-carnitine criteria. In our entire research population (irrespective of symptomology), the incidence of patients with normal L-carnitine levels is very low, with only 6/144 patients who have received dialysis treatment for at least 6 months, and 2/107 patients who have received dialysis treatment for at least 12 months exhibiting levels >40 µmol/L (Figure 8.2). More importantly, in the subgroups of patients who were classified as erythropoietin-resistant (defined as ERI >0.02 µg/kg/week/gHb) and patients who experienced intradialytic hypotension[23], plasma L-carnitine levels were 17.0 ± 5.47 µmol/L and 19.8 ± 6.17 µmol/L respectively, and in all cases patients who displayed symptomology had plasma L-carnitine levels below the Medicare 40 µmol/L cut-off, as well as a proportion of acylcarnitines within the total carnitine pool which was substantially higher than that observed in healthy subjects[21, 22]. However, it is worth noting that this is based on data from carnitine-naive patients; the composition of the carnitine pool and the time-course of carnitine washout in patients who have previously received carnitine supplementation are not known.

Based on these findings, it seems that carnitine profiling adds an additional unjustified expense to the management of these patients. In fact, if we assume that all of our 107 patients who have received dialysis treatment for >12 months displayed the necessary symptomology to meet the Centers for Medicare and Medicaid Services requirements, the associated direct costs of testing these patients and treating only those with levels <40 µmol/L (i.e. 105 patients) with intravenous L-carnitine for 6 months would be greater than the costs of treating all of these patients without testing, based on median test cost of $32.08[24] and treatment costs of $1000 per patient[25].

Taking into consideration the more complex pattern of carnitine deficiency which has been established in haemodialysis patients and the lack of a clear association between absolute L-carnitine levels and the severity and/or incidence of dialysis-related conditions, we propose that the requirement for patients to document plasma L-carnitine levels <40 µmol/L in order to be eligible for L-carnitine reimbursement is unjustified. Given the excellent safety profile of L-carnitine and the high incidence of carnitine deficiency in haemodialysis patients, we suggest that Medicare coverage of treatment based on the presence of symptomology in conjunction with patient dialysis age can

have significant economic and clinical implications; however, additional research establishing the most appropriate dosing regimen in these patients is needed.

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Figure 8.1 Dialysis age and endogenous plasma carnitine pool composition

Impact of haemodialysis on the composition of the plasma carnitine pool[16]. The proportion of endogenous L-carnitine (□), acetyl-L-carnitine (▒) and non-acetyl acylcarnitines (■) within the total carnitine pool was examined in healthy subjects, end-stage renal disease (ESRD) patients not receiving haemodialysis treatment, and ESRD patients undergoing haemodialysis treatment for various lengths of time. An increase in dialysis age is associated with a decrease in the proportion of L-carnitine and an increase in the proportion of non-acetyl acylcarnitines within the total carnitine pool. HD = haemodialysis.

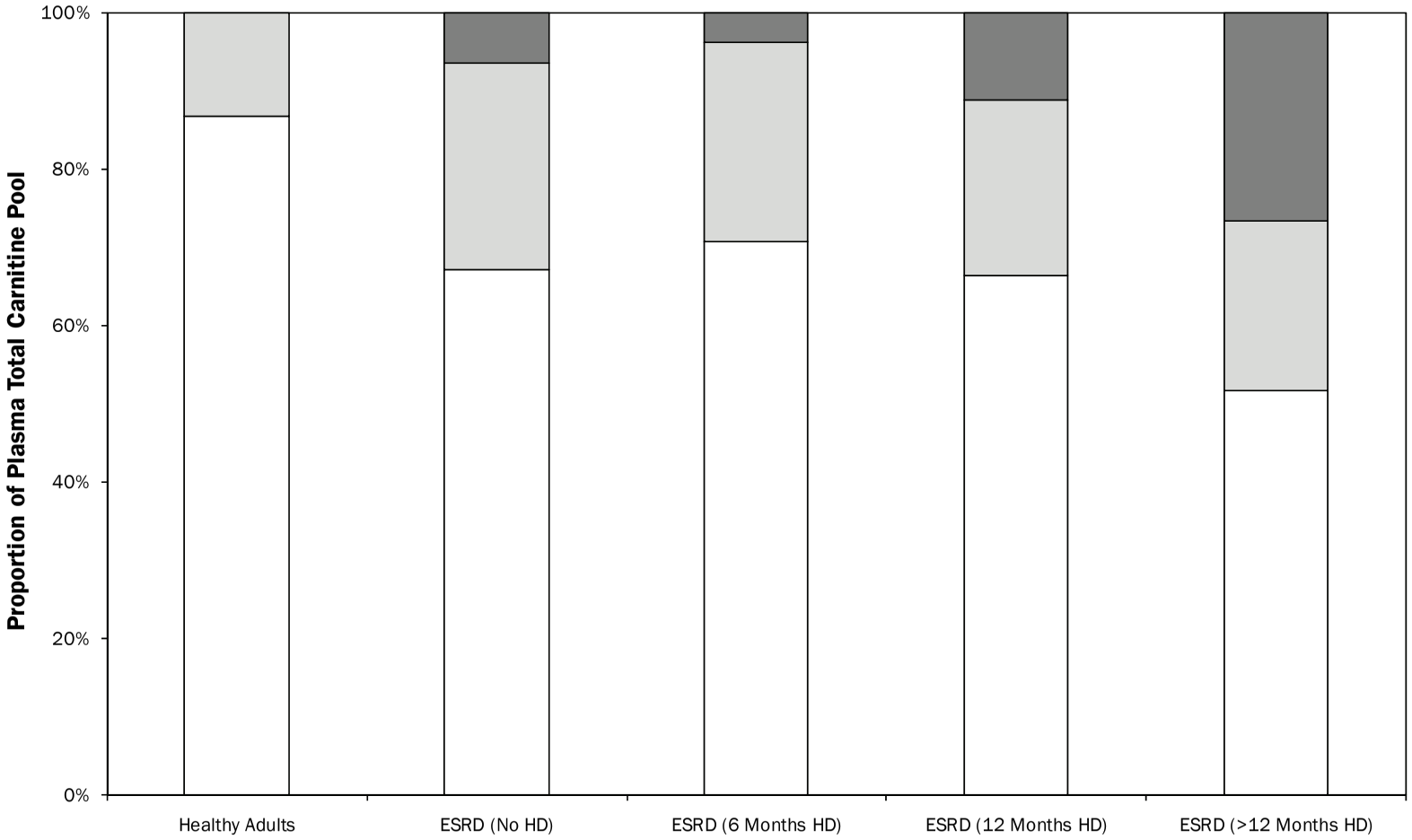
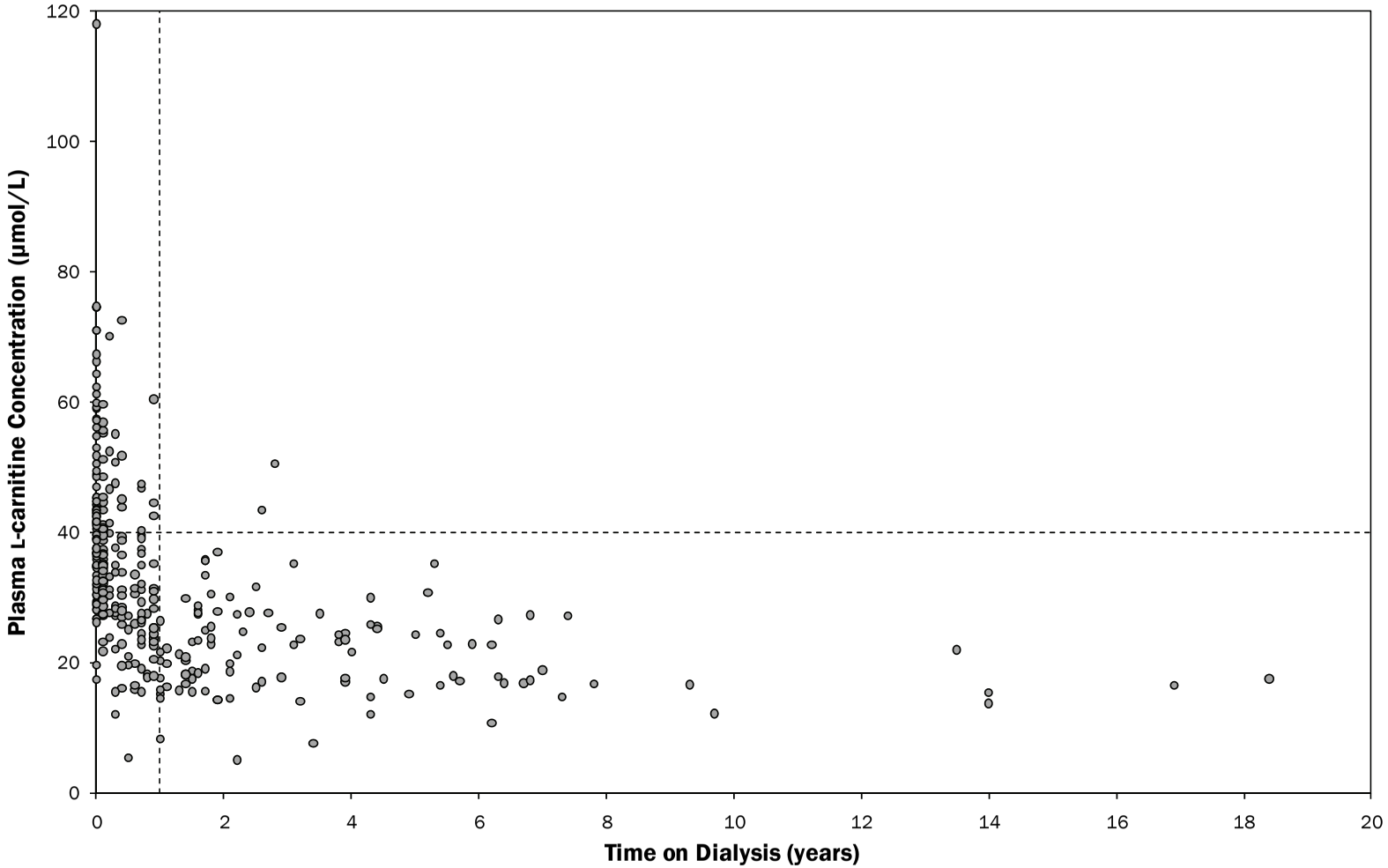


Figure 8.2 Dialysis age and endogenous plasma L-carnitine concentrations

Endogenous plasma L-carnitine levels in end-stage renal disease patients undergoing long-term haemodialysis treatment not receiving carnitine supplementation. A significant relationship between dialysis age and L-carnitine concentrations can be seen and, for the most part, L-carnitine concentrations in patients who had received dialysis treatment for at least 12 months were less than the 40 µmol/L cut-off for Medicare reimbursement.



CHAPTER 9

Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients

Stephanie E Reuter, Allan M Evans

Long-chain Acylcarnitine Deficiency in Patients with Chronic Fatigue Syndrome. Potential Involvement of Altered Carnitine Palmitoyltransferase-I Activity

Journal of Internal Medicine; In Press

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

The research presented in this chapter assessed the potential involvement of disturbed carnitine homeostasis in chronic fatigue syndrome and forms the basis of Section III of the thesis. This study demonstrated that patients with chronic fatigue syndrome exhibit significantly altered concentrations of specific acylcarnitines, in particular substantially reduced levels of oleyl-L-carnitine and linoleyl-L-carnitine. Significant correlations between acylcarnitine concentrations and clinical symptomology were also demonstrated. It is proposed that this disturbance in carnitine homeostasis is reflective of a reduction in carnitine palmitoyltransferase activity, possibly associated with the accumulation of omega-6 fatty acids previously reported in this patient population.

9.1 Summary

Objective: The underlying aetiology of chronic fatigue syndrome is currently unknown; however, in light of carnitine's critical role in mitochondrial energy production, it has been suggested that chronic fatigue syndrome may be associated with altered carnitine homeostasis. This study was conducted to comparatively examine full endogenous carnitine profiles in patients with chronic fatigue syndrome and healthy controls.

Design: A cross-sectional, observational study.

Setting and Subjects: Forty-four patients with chronic fatigue syndrome and 49 age- and gender-matched healthy controls were recruited from the community and studied at the School of Pharmacy and Medical Sciences, University of South Australia.

Main Outcome Measures: All participants completed a fatigue severity scale questionnaire and had a single fasting blood sample collected which was analysed for L-carnitine and 35 individual acylcarnitine concentrations in plasma by tandem mass spectrometry.

Results: Patients with chronic fatigue syndrome exhibited significantly altered concentrations of C8:1, C12DC, C14, C16:1, C18, C18:1, C18:2 and C18:1-OH acylcarnitines; of particular note, oleyl-L-carnitine (C18:1) and linoleyl-L-carnitine (C18:2) were, on average, 30 - 40% lower in patients than controls ($p<0.0001$). Significant correlations between acylcarnitine concentrations and clinical symptomology were also demonstrated.

Conclusions: It is proposed that this disturbance in carnitine homeostasis is reflective of a reduction in carnitine palmitoyltransferase-I activity, possibly a result of the accumulation of omega-6 fatty acids previously observed in this patient population. It is hypothesised that the administration of omega-3 fatty acids in combination with L-carnitine would increase carnitine palmitoyltransferase-I activity and improve chronic fatigue syndrome symptomology.

9.2 Introduction

Chronic fatigue syndrome (also known as myalgic encephalomyletis) is a term used to describe a heterogeneous condition that is primarily characterised by persistent debilitating fatigue that cannot be attributed to any alternative condition [1-4]. Despite being widely recognised, it remains one of the most perplexing conditions in medical science as there is little understanding of its pathogenesis and aetiology. Consequently, there remains no universally effective treatment option and diagnosis is based on the presence of a number of poorly understood signs and symptoms. Additional research to ascertain the underlying pathology of chronic fatigue syndrome is vital to establish appropriate diagnostic criteria as well as effective treatment protocols.

It is estimated that the prevalence of chronic fatigue syndrome is approximately 0.2 - 0.7% in the community and 0.5 - 2.5% in primary care [4] with the condition predominantly affecting young adults (onset 20 - 40 years) with a higher incidence in women (female-male ratio 3:1) [1-4]. Chronic fatigue syndrome has been associated with a significant loss in productivity [5] and a high total annual cost burden [4, 6, 7]. However, it is important to note that the impact of chronic fatigue syndrome is not merely economic with reports that sufferers experience a heavy psychosocial burden and a substantially reduced quality-of-life [8].

A variety of factors have been attributed to the pathophysiology of chronic fatigue syndrome, including immunologic, biochemical, psychiatric and neurological [2, 3]; however, at this time, no firm associations can be drawn. Numerous studies have emphasised a viral cause of chronic fatigue syndrome [2, 3] and recently a link between chronic fatigue syndrome and infection with certain retroviruses has been reported [9], although this result remains controversial [10-14].

L-carnitine is an endogenous compound, found in all mammalian species [15]. Adequate levels of L-carnitine are achieved from dietary sources, in particular from red meat, as well as via biosynthesis in the kidneys, liver and to some extent in the brain [15]. The principal biological role of carnitine is in the transport of fatty acids across the inner mitochondrial membrane for fatty acid oxidation via the reversible binding of acyl groups from coenzyme A (CoA) (Figure 9.1) [16]. It has also been established that carnitine has a role in regulating the cellular to mitochondrial ratio of free CoA to acyl-CoA, in the transport of short and medium acyl groups from the peroxisome to the mitochondria and in the removal of unwanted acyl groups from the body [15, 17]. Alterations in carnitine homeostasis can have a detrimental effect on human health [18, 19]. In its severest form,

carnitine deficiency is associated with progressive cardiomyopathy, encephalopathy and muscle weakness, resulting in death from heart failure [19, 20].

In light of carnitine's critical role in fatty acid oxidation, it has been suggested that chronic fatigue syndrome may be associated with alterations in L-carnitine and acylcarnitine homeostasis [21]. Previous studies have reported a reduction in endogenous plasma L-carnitine (free carnitine), total carnitine and/or acylcarnitine levels in patients with chronic fatigue syndrome [22 - 26]; however, these results have not been replicated in other studies [27, 28]. Whilst these studies have provided useful information on the composition of the endogenous carnitine pool in chronic fatigue syndrome, they have only examined free carnitine and total acylcarnitine levels (i.e. the sum of all individual acylcarnitines) and consequently alterations in the levels of some individual acylcarnitines may be effectively 'cancelled out' by relatively normal levels of other acylcarnitines in these patients. Recently, tandem mass spectrometry methods have been developed which are capable of quantifying individual acylcarnitine levels in human plasma [29, 30] and therefore provide a more adequate representation of the full carnitine profile.

One previous study examined individual plasma acylcarnitine concentrations in patients with chronic fatigue syndrome [31] in which the authors reported no significant differences in acylcarnitine levels between 25 patients and 25 healthy controls. However, it should be noted that in this study, only 20 individual acylcarnitines were quantified, and consequently, the individual acylcarnitines reported accounted for less than two-thirds of the reported total acylcarnitine levels. In addition, the lower limit of quantification of the assay used was well above the levels reported for all of the medium- and long-chain acylcarnitines quantified. Consequently, no firm conclusions regarding carnitine pool composition in patients with chronic fatigue syndrome can be drawn from this study.

The present study was conducted to examine the levels of endogenous plasma L-carnitine and 35 individual acylcarnitines in patients with chronic fatigue syndrome compared with age- and gender-matched healthy controls.

9.3 Methods

9.3.1 Study Design

This study was reviewed and approved by the Human Research Ethics Committee of the University of South Australia. Participants 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).

Patients with chronic fatigue syndrome were recruited via the Chronic Fatigue Syndrome Society of South Australia [ME/CFS Society (SA) Inc]. Patients had been previously diagnosed with chronic fatigue syndrome by a physician according to the standard diagnostic criteria [4]. Age- and gender-matched healthy subjects, with no significant illnesses, were recruited from the general population via advertising. Neither patients nor subjects had received any carnitine supplementation within two months of assessment.

On the study assessment day, each participant completed a fatigue severity scale questionnaire and had a single fasting blood sample collected via venepuncture for carnitine profiling.

9.3.2 Fatigue Severity Scale

The fatigue severity scale is a validated functional measure which comprises nine items that are rated according to a Likert-type rating scale from 1 to 7, with '1' indicating no impairment and '7' indicating severe impairment (Table 9.1) [32]. The fatigue severity scale has been shown to be an appropriate and accurate measure of fatigue severity and symptomology and is able to distinguish between individuals with chronic fatigue syndrome-like symptomology and those individuals with no or varying levels of general fatigue [33].

9.3.3 Carnitine Profiling

A single fasting blood sample was collected from each study participant for analysis of plasma L-carnitine and individual acylcarnitine concentrations. Analysis was conducted by the Department of Genetic Medicine, Women's and Children's Hospital (Adelaide, SA, Australia) using a 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 (synthesised in-house). After a 15 minute 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 precursor scan of 85.1 amu. The levels of acylcarnitines were determined against the respective deuterated stable isotope using Analyst® software (Applied Biosystems Inc).

Individual carnitines analysed are given in Table 9.2. Total acylcarnitine levels were taken as the sum of all individual acylcarnitine concentrations and total carnitine levels were calculated as the sum of L-carnitine and total acylcarnitine concentrations. It should be noted that, because of the nature of the tandem mass spectrometry method, some assay results represent the sum of 2 or 3 carnitine esters; for example, C4 represents the sum of two structural isomers with a 4 carbon-chain acyl group (butyryl-L-carnitine and isobutyryl-L-carnitine).

9.3.4 Statistical Analysis

Unless otherwise indicated, data are expressed as mean ± standard deviation. Carnitine concentrations and demographic characteristics (i.e. age and fatigue severity scale results) obtained from patients with chronic fatigue syndrome were statistically compared to those obtained from healthy subjects using an analysis of variance (ANOVA). Statistical examination of the relationship between carnitine pool composition and fatigue severity was conducted using linear regression of fatigue severity scale results versus endogenous carnitine levels. Gender distribution between the groups was compared using Pearson's Chi-Squared (χ^2) cross-tabulation analysis.

Significance was set at an α-level of 0.05. WinNonlin® Professional Version 5.3 (Pharsight Corporation, Mountain View, CA, USA) was used for ANOVA analysis. SPSS for Windows Version 16.0 (SPSS Inc, Chicago, IL, USA) was used for conduct of the linear regression and Pearson's Chi-Squared (χ^2) cross-tabulation analyses.

9.4 Results

Forty-four patients with chronic fatigue syndrome (17 men; 27 women), with an average age of 49.9 ± 15.0 years, participated in the study. In addition, 49 healthy subjects (20 men; 29 women), aged 45.6 ± 11.6 years, were recruited to serve as controls. Average fatigue severity scale scores for the chronic fatigue syndrome group were 6.22 ± 0.660, compared with scores of 3.04 ± 1.23 for the healthy control group ($p<0.0001$). There were no significant differences in age or gender distribution between the groups.

Endogenous plasma L-carnitine, total carnitine, total acylcarnitine and individual acylcarnitine concentrations for the chronic fatigue syndrome and healthy control groups are presented in Table 9.2. There were no significant differences in L-carnitine, total carnitine or total acylcarnitine levels between the groups, whereas patients with chronic fatigue syndrome had significantly lower C8:1, C14, C16:1, C18, C18:1 and C18:2 concentrations and significantly higher C12DC and C18:1-OH levels than healthy subjects. In most of these cases, endogenous acylcarnitine levels differed by approximately 20% between the groups; however, of particular note, C18:1 and C18:2 were 30 - 40% lower in patients with chronic fatigue syndrome than in healthy controls ($p<0.0001$) (Figure 9.2).

Significant negative correlations between the results of the fatigue severity scale and endogenous plasma concentrations of C8:1, C14, C16:1, C18:1 and C18:2 were observed. Importantly, there were highly significant associations between fatigue severity and C18:1 ($p = 0.0009$, $R = -0.3547$) and C18:2 ($p<0.0001$, $R = -0.4191$) levels. Significant positive correlations between fatigue severity and concentrations of C12DC, C16 and C18:1-OH were demonstrated; however, it should be noted that the data for C12DC was skewed because of two chronic fatigue syndrome patients who exhibited highly elevated levels.

9.5 Discussion

On account of carnitine's critical role in energy metabolism, a number of previous studies have investigated endogenous plasma carnitine levels in patients with chronic fatigue syndrome, with equivocal results. Some studies have suggested that chronic fatigue syndrome is associated with a reduction in endogenous plasma L-carnitine [22, 25, 26] and total carnitine levels [26]; however, these findings have not been replicated in other studies [23, 24, 27, 28, 31]. Similarly, chronic fatigue syndrome has also been associated with a reduction in endogenous plasma (total) acylcarnitine

concentrations[22 - 24, 26], whilst other studies have reported no differences in levels between patients with chronic fatigue syndrome and healthy controls[27, 28, 31]. For instance, Plioplys & Pliopys[26] reported significantly lower endogenous levels of L-carnitine, total carnitine and total acylcarnitines in 35 patients with chronic fatigue syndrome compared with normative data obtained from the Mayo Clinic, whereas in a series of experiments conducted by Kuratsune et al.[22 - 24], the authors reported no differences in L-carnitine and total carnitine levels between patients with chronic fatigue syndrome and healthy controls in their earlier studies whilst their later study noted a significant reduction in L-carnitine levels in the patient population. Significantly lower total acylcarnitine levels in patients with chronic fatigue syndrome were reported in all three studies conducted by Kuratsune et al.[22 - 24]. In contrast, Jones et al.[27] reported no significant differences in L-carnitine, total carnitine and total acylcarnitine levels between 31 patients with chronic fatigue syndrome and 31 healthy controls.

Previous studies have predominantly used enzymatic assays for the quantification of L-carnitine, total carnitine and total acylcarnitine levels. This analysis, whilst useful, does not provide information on individual acylcarnitine levels; it is therefore possible that chronic fatigue syndrome is associated with alterations in the levels of only some acylcarnitines and consequently potential differences may be missed in the examination of total acylcarnitine levels in these studies. Only one previous study has investigated individual acylcarnitine levels in patients with chronic fatigue syndrome[31]. The study reported no significant differences in individual acylcarnitine levels between 25 female patients with chronic fatigue syndrome and 25 female healthy controls; however, only a limited number of individual acylcarnitines were quantified and no firm conclusions can be made. The present study was therefore conducted to examine the levels of endogenous plasma L-carnitine and 35 individual acylcarnitines in patients with chronic fatigue syndrome compared with age- and gender-matched healthy controls.

The present study confirmed that chronic fatigue syndrome is not associated with alterations in L-carnitine, total carnitine or total acylcarnitine levels; however, significant differences in levels between patients and healthy subjects were demonstrated for eight of the 35 of individual acylcarnitines quantified. Of particular note was the substantial reduction in oleyl-L-carnitine (C18:1) and linoleyl-L-carnitine (C18:2) levels in chronic fatigue syndrome, a result which has not been previously demonstrated. Soetekouw et al.[31] failed to find a significant difference in levels between patients and controls for five of the eight acylcarnitines which were found to be altered in this study (the remaining 3/8 were not analysed); this may be contributed to a lack of power. For example, whilst the levels reported for C8:1 are similar to those observed in this study, Soetekouw et al.[31] examined carnitine profiles in just 25 patients and 25 controls and,

based on these subject numbers, we estimate that their study only had 45% power to determine a difference between the groups.

This study has also demonstrated significant relationships between severity of fatigue and endogenous plasma acylcarnitine levels. Importantly, highly significant associations were demonstrated for oleyl-L-carnitine and linoleyl-L-carnitine, with lower levels associated with greater fatigue severity as estimated by the fatigue severity scale. The relationship between fatigue severity and individual acylcarnitine levels in chronic fatigue syndrome has not been previously investigated. Plioplys & Plioplys[26] demonstrated significant negative relationships between L-carnitine and total carnitine concentrations and fatigue severity, but not total acylcarnitine levels. In keeping with the findings of our study, Kuratsune et al.[21] reported that an improvement in the condition of chronic fatigue syndrome patients was associated with a concurrent increase in endogenous plasma acylcarnitine levels, indicating the importance of carnitine homeostasis in the symptomology of chronic fatigue syndrome.

Based on the L-carnitine/acylcarnitine pathway in fatty acid oxidation, it is conceivable that the observed deficiency in long-chain acylcarnitines in chronic fatigue syndrome may be reflective of either (i) an increase in the activity of L-carnitine/acylcarnitine translocase or (ii) a reduction in the activity of carnitine palmitoyltransferase-I (Figure 9.1). An increase in L-carnitine/acylcarnitine translocase activity would result in enhanced long-chain acylcarnitine transfer across the inner mitochondrial membrane and hence an increase in substrate availability for muscle β-oxidation; a scenario that is improbable given the symptomology of chronic fatigue syndrome. More likely is that the deficiency in long-chain acylcarnitines in these patients is suggestive of reduced formation via carnitine palmitoyltransferase-I; in principle, this would result in less acylcarnitines available for transport across the inner mitochondrial membrane. Consequently, there would be a reduction in the amount of acylcarnitines within the mitochondria available for reverse transesterification by carnitine palmitoyltransferase-II and hence a reduction in long-chain fatty acid oxidation (Figure 9.1). In keeping with this, previous research has established that mitochondrial long-chain fatty acid oxidation is reduced in patients with chronic fatigue syndrome[34, 35]. In further support, patients diagnosed with carnitine palmitoyltransferase-II deficiency (in which less substrate is available for β-oxidation within the mitochondria) exhibit remarkably similar symptoms to chronic fatigue syndrome patients including myalgia, cramps, muscle stiffness, painful muscles and muscle weakness[19]. As long-chain fatty acids are the most energy rich substrates for β-oxidation, it is plausible that small changes in acylcarnitine levels would have a significant impact on energy production, leading to fatigue.

The findings of a previous study by Maes et al.[36] further support our hypothesis. In their study, endogenous levels of fatty acids were examined in 22 patients with chronic fatigue syndrome and 12 healthy controls, and it was demonstrated that chronic fatigue syndrome was accompanied by increased levels of omega-6 poly-unsaturated fatty acids and mono-unsaturated fatty acids. Interestingly, of the fatty acids measured by Maes et al.[36] for which the corresponding acylcarnitine was quantified in the present study (C14, C16, C16:1, C18, C18:1, C18:2), for five of the six cases there was a significant reduction in acylcarnitine levels and an increase in corresponding free fatty acid levels (C14, C16:1, C18, C18:1, C18:2). In fact, when our findings are linked with those of Maes et al.[36], it can be speculated that the ratio of free fatty acid to acylcarnitine for these acyl groups is approximately two- to three-fold higher in patients with chronic fatigue syndrome than in healthy controls, indicating a substantial disruption in fatty acid/carnitine homeostasis in these patients. This may because of either (i) a reduction in the activity of acyl-CoA synthase required for the conversion of free fatty acid to acyl-CoA or (ii) reduced activity of carnitine palmitoyltransferase-I. As carnitine palmitoyltransferase-I is the rate-controlling enzyme in mitochondrial fatty acid oxidation[37], it is hypothesised, based on these findings that a reduction in carnitine palmitoyltransferase-I activity contributes to the symptomology of chronic fatigue syndrome.

In keeping with this, high levels of omega-6 fatty acids (such as C18:2 seen in this patient group) have been shown to inhibit carnitine palmitoyltransferase-I activity in rats[38], whereas an increase in the ratio of omega-3 to omega-6 has been shown to increase carnitine palmitoyltransferase-I activity in both rats[39] and healthy volunteers[40, 41]. As L-carnitine is also known to increase carnitine palmitoyltransferase-I activity[42], it is proposed that administration of omega-3 fatty acids in combination with L-carnitine would improve carnitine palmitoyltransferase-I activity in chronic fatigue syndrome, thereby decreasing the ratio of free fatty acid to acylcarnitine and theoretically normalising mitochondrial fatty acid oxidation in these patients.

No studies have been conducted to investigate the impact of administration of L-carnitine in combination with essential fatty acids on chronic fatigue syndrome symptomology. Preliminary exploratory studies have determined the impact of carnitine supplementation (alone) on chronic fatigue syndrome severity; however, no double-blind, placebo-controlled studies have been conducted. In an open-label study, Plioplys & Plioplys[43] demonstrated that L-carnitine supplementation (1 g t.i.d. p.o.) resulted in significant improvements in fatigue severity after 2 months of supplementation. Similarly, in an open-label, randomised study, investigating the effect of administration of acetyl-L-carnitine and/or propionyl-L-carnitine in chronic fatigue syndrome, Vermeulen

& Scholte [44] noted that supplementation with acetyl-L-carnitine (2 g daily p.o. for 24 weeks) resulted in significant improvements in mental fatigue and attention concentration, whilst administration of propionyl-L-carnitine (2 g daily p.o. for 24 weeks) resulted in significant improvements in general and physical fatigue. Although the present study has demonstrated that endogenous levels of these carnitines (i.e. L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine) are not altered in chronic fatigue syndrome, we propose that their administration alters the equilibrium balance between L-carnitine and acylcarnitines via carnitine palmitoyltransferase-I, thereby impacting positively on the levels of acylcarnitines which have been shown to be depleted in this patient population. Previous studies have also demonstrated that administration of essential fatty acids (alone) results in a significant improvement in chronic fatigue syndrome symptomology [45 - 47].

Whilst the cause of this proposed impairment of mitochondrial fatty acid oxidation in patients with chronic fatigue syndrome is unknown, it is possible that this may be linked to the recent finding that chronic fatigue syndrome is associated with an increase in the incidence of viral infection [2, 3, 9]. Previous research has indicated that viral infection may result in altered fatty acid oxidation [48] and therefore, it is feasible that the condition may be triggered by a viral infection which leads to a disruption in free fatty acid and carnitine homeostasis, thereby impacting on the clinical condition of these patients. This hypothesis warrants further investigation.

9.6 Acknowledgements

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Table 9.1 Subjective assessment of fatigue

Fatigue severity scale [32].

Indicate your agreement/disagreement with the following statements on a scale of 1 to 7, with a score of 1 indicating STRONGLY DISAGREE and a score of 7 indicating STRONGLY AGREE.		
1.	My motivation is lower when I am fatigued.	1 2 3 4 5 6 7
2.	Exercise brings on my fatigue.	1 2 3 4 5 6 7
3.	I am easily fatigued.	1 2 3 4 5 6 7
4.	Fatigue interferes with my physical functioning.	1 2 3 4 5 6 7
5.	Fatigue causes frequent problems for me.	1 2 3 4 5 6 7
6.	My fatigue prevents sustained physical functioning.	1 2 3 4 5 6 7
7.	Fatigue interferes with me carrying out certain duties and responsibilities.	1 2 3 4 5 6 7
8.	Fatigue is among my three most disabling symptoms.	1 2 3 4 5 6 7
9.	Fatigue interferes with my work, family or social life.	1 2 3 4 5 6 7

Table 9.2 Endogenous plasma carnitine concentrations and chronic fatigue syndrome

Endogenous plasma carnitine concentrations (μmol/L) for patients with chronic fatigue syndrome (n = 44) and healthy controls (n = 49). Data expressed as mean ± standard deviation. † $p<0.05$ compared to healthy controls.

Endogenous Plasma Carnitine		Healthy Controls	Chronic Fatigue Syndrome Patients	
LC	L-Carnitine	45.2 ± 9.79	45.0 ± 11.3	
TC	Total Carnitine	59.5 ± 12.9	58.8 ± 13.6	
AcylLC	Total Acylcarnitines	14.3 ± 4.13	13.8 ± 3.45	
C2	Acetyl-L-Carnitine	10.6 ± 3.37	10.2 ± 2.72	
C3	Propionyl-L-Carnitine	0.502 ± 0.153	0.489 ± 0.199	
C3DC	Malonyl-L-Carnitine	0.0447 ± 0.0217	0.0416 ± 0.0166	
C4	Butyryl-L-Carnitine	0.255 ± 0.0926	0.250 ± 0.107	
C4-OH	Hydroxy-Butyryl-L-Carnitine	0.0239 ± 0.0117	0.0252 ± 0.0130	
C4DC	Succinyl-L-Carnitine	0.0750 ± 0.0135	0.104 ± 0.135	
C5	Isovaleryl-L-Carnitine	0.111 ± 0.0408	0.103 ± 0.0424	
C5:1	Tiglyl-L-Carnitine	0.0276 ± 0.00787	0.0285 ± 0.0110	
C5-OH	Hydroxy-Isovaleryl-L-Carnitine	0.0352 ± 0.00789	0.0392 ± 0.0103	
C5DC	Glutaryl-L-Carnitine	0.129 ± 0.0419	0.132 ± 0.0575	
C6	Hexanoyl-L-Carnitine	0.0602 ± 0.0249	0.0605 ± 0.0213	
C6:1	Hexenoyl-L-Carnitine	0.0211 ± 0.00818	0.0222 ± 0.00988	
C6DC	Adipyl-L-Carnitine	0.0947 ± 0.0121	0.0971 ± 0.0277	
C8	Octanoyl-L-Carnitine	0.0994 ± 0.0655	0.0953 ± 0.0553	
C8:1	Octenoyl-L-Carnitine	0.178 ± 0.117	0.132 ± 0.0752 †	p = 0.0201
C8DC	Suberyl-L-Carnitine	0.0549 ± 0.00773	0.0601 ± 0.0177	
C10	Decanoyl-L-Carnitine	0.161 ± 0.113	0.154 ± 0.106	
C10:1	Decenoyl-L-Carnitine	0.105 ± 0.0520	0.109 ± 0.0536	
C10:2	Decadienoyl-L-Carnitine	0.0374 ± 0.0184	0.0395 ± 0.0216	
C10DC	Sebacyl-L-Carnitine	0.0970 ± 0.0118	0.0999 ± 0.0162	
C12	Lauroyl-L-Carnitine	0.0668 ± 0.0364	0.0633 ± 0.0276	
C12:1	Dodecenoyl-L-Carnitine	0.0681 ± 0.0424	0.0707 ± 0.0437	
C12DC	Dodecanedioyl-L-Carnitine	0.0782 ± 0.0124	0.108 ± 0.0489 †	p < 0.0001
C14	Myristoyl-L-Carnitine	0.0751 ± 0.0248	0.0612 ± 0.0269 †	p = 0.0023
C14:1	Myristoleyl-L-Carnitine	0.0694 ± 0.0435	0.0651 ± 0.0324	
C14:2	Tetradecadienoyl-L-Carnitine	0.0390 ± 0.0164	0.0398 ± 0.0177	
C14-OH	Hydroxy-Myristoyl-L-Carnitine	0.0149 ± 0.00667	0.0157 ± 0.00605	
C16	Palmitoyl-L-Carnitine	0.352 ± 0.148	0.404 ± 0.220	
C16:1	Palmitoleyl-L-Carnitine	0.0586 ± 0.0276	0.0472 ± 0.0212 †	p = 0.0383
C16-OH	Hydroxyl-Palmitoyl-L-Carnitine	0.0104 ± 0.00361	0.0114 ± 0.00570	
C16:1-OH	Hydroxy-Palmitoleyl-L-Carnitine	0.0193 ± 0.00764	0.0211 ± 0.0111	
C18	Stearoyl-L-Carnitine	0.103 ± 0.0350	0.0874 ± 0.0326 †	p = 0.0104
C18:1	Oleyl-L-Carnitine	0.401 ± 0.170	0.279 ± 0.159 †	p < 0.0001
C18:2	Linoleyl-L-Carnitine	0.232 ± 0.111	0.146 ± 0.0911 †	p < 0.0001
C18:1-OH	Hydroxy-Oleyl-L-Carnitine	0.0178 ± 0.00858	0.0228 ± 0.0116 †	p = 0.0191

Figure 9.1 Role of L-carnitine in fatty acid metabolism

The role of L-carnitine in fatty acid oxidation. CoA = coenzyme A.

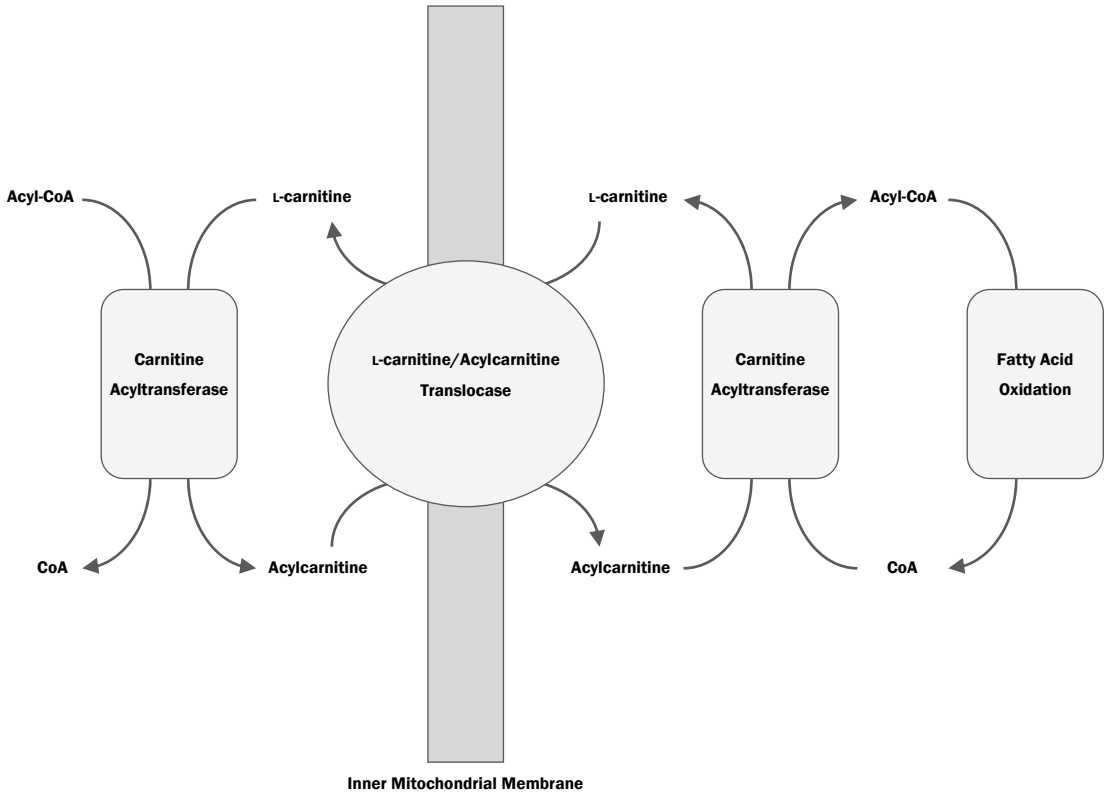
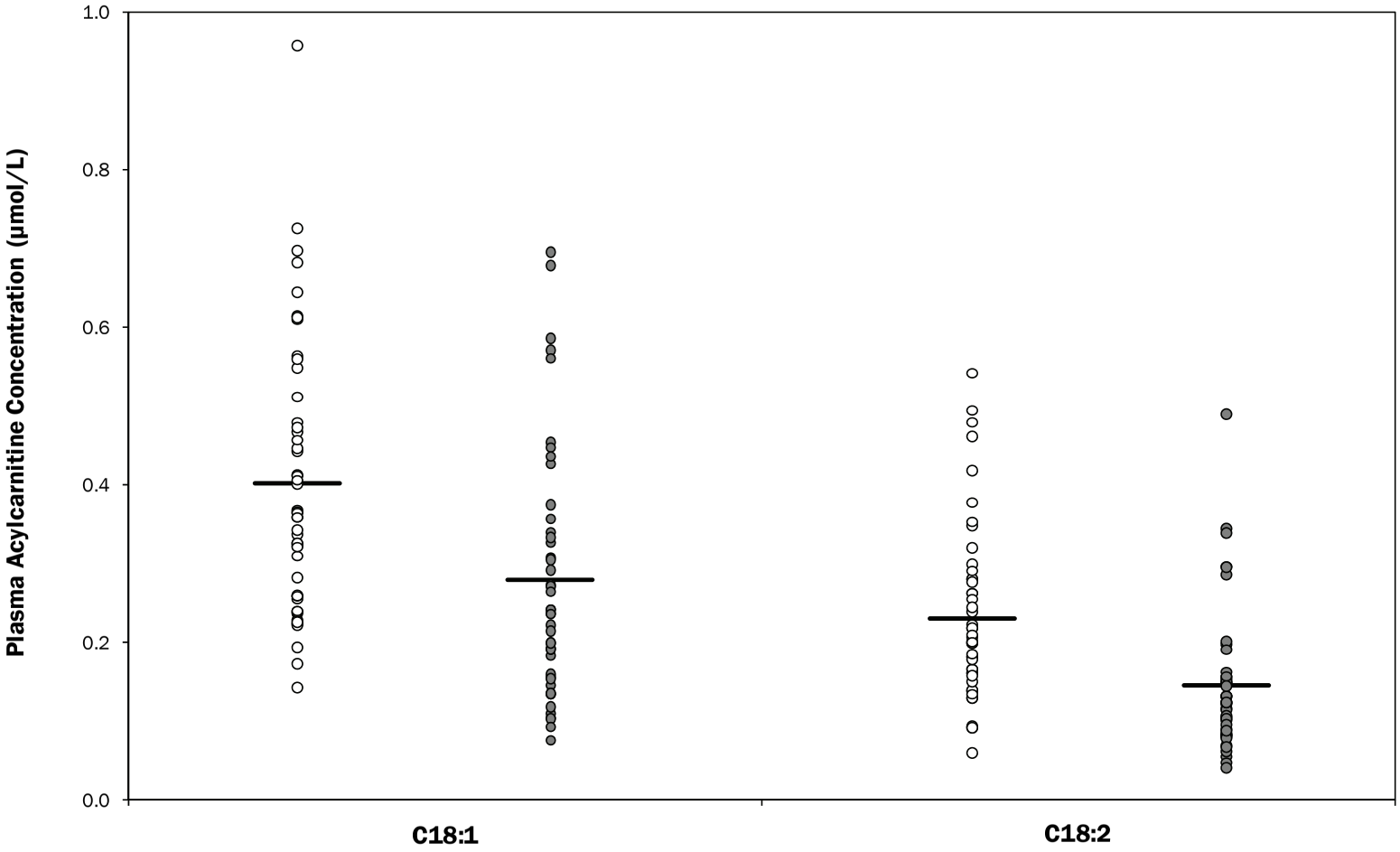


Figure 9.2 Endogenous plasma oleyl-L-carnitine and linoleyl-L-carnitine concentrations and chronic fatigue syndrome

Endogenous plasma oleyl-L-carnitine (C18:1) and linoleyl-L-carnitine (C18:2) concentrations (μmol/L) in patients with chronic fatigue syndrome (●) and healthy controls (○).



CHAPTER 10

General Summary

	CHAPTER 1 Carnitine and Carnitine Esters	**Invited Review** Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.
SECTION I	**CHAPTER 2** Endogenous Plasma Carnitine Levels in Healthy Adults	**Original Publication** Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.
SECTION II	**CHAPTER 3** Endogenous Plasma Carnitine Levels in Haemodialysis Patients	**Original Publication** Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.
	CHAPTER 4 Carnitine and rHuEPO Requirements in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.
	CHAPTER 5 Assessment of Muscle Fatigue	**Original Publication** Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.
	CHAPTER 6 Carnitine and Functionality in Haemodialysis Patients	**Original Publication** Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.
	CHAPTER 7 Carnitine Therapy in Haemodialysis	**Invited Review** Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.
	CHAPTER 8 Carnitine Supplementation Guidelines for Haemodialysis Patients	**Editorial Comment** Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.
SECTION III	**CHAPTER 9** Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients	**Original Publication** Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.
	CHAPTER 10 General Summary	

10.1 Summary

L-carnitine is an endogenous compound found in all mammalian species. The principal biological role of L-carnitine is in the transport of fatty acids into the mitochondrial matrix for β-oxidation, a process that involves the transesterification of activated fatty acids with L-carnitine via carnitine acyltransferases to form free coenzyme A (CoA) and the corresponding acylcarnitine [1-3]. The acylcarnitine can then be transported across the inner mitochondrial membrane by L-carnitine/acylcarnitine translocase [4, 5], where it undergoes reverse transesterification with free CoA to form L-carnitine and acyl-CoA which is then able to enter the fatty acid β-oxidation pathway [2, 6].

More recently, additional functions of the carnitine system have been described [2, 6, 7], including the transport of short- and medium-chain acyl groups from the peroxisome to the mitochondria, the removal of excess acyl groups from the body and modulating intracellular CoA homeostasis such that, by virtue of the dynamic equilibrium between the acylcarnitine/L-carnitine and acyl-CoA/free CoA systems, the intramitochondrial relationship between free CoA and acyl-CoA is reflected in the extramitochondrial ratio of acylcarnitine to L-carnitine [8, 9]. In light of this, acylcarnitines cannot simply be considered by-products of the enzymatic carnitine transfer system, but provide indirect evidence of altered mitochondrial metabolism. Consequently, examination of the contribution of L-carnitine and acylcarnitines to the endogenous carnitine pool (i.e. carnitine pool composition) is critical in order to adequately characterise metabolic status.

The endogenous carnitine pool is comprised of L-carnitine and various acylcarnitines ranging from the short-chain acetyl-L-carnitine to the long-chain stearoyl-L-carnitine [10]. Levels of L-carnitine and acylcarnitines are maintained within relatively narrow limits via a number of homeostatic mechanisms including absorption from dietary sources, *de novo* biosynthesis, active tissue distribution and extensive renal tubular reabsorption of L-carnitine and preferential urinary excretion of acylcarnitines [2, 6, 7].

Carnitine deficiency has been associated with a number of conditions, either the result of disturbances in the dietary intake, biosynthesis, distribution, elimination or utilisation of L-carnitine [7, 9, 11-14]. Despite widespread research examining altered L-carnitine levels in these conditions, relatively few studies have examined the involvement of acylcarnitines and carnitine pool composition.

Recently, tandem mass spectrometry methods have been developed which allow for the accurate and precise quantification of L-carnitine and individual short-, medium- and long-chain carnitine esters, thereby fully characterising carnitine profiles [15]. Consequently, this body of work has investigated disturbances in the relative

composition of the endogenous carnitine pool in patient groups associated with carnitine insufficiency; specifically, end-stage renal disease patients undergoing long-term haemodialysis treatment and patients diagnosed with chronic fatigue syndrome. The relationships between carnitine pool composition and symptomology in these patient groups were also examined.

The impact of chronic haemodialysis on endogenous carnitine levels has been extensively studied [16-20]. Our group and others have previously demonstrated that end-stage renal disease patients undergoing long-term haemodialysis treatment exhibit alterations in carnitine homeostasis, characterised by low L-carnitine levels and accumulation of acylcarnitines [19-25]. This novel project determined the exact nature of these accumulated carnitine esters and measured the efficiency of their removal by the haemodialysis procedure.

In keeping with the findings of previous studies, this research demonstrated that chronic haemodialysis patients, not receiving L-carnitine supplementation, have significantly lower endogenous plasma L-carnitine levels compared to reference data from healthy adult subjects. However, for the first time this study was also able to demonstrate that haemodialysis treatment is associated with substantial elevations in almost all acylcarnitines quantified. While, for the most part, these acylcarnitines were eliminated into the dialysate over the course of a haemodialysis session, post-dialysis concentrations invariably remained higher than levels in the healthy population. The extent of this removal correlated with the carbon-chain length of the acyl group, suggesting that removal of the longer chain esters was impaired due to the increased molecular weight, lipophilicity and protein-binding that accompanies increasing chain length. This inefficient removal by the dialysis procedure is thought to account, at least in part, for the accumulation of medium- and long-chain esters in long-term haemodialysis patients.

The clinical impact of the accumulation of acylcarnitines in haemodialysis patients was examined with respect to common disorders experienced by this patient group. This project was able to demonstrate significant relationships between the relative composition of the endogenous plasma carnitine pool and the severity/incidence of these dialysis-related disorders, including erythropoietin-resistant anaemia, muscle fatigue, cardiac dysfunction, muscle cramps and intradialytic hypotension. Interestingly, in all cases these dialysis symptoms were associated with alterations in the proportion of L-carnitine and acylcarnitines, in particular medium- and long-chain esters, as part of the total plasma carnitine pool rather than the absolute levels of these carnitines, thereby suggesting that the relative composition of the endogenous carnitine pool is

more important than the levels of carnitines alone. These more complex indicators of carnitine pool composition have not been previously investigated.

We hypothesise that these associations between patient symptomology and carnitine profiles may be a result of alterations in carnitine palmitoyltransferase activity. Acylcarnitines, in particular long-chain acylcarnitines, have been shown to inhibit carnitine palmitoyltransferase activity[26 - 32]. Early work conducted by Marquis et al.[28] established that addition of long-chain acylcarnitines to an *in vitro* experimental system resulted in the inhibition of the action of citrate lysase and an increase in the activity of citrate synthase, thus resulting in an increase in citrate levels. Given that citrate regulates that activity of acetyl-CoA carboxylase which catalyses the production of malonyl-CoA from acetyl-CoA, this increase in citrate levels would conceivably result in an increase in the cytosolic content of malonyl-CoA, a known inhibitor of carnitine palmitoyltransferase activity[33 - 37]. On the other hand, L-carnitine has been shown to enhance carnitine palmitoyltransferase activity[38 - 40].

In view of our finding that there is limited removal of medium- and long-chain acylcarnitines over the course of the haemodialysis session, thus leading to accumulation of these esters, we suggest that long-term haemodialysis treatment may be likened to chronic administration of acylcarnitines, or equivalent to the addition of acylcarnitines to an experimental system as examined by Marquis et al[28]. Given that there is also significant loss of L-carnitine with haemodialysis treatment, the action of these accumulated acylcarnitines cannot be sufficiently counteracted by the action of L-carnitine. Accordingly, the progressive accumulation of acylcarnitines and loss of L-carnitine associated with haemodialysis treatment would result in reduced carnitine palmitoyltransferase activity. This reduction in carnitine palmitoyltransferase activity could theoretically lead to impaired utilisation of acyl groups in mitochondrial fatty acid oxidation and membrane turnover which would in-turn contribute to dialysis-related symptomology.

Based on our proposed mechanism of the involvement of carnitine pool composition in the symptomology of dialysis-related disorders, we suggest that L-carnitine supplementation may be beneficial for the treatment of these conditions via an increase in carnitine palmitoyltransferase activity. Theoretically, administration of L-carnitine would result in a normalisation in the proportion of L-carnitine and acylcarnitines within the total carnitine pool and thus providing a better 'balance' between acylcarnitine inhibition and L-carnitine activation of carnitine palmitoyltransferase.

A large number of clinical trials have been conducted to assess the efficacy of supplemental L-carnitine in haemodialysis patients; however, many of these studies have been small, retrospective trials and few blinded, placebo-controlled, large-scale trials have been conducted. Interestingly, while studies have demonstrated an overall improvement with L-carnitine supplementation, a number have studies have indicated that the patient population appears to be divided into 'responders' and 'non-responders'; however, no clear distinction between these two groups has been proposed [41-44]. It is possible that an individual's responsiveness to L-carnitine supplementation may be related to extent of normalisation of carnitine pool composition and therefore improvement in carnitine palmitoyltransferase activity. The relationship between the composition of the carnitine pool and the efficacy of exogenous L-carnitine for the treatment of erythropoietin-resistant anaemia, muscle and cardiac dysfunction and poor quality-of-life in haemodialysis patients is currently being investigated in a randomised, double-blind, placebo-controlled clinical trial supported by the National Health and Medical Research Council (Australia).

Intravenous L-carnitine supplementation is currently approved for use and covered by the Centers for Medicare and Medicaid Services (USA) for the treatment of erythropoietin-resistant anaemia and/or intradialytic hypotension in end-stage renal disease patients undergoing long-term haemodialysis treatment. However, in order to be eligible for reimbursement of treatment by Medicare, patients must have 'documented carnitine deficiency, defined as a plasma free carnitine level <40 μmol/L'. Taking into consideration the more complex pattern of carnitine deficiency in haemodialysis patients and the lack of clear association between absolute L-carnitine levels and patient symptomology we have established as part of this project, we propose that the requirement of documented plasma L-carnitine concentrations <40 μmol/L for Medicare reimbursement in this population is not warranted. Based on our findings, it seems that carnitine profiling adds an additional unjustified expense to the management of these patients and coverage based on the presence of symptomology in conjunction with patient dialysis age can have significant economic and clinical implications.

Whilst numerous studies have determined that chronic haemodialysis treatment is associated with carnitine insufficiency, the involvement of disturbances in carnitine homeostasis in chronic fatigue syndrome is more controversial. Previous studies have reported that patients with chronic fatigue syndrome have significantly lower L-carnitine, total carnitine and/or (total) acylcarnitine levels than healthy subjects [45-49]; however, these results have not been replicated in other studies [50, 51]. This project examined the levels of endogenous plasma L-carnitine and individual acylcarnitines in patients with chronic fatigue syndrome utilising reference data from healthy adult subjects. No

significant differences in L-carnitine, total carnitine and total acylcarnitine concentrations were found; however, for the first time, this study demonstrated that patients with chronic fatigue syndrome exhibit significantly lower levels of octanoyl-L-carnitine, myristoyl-L-carnitine, palmitoleyl-L-carnitine, stearoyl-L-carnitine, oleyl-L-carnitine and linoleyl-L-carnitine and elevated levels of dodecanedioyl-L-carnitine and hydroxyl-oleoyl-L-carnitine compared to age- and gender-matched healthy controls. Significant correlations between the levels of these acylcarnitines and fatigue severity were demonstrated.

Previous research has demonstrated that chronic fatigue syndrome is accompanied by increased levels of omega-6 poly-unsaturated fatty acids and mono-unsaturated fatty acids[52]. Interestingly, of the fatty acids measured for which the corresponding acylcarnitine was quantified in our study, for five of the six cases there was a significant reduction in acylcarnitine levels and an increase in corresponding free fatty acid levels. In fact, when the findings of these studies are linked, it can be speculated that the ratio of free fatty acid to acylcarnitine for these acyl groups is approximately two- to three-fold higher in patients with chronic fatigue syndrome than in healthy controls, indicating a substantial disruption in the conversion of fatty acids to acylcarnitines in these patients. As carnitine palmitoyltransferase is the rate-controlling enzyme in mitochondrial fatty acid oxidation[53], it is hypothesised that a reduction in the activity of carnitine palmitoyltransferase contributes to the symptomology of chronic fatigue syndrome. In keeping with this, high levels of omega-6 fatty acids (as documented in this patient group) have been shown to inhibit carnitine palmitoyltransferase activity in rats. Conversely, an increase in the ratio of omega-3 to omega-6 has been shown to increase carnitine palmitoyltransferase activity in both rats and healthy volunteers. As L-carnitine is also known to increase carnitine palmitoyltransferase activity, it is proposed that administration of omega-3 fatty acids in combination with L-carnitine would improve carnitine palmitoyltransferase activity in chronic fatigue syndrome, thereby decreasing the ratio of free fatty acid to acylcarnitine and theoretically normalising mitochondrial fatty acid oxidation in these patients. Studies investigating the potential benefit of combined omega-3 and L-carnitine treatment in chronic fatigue syndrome are currently being designed.

It is important to point out that although the patient groups examined as part of the project display vastly different endogenous carnitine profiles (one group with elevated acylcarnitines and low L-carnitine levels and the other with low acylcarnitine concentrations), we have hypothesised that patient symptomology in both of these populations can be attributed to reduced carnitine palmitoyltransferase activity. However, it is important to note that the mechanism by which we propose that this

occurs is different between the patient groups. In chronic haemodialysis patients we suggest that disturbed carnitine homeostasis, a result of the extensive clearance of L-carnitine and limited removal of acylcarnitines by the dialysis procedure, results in a reduction in the activity of carnitine palmitoyltransferase. On the other hand, we propose that the deficiency of acylcarnitines observed in patients with chronic fatigue syndrome is reflective of reduced carnitine palmitoyltransferase activity. That is, altered carnitine pool composition in haemodialysis patients causes a reduction in carnitine palmitoyltransferase activity, whereas reduced carnitine palmitoyltransferase activity causes altered carnitine pool composition in patients with chronic fatigue syndrome. Further investigations of these proposed mechanisms are needed.

While this series of studies has demonstrated that disturbances in carnitine pool composition identified in patients undergoing haemodialysis treatment and patients with chronic fatigue syndrome display significant associations with clinical symptomology, these results should be interpreted with caution. It should be noted that this project has not demonstrated that alterations in carnitine profiles result in patient symptoms and other factors may account for these relationships. For example, previous research in haemodialysis patients has demonstrated a negative correlation between endogenous carnitine levels and duration of dialysis treatment (i.e. dialysis age)[20]; given that clinical symptoms are likely to worsen with increasing time on dialysis treatment, a relationship between carnitine profile and patient functionality could be accounted for by dialysis age. Although the relationships between carnitine pool composition and dialysis-related conditions in this project were found to be independent of dialysis age, other factors such as diet and underlying conditions have not been taken into account and cannot be discounted. Nonetheless, while the relationships demonstrated as part of this research project do not imply causality, the results of these studies form a basis from which further investigations into the benefits of treatment for these patient groups can be investigated.

Future studies should determine whether some aspect of carnitine profile can be a useful predictor of responsiveness to specific interventions such as L-carnitine replacement and/or supplementation of certain fatty acids. One such study, interrogating the impact of intravenous L-carnitine on the composition of the carnitine pool and patient response in chronic haemodialysis patients is currently being conducted by our group.

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Appendix 1: Authorship and Contributorship

CHAPTER 1: Carnitine and Carnitine Esters

Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. [Invited Review] *Curr Drug Metab*: prepared for submission.

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CHAPTER 2: Endogenous Plasma Carnitine Levels in Healthy Adults

Reuter SE, Evans AM, Chace DH, Fornasini G. Determination of the reference range of endogenous plasma carnitines in healthy adults. *Ann Clin Biochem* 2008; 45(6): 585-592.

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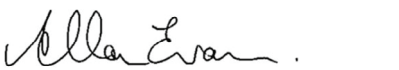
- Substantial contribution to conception and design
- Critical revision of the manuscript for important intellectual content
- Final approval of the manuscript

Declared:



Stephanie E REUTER LANGE

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CHAPTER 3: Endogenous Plasma Carnitine Levels in Haemodialysis Patients

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.

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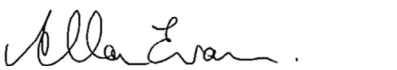
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CHAPTER 4: Carnitine and rHuEPO Requirements in Haemodialysis Patients

Reuter SE, Faull RJ, Ranieri E, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(3): 990-996.

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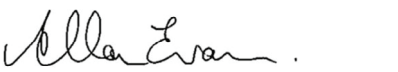
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- Final approval of the manuscript

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CHAPTER 5: Assessment of Muscle Fatigue

Reuter SE, Massy-Westropp N, Evans AM. Reliability and validity of indices of hand-grip strength and endurance. *Aust Occup Ther J* 2011; 58(2): 82-87.

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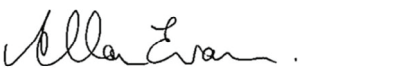
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CHAPTER 6: Carnitine and Functionality in Haemodialysis Patients

Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.

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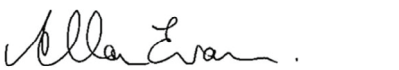
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- Critical revision of the manuscript for important intellectual content
- Final approval of the manuscript

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CHAPTER 7: Carnitine Therapy in Haemodialysis

Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the haemodialysis population - Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.

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- Substantial contribution to conception and design
- Analysis and interpretation of data
- Drafting of the manuscript
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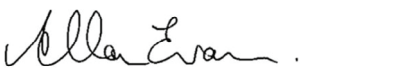
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CHAPTER 8: Carnitine Supplementation Guidelines for Haemodialysis Patients

Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.

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- Analysis and interpretation of data
- Drafting of the manuscript
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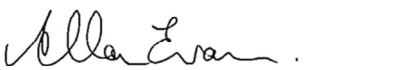
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- Final approval of the manuscript

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CHAPTER 9: Endogenous Plasma Carnitine Levels in Chronic Fatigue Syndrome Patients

Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in chronic fatigue syndrome patients. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.

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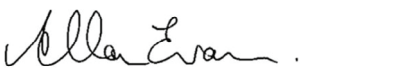
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Appendix 2: Publications in Support of Thesis

ORIGINAL PUBLICATION

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease. *Ann Clin Biochem* 2005; 42(5): 387-393.

Impact of haemodialysis on individual endogenous plasma acylcarnitine concentrations in end-stage renal disease

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Abstract

Background Patients with end-stage renal disease (ESRD) undergoing long-term haemodialysis exhibit low L-carnitine and elevated acylcarnitine concentrations. This study evaluated endogenous concentrations of an array of acylcarnitines (carbon chain length up to 18) in healthy individuals and ESRD patients receiving haemodialysis, and examined the impact of a single haemodialysis session on acylcarnitine concentrations.

Methods Blood samples were collected from 60 healthy subjects and 50 ESRD patients undergoing haemodialysis (pre- and post-dialysis samples). Plasma samples were analysed for individual acylcarnitine concentrations by electrospray MS/MS.

Results Of the 31 acylcarnitines, 29 were significantly ($P < 0.05$) elevated in ESRD patients compared with healthy controls; in particular, C5 and C8:1 concentrations were substantially elevated. For acylcarnitines with a carbon chain length less than eight, plasma acylcarnitine concentrations decreased significantly over the course of a single dialysis session; however, post-dialysis concentrations invariably remained significantly higher than those in healthy subjects. Dialytic removal of acylcarnitines diminished once the acyl chain length exceeded eight carbons.

Conclusions The accumulation of acylcarnitines during long-term haemodialysis suggests that removal by haemodialysis is less efficient than removal from the body by the healthy kidney. Removal is significantly correlated to acyl chain length, most likely due to the increased molecular weight and lipophilicity that accompanies increased chain length.

Ann Clin Biochem 2005; **42**: 387–393

Introduction

L-Carnitine (free carnitine) is a low-molecular weight endogenous compound present in all mammalian species.[1] The primary role of L-carnitine is to facilitate the transfer of fatty acids across the inner mitochondrial membrane for β-oxidation and consequently to produce energy.[2] L-Carnitine also acts to maintain inner mitochondrial concentrations of free CoA by accepting short-chain acyl groups from the corresponding acyl-CoA and transporting them out of the mitochondria.[1,3] Due to this critical role in fatty acid oxidation, endogenous concentrations of L-carnitine and its acyl derivatives are maintained within relatively narrow limits.[4,5] The majority of the endogenous carnitine pool is located within the skeletal and cardiac muscle (approximately 98%), which is reflective of the importance of L-carnitine in these primary sites of fatty acid oxidation.[4–6] Less than 1% of the body's total carnitine pool is located within the plasma.

L-Carnitine concentrations are achieved by absorption from dietary sources, in particular red meat, and by endogenous biosynthesis from lysine.[1] Most human tissues contain the necessary enzyme to convert lysine to trimethyllysine and subsequently butyrobetaine; however, the enzymes required for the final stage of L-carnitine biosynthesis are only present in the liver, kidney and brain.[1] The plasma is therefore of critical

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importance for the delivery of L-carnitine from the site of synthesis to the skeletal and cardiac muscle for storage and use.[4,5]

Given that urinary excretion is the primary route of loss of L-carnitine and its esters from the body, patients with end-stage renal disease (ESRD) not receiving dialysis treatment exhibit significantly higher plasma concentrations of L-carnitine and its acyl derivatives than the healthy population.[4,5,7–10] Bartel *et al.*[7] examined the composition of the endogenous serum carnitine pool in four male and four female ESRD patients not receiving dialysis treatment (age 19–42 years), and 64 healthy subjects (age 18–62 years; 30 men and 34 women). Serum L-carnitine concentrations were significantly higher in the ESRD patient population, with concentrations approximately two-fold greater than those measured in the healthy controls. However, no significant differences in total acylcarnitine concentrations were reported between the two subject groups. Rodriguez-Segade *et al.*[9] published a similar study in which plasma carnitine concentrations in 34 ESRD patients aged 22–60 years (23 men and 11 women) were compared with those in 49 healthy subjects aged 20–56 years (30 men and 19 women). This study reported significantly higher L-carnitine and total carnitine concentrations in the ESRD group, with concentrations 35% and 74% greater than those measured in the healthy controls. In contrast to Bartel *et al.*,[7] however, total acylcarnitine concentrations were markedly elevated, with concentrations 4.6 times greater in the ESRD patients than the healthy subjects (28.7 ± 16.5 μmol/L compared with 6.2 ± 2.4 μmol/L). Comparable results were reported by Wanner *et al.*,[10] in which carnitine concentrations were 1.7 and 1.9 times higher (for L-carnitine and total carnitine, respectively) in ESRD patients not undergoing haemodialysis treatment than in the healthy population. Both short-chain and long-chain acylcarnitine concentrations were significantly elevated, with ESRD patients exhibiting concentrations 2.6 and 3.2 times higher (for short-chain and long-chain acylcarnitines, respectively) than the healthy subjects.

Conversely, chronic haemodialysis treatment has been shown to be associated with a reduction in plasma and tissue L-carnitine concentrations and accumulation of acylcarnitines.[4–20] Bohmer *et al.*[14] were the first to demonstrate that L-carnitine is extensively removed by the dialyser during a single dialysis session, probably due to the compound's low molecular weight and high hydrophilicity in combination with its lack of protein binding. It was later described that the dialytic extraction ratio for L-carnitine is approximately 74%.[15] Changes in endogenous carnitine pool composition in this patient group have been widely investigated. Golper *et al.*[16] found no significant differences in total carnitine concentrations between ESRD patients on haemodialysis and the healthy population; however, L-carnitine concentrations were reduced by approximately 35% and both short-chain and long-chain acylcarnitine concentrations were significantly elevated (3.5 and 1.5 times higher for short-chain and long-chain acylcarnitines, respectively). Rodriguez-Segade *et al.* reported in two papers[9,11] that total carnitine concentrations were slightly elevated in the ESRD patients receiving haemodialysis treatment (although not significantly); however, L-carnitine concentrations were diminished by approximately 17% and total acylcarnitine concentrations were almost four-fold higher in this patient group. Similar results were reported by Wanner *et al.*,[10,19] with no differences in total carnitine concentrations between the two study populations but significantly lower L-carnitine and significantly higher total acylcarnitine concentrations in the haemodialysis patients. In these studies, both short- and long-chain acylcarnitines were found to be elevated in the ESRD patients, with a two-fold increase in both instances. A number of other research groups have investigated the effect of haemodialysis on endogenous plasma carnitine concentrations, with comparable results.[13,15,17,18]

Recent research by our group has investigated the relationship between the duration of haemodialysis treatment (dialysis age) and carnitine status.[21] The study demonstrated a significant negative correlation between plasma L-carnitine concentrations and dialysis age, with the majority of the reduction in L-carnitine concentrations occurring within the first few months of haemodialysis treatment. The decline in plasma L-carnitine with increasing dialysis age was accompanied by a similar reduction in plasma acetyl-L-carnitine concentrations (acetyl-L-carnitine is the most abundant acylcarnitine in the body); however, acetyl-L-carnitine concentrations remained above the reference range for healthy individuals. Compared with the healthy population, acylcarnitine concentrations (calculated as total carnitine subtracted by L-carnitine concentrations) were shown to be significantly higher in ESRD patients, particularly in those patients who had been receiving haemodialysis treatment for more than 12 months.

Until recently, measurements of acylcarnitines have given values for the sum of all of the carnitine esters or the sum of all short-chain and/or long-chain acylcarnitines. Chace *et al.*[22] has developed an electrospray tandem mass spectrometry method in which individual acylcarnitines can be quantified in human blood. The present study evaluated the concentrations of L-carnitine and an array of short-, medium- and long-chain carnitine esters in healthy individuals and ESRD patients receiving haemodialysis. For the first time, the impact of a single haemodialysis session on individual plasma acylcarnitine concentrations was examined.

Methods

Study design

This study was reviewed and approved by the Royal Adelaide Hospital Research Ethics Committee and the Human Research Ethics Committee of the University of South Australia. Patients were recruited from the Royal Adelaide Hospital Dialysis Unit and its associated satellite dialysis centres. Healthy subjects were recruited from the general population via advertisement, and were assessed at the Centre for Pharmaceutical Research of the University of South Australia. All subjects were enrolled into the study once it had been established that they were not receiving any carnitine supplementation. Patients and healthy subjects were fully informed of the study procedures and provided written informed consent prior to study participation.

Healthy subjects

Sixty healthy subjects (Table 1) were selected for study participation. Subjects attended the study centre on one occasion for collection of a single 20 mL blood sample.

ESRD patients

Fifty ESRD patients receiving haemodialysis treatment (Table 1) were recruited to participate in the study. Arterial blood samples (5 mL) were collected from the arteriovenous fistula on one occasion prior to commencement of dialysis (pre-dialysis) and after completion of the dialysis session (post-dialysis).

Analytical methods

Plasma samples were analysed for L-carnitine and acylcarnitine profiles using an electrospray tandem mass spectrometry (API/Sciex MDS 3000) assay, based on the method reported by Chace *et al.*[22] but with modifications. The target biological matrix was modified to plasma rather than blood spots. An improved quantitative analysis was achieved using dried plasma spots as haematocrit and spot diameter have less effect on variations in the actual volume of the matrix for plasma compared with blood. Dialysed plasma (prepared as described[15,23]) was used for the preparation of calibration curves. Overall, from repeat analysis, the assay was shown to have a precision and accuracy within 9%.

Individual acylcarnitines analysed are detailed in Table 2. It should be noted that, due to the nature of the tandem mass spectrometry method, some assay results represent the sum of two or three carnitine esters; for example, C4 represents the sum of two structural isomers with a four-carbon chain acyl group (butyryl-L-carnitine and isobutyryl-L-carnitine) (Table 2).

Table 1 *Subject demographic and dialysis data: mean ± SD (range)*

	ESRD patients	Healthy subjects
Subjects (*n*)	50	60
Age (years)	56 ± 15 (22–71)	39 ± 14 (18–76)
Gender	30 men/20 women	30 men/30 women
Dialysis age (years)	3.15 ± 3.07 (0.13–18.43)	NA
Dialysis session duration (h)	4.00 ± 0.59 (2.50–5.15)	NA

Table 2 *Acylcarnitines measured as part of the electrospray tandem mass spectrometry method*

Abbreviation	Carnitine name
LC	L-carnitine
TC	Total carnitine
C2	Acetyl-L-carnitine
C3	Propionyl-L-carnitine
C4	Butyryl-L-carnitine/isobutyryl-L-carnitine
C4-OH	3-OH-butyryl-L-carnitine
C4DC	2-methylmalonyl-L-carnitine
C5	Valeryl-L-carnitine/isovaleryl-L-carnitine/2-methylbutyryl-L-carnitine
C5-OH	3-OH-valeryl-L-carnitine
C5:1	Pentenoyl-L-carnitine
C5DC (C10-OH)	Glutaryl-L-carnitine (minor contribution of 3-OH-decanoyl-L-carnitine)
C6	Hexanoyl-L-carnitine
C6-OH	3-OH-hexanoyl-L-carnitine
C6DC	Adipoyl-L-carnitine
C8	Octanoyl-L-carnitine
C8:1	Octenoyl-L-carnitine (?)
C8-OH (C3DC)	3-OH-octanoyl-L-carnitine (minor contribution of malonyl-L-carnitine)
C10	Decanoyl-L-carnitine
C10:1	Decenoyl-L-carnitine
C10:2	Decandienoyl-L-carnitine
C12	Lauroyl-L-carnitine
C12:1	Dodecenoyl-L-carnitine
C12-OH	3-OH-dodecanoyl-L-carnitine
C14	Myristoyl-L-carnitine
C14:1	Tetradecenoyl-L-carnitine
C14:2	Tetradidecenoyl-L-carnitine
C14-OH	3-OH-myristoyl-L-carnitine
C16	Palmitoyl-L-carnitine
C16:1	Palmitoeoyl-L-carnitine
C16-OH	3-OH-palmitoyl-L-carnitine
C18	Stearoyl-L-carnitine
C18:1	Oleoyl-L-carnitine
C18:2	Linoleoyl-L-carnitine

Table 3 *Mean ± SD plasma acylcarnitine concentrations (μmol/L) for healthy subjects and ESRD patients receiving chronic haemodialysis treatment*

Abbreviation	Healthy subjects	ESRD patients	
		Pre-dialysis	Post-dialysis
LC	41.16 ± 10.13	24.16 ± 9.95*	9.93 ± 6.04*,†
TC	49.85 ± 11.03	42.22 ± 16.12*	17.39 ± 9.80*,†
C2	6.43 ± 2.01	9.23 ± 4.76*	3.32 ± 2.35*,†
C3	0.52 ± 0.17	0.57 ± 0.37	0.21 ± 0.14*,†
C4	0.24 ± 0.09	0.68 ± 0.51*	0.19 ± 0.15†
C4-OH	0.02 ± 0.01	0.08 ± 0.04*	0.03 ± 0.02†
C4DC	0.03 ± 0.01	0.31 ± 0.14*	0.10 ± 0.07*,†
C5	0.12 ± 0.04	0.82 ± 1.40*	0.32 ± 0.74†
C5-OH	0.03 ± 0.01	0.08 ± 0.04*	0.03 ± 0.02†
C5:1	0.03 ± 0.01	0.11 ± 0.05*	0.04 ± 0.02†
C5DC (C10-OH)	0.04 ± 0.02	0.65 ± 0.29*	0.18 ± 0.15*,†
C6	0.05 ± 0.02	0.12 ± 0.09*	0.05 ± 0.03†
C6-OH	0.02 ± 0.01	0.08 ± 0.04*	0.03 ± 0.02*,†
C6DC	0.02 ± 0.01	0.63 ± 0.43*	0.16 ± 0.18*,†
C8	0.10 ± 0.07	0.29 ± 0.16*	0.11 ± 0.06†
C8:1	0.15 ± 0.08	1.00 ± 0.57*	0.41 ± 0.36*,†
C8-OH (C3DC)	0.03 ± 0.02	0.23 ± 0.11	0.06 ± 0.07*,†
C10	0.15 ± 0.11	0.37 ± 0.19*	0.18 ± 0.09†
C10:1	0.09 ± 0.04	0.29 ± 0.14*	0.15 ± 0.08*,†
C10:2	0.05 ± 0.02	0.16 ± 0.08*	0.10 ± 0.04*,†
C12	0.06 ± 0.03	0.18 ± 0.07*	0.10 ± 0.05*,†
C12:1	0.06 ± 0.03	0.11 ± 0.05*	0.07 ± 0.04†
C12-OH	0.01 ± 0.005	0.14 ± 0.14*	0.05 ± 0.05*,†
C14	0.03 ± 0.01	0.16 ± 0.07*	0.09 ± 0.05*,†
C14:1	0.05 ± 0.03	0.12 ± 0.06*	0.09 ± 0.06*,†
C14:2	0.02 ± 0.01	0.05 ± 0.02*	0.03 ± 0.02*,†
C14-OH	0.01 ± 0.005	0.22 ± 0.30*	0.10 ± 0.20*,†
C16	0.09 ± 0.03	0.35 ± 0.13*	0.27 ± 0.12*,†
C16:1	0.02 ± 0.01	0.07 ± 0.04*	0.05 ± 0.03*,†
C16-OH	0.03 ± 0.01	0.06 ± 0.18	0.05 ± 0.19
C18	0.05 ± 0.02	0.17 ± 0.11*	0.14 ± 0.07*
C18:1	0.09 ± 0.03	0.51 ± 0.25*	0.53 ± 0.32*
C18:2	0.03 ± 0.01	0.22 ± 0.12*	0.20 ± 0.13*

*$P < 0.05$ compared with healthy subjects. †$P < 0.05$ compared with ESRD patients pre-dialysis.

Statistical methods

Acylcarnitine concentrations obtained from healthy subject samples were statistically compared with pre- and post-dialysis concentrations in ESRD patients using an analysis of variance (ANOVA). Pre- and post-dialysis acylcarnitine concentrations were compared with a paired Student's *t*-test. Significance was set at an α-level of 0.05. WinNonlin® Professional, Version 4.0 (Pharsight Corporation, Mountain View, CA, USA) was used for all statistical analyses.

Acylcarnitines were grouped according to carbon chain length as short- (C2–C6), medium- (C8–C12) and long-chain (C14–C18) acylcarnitines.

Results

Sixty healthy subjects and 50 ESRD patients receiving chronic haemodialysis treatment on a regular basis (approximately three times per week) participated in the study. The demographic and dialysis details are presented in Table 1. Although there is a notable difference in the mean age for each study group, previous investigations by our group have demonstrated that there is no significant effect of age on carnitine concentrations.

Mean L-carnitine and total carnitine pre-dialysis concentrations in ESRD patients were 24.16 ± 9.95 and 42.22 ± 16.12 μmol/L, respectively. In comparison,

mean concentrations in the healthy population were 41.16 ± 10.13 and 49.85 ± 11.03 μmol/L, respectively, with the difference being statistically significant ($P < 0.05$) in both cases. It should be noted that, despite the fact that plasma L-carnitine concentrations were 41.3% lower in the ESRD population than in normal volunteers, total carnitine concentrations were only 15.3% lower.

ESRD patients displayed significantly higher concentrations of total short-, medium- and long-chain acylcarnitines ($P < 0.05$), with concentrations of 11.77 ± 5.78, 4.36 ± 1.44 and 1.93 ± 0.79 μmol/L compared with normal concentrations of 7.47 ± 2.10, 0.80 ± 0.33 and 0.43 ± 0.12 μmol/L respectively.

Upon examination of the individual L-carnitine acyl derivatives, it can be seen that in all cases, with the exception of C3 and C16-OH, the carnitine esters were significantly elevated in the ESRD patient population compared to their healthy counterparts (Table 3). Most notably, carnitine esters of 18-carbon chain length were much higher in the ESRD population, with concentrations of 0.17 ± 0.11, 0.51 ± 0.25 and 0.22 ± 0.12 μmol/L for C18, C18:1 and C18:2, respectively, compared with 0.05 ± 0.02, 0.09 ± 0.03 and 0.03 ± 0.01 μmol/L in the healthy subjects. In addition, C5 and C8:1 concentrations were almost seven times higher in the ESRD population, with values of 0.82 ± 1.40 and 1.00 ± 0.57 μmol/L, respectively, compared with concentrations of 0.12 ± 0.04 and 0.15 ± 0.08 μmol/L in healthy subjects. Despite the high variability in C5 and C8:1 concentrations in the ESRD population, the difference in the endogenous concentrations of these compounds is statistically significant between the two populations.

L-carnitine and total carnitine concentrations were significantly reduced over the course of a single dialysis session, with mean post-dialysis concentrations of 9.93 ± 6.04 and 17.39 ± 9.80 μmol/L, respectively, compared with concentrations of 24.16 ± 9.95 and 42.22 ± 16.12 μmol/L prior to dialysis. Statistical comparisons of the pre- and post-dialysis acylcarnitine concentrations revealed that all of the carnitine esters were significantly removed during the dialysis session, with the exception of the 18-carbon chain esters (C18, C18:1 and C18:2) and C16-OH. There was a significant correlation ($P < 0.05$) between the ratio of post-dialysis to pre-dialysis acylcarnitine concentrations and carbon chain length of the acyl group (indicating decreasing dialytic removal with increasing chain length), with a notable reduction in the amount of acylcarnitine removed once the acyl chain length exceeded eight carbons (Figure 1). Despite significant removal of acylcarnitines during a single dialysis session, for the majority of the carnitine esters, post-dialysis

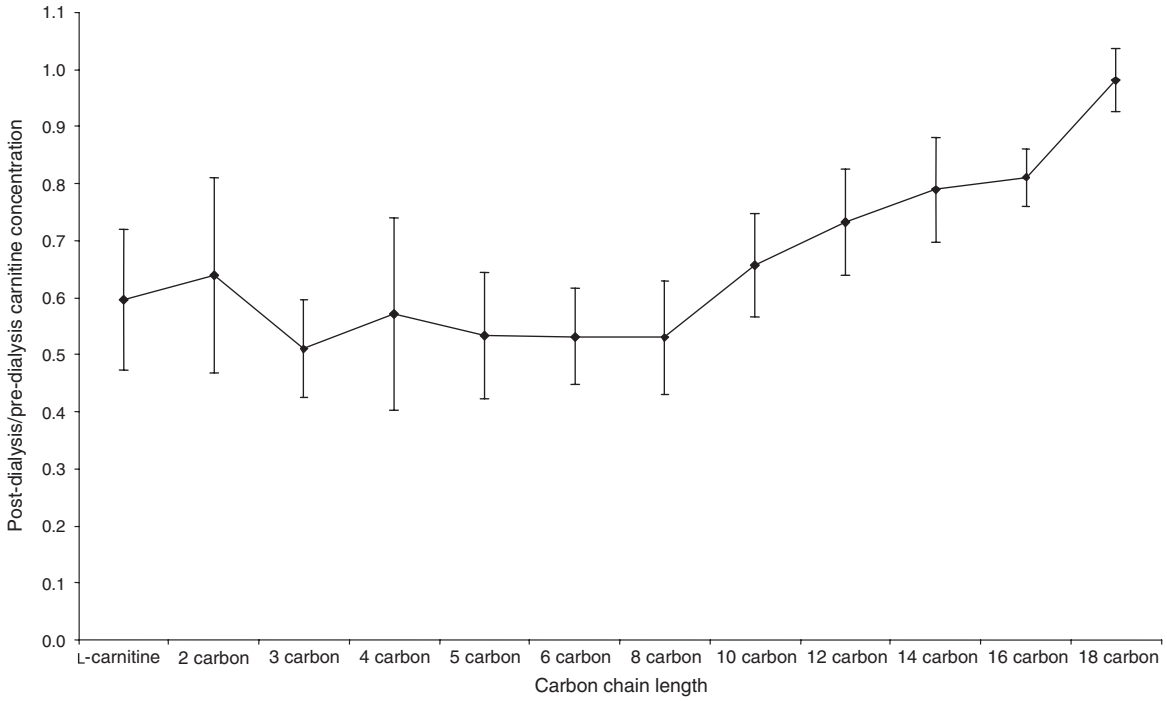


Figure 1 *Relationship between mean (± SEM) ratio of post-dialysis/pre-dialysis acylcarnitine concentrations and carbon chain length of the acyl group.*

concentrations remained significantly higher than those in the healthy population, in particular the long-chain acylcarnitines (Table 3).

Discussion

Disturbances in carnitine pool homeostasis during long-term haemodialysis have been implicated as a contributing factor for certain clinical problems in ESRD patients, including skeletal myopathies, poor exercise performance, cardiomyopathy and intradialytic complications such as hypotension, cramps, weakness and fatigue.[12,13,20]

It has been widely established that long-term haemodialysis treatment results in alterations in the endogenous carnitine pool, with a large number of studies reporting significantly lower plasma endogenous L-carnitine concentrations in haemodialysis patients compared with healthy individuals.[4–21] Despite significant reductions in L-carnitine concentrations in the haemodialysis population, total carnitine concentrations remain relatively unchanged. This is due to a significant accumulation of the acylated forms of L-carnitine, with acylcarnitine concentrations between 33% and 47.6% of total concentrations in haemodialysis patients, compared with 11.6–17.4% in healthy subjects.[8–11,16,17,19,21] However, the pattern of this accumulation, in terms of the composition of the accumulated acyl pool, has not been studied. To our knowledge, this is the first study which has quantified individual acylcarnitines in ESRD patients receiving haemodialysis.

In this study, total carnitine concentrations in the ESRD patients were 15.3% lower than the healthy population, a relatively small reduction considering that L-carnitine concentrations were reduced by 41.3%. Upon examination of the data, it can be seen that the carnitine pool composition is considerably different between the two study populations. L-Carnitine concentrations in the healthy population make up 82.6% of the total carnitine pool (17.4% acylcarnitine), whereas in the ESRD patients on haemodialysis, the L-carnitine and acylcarnitine proportions of the carnitine pool are 57.2% and 42.8%, respectively. Haemodialysis patients exhibited significantly higher acylcarnitine concentrations than healthy individuals for 29 out of the 31 individual acylcarnitines measured. A result of particular interest is the considerable accumulation noted for the C5 and C8:1 acylcarnitines.

Comparison of the pre- and post-dialysis plasma acylcarnitine concentrations indicated that dialytic removal of acylcarnitine is significantly correlated to carbon chain length of the acyl group, with no significant removal of the 18-carbon chain length esters. Removal of the longer chain esters across the dialysis membrane is obviously impaired due to the increased molecular weight and lipophilicity that accompanies increasing chain length.

Interestingly, despite the fact that the short and medium-chain esters are removed by haemodialysis, the concentrations of the majority of carnitine esters remain significantly higher after the dialysis session than concentrations found in the healthy individuals. The accumulation of acylcarnitines during long-term haemodialysis suggests that removal by haemodialysis is less efficient than removal from the body by the healthy kidney. Alternatively, enhanced formation of acylcarnitines or diminished utilization of fatty acids may also contribute to these alterations to the carnitine pool in this population.

The clinical and toxicological implications of the accumulation of acylcarnitines in ESRD patients receiving long-term haemodialysis are yet to be determined. However, Yamada *et al.*[24] have demonstrated that palmitoyl-L-carnitine and stearoyl-L-carnitine enhance calcium release from rabbit cardiac sarcoplasmic-reticulum-enriched membrane vesicles, an observation that was found to be both concentration dependent and carbon chain length dependent, a finding which suggests that these compounds could contribute to the development of ventricular arrhythmias. This, coupled with the high incidence of cardiomyopathy and other cardiac disorders in haemodialysis patients, gives us a basis from which future research can be conducted investigating the clinical implications of this altered carnitine profile, including the impact of L-carnitine supplementation on the pattern of accumulation of acylcarnitines, in particular long-chain acylcarnitines.

Acknowledgements

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INVITED REVIEW

Reuter SE, Faull RJ, Evans AM. L-carnitine supplementation in the dialysis population: Are Australian patients missing out? *Nephrology* 2008; 13(1): 3-16.

L-carnitine supplementation in the dialysis population: Are Australian patients missing out?

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SUMMARY: It has been widely established that patients with end-stage renal disease undergoing chronic haemodialysis therapy exhibit low endogenous levels of L-carnitine and elevated acylcarnitine levels; however, the clinical implication of this altered carnitine profile is not as clear. It has been suggested that these disturbances in carnitine homeostasis may be associated with a number of clinical problems common in this patient population, including erythropoietin-resistant anaemia, cardiac dysfunction, and dialytic complications such as hypotension, cramps and fatigue. In January 2003, the Centers for Medicare and Medicaid Services (USA) implemented coverage of intravenous L-carnitine for the treatment of erythropoietin-resistant anaemia and/or intradialytic hypotension in patients with low endogenous L-carnitine concentrations. It has been estimated that in the period of 1998–2003, 3.8–7.2% of all haemodialysis patients in the USA received at least one dose of L-carnitine, with 2.7–5.2% of patients receiving at least 3 months of supplementation for one or both of these conditions. The use of L-carnitine within Australia is virtually non-existent, which leads us to the question: Are Australian haemodialysis patients missing out? This review examines the previous research associated with L-carnitine administration to chronic dialysis patients for the treatment of anaemia, cardiac dysfunction, dyslipidaemia and/or dialytic symptoms, and discusses whether supplementation is warranted within the Australian setting.

KEY WORDS: **anaemia, cardiac function, dialytic symptom, dyslipidaemia, haemodialysis, L-carnitine.**

L-carnitine is an endogenous compound critical for the transfer of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation.[1,2] Plasma L-carnitine concentrations are regulated by the healthy kidney, with active tubular reabsorption and preferential excretion of acylcarnitines.[3–5] It has been widely established that patients with end-stage renal disease (ESRD) undergoing chronic haemodialysis treatment exhibit altered carnitine levels.[6,7]

In light of this alteration in carnitine homeostasis and evidence for its impact on a number of clinical conditions, the Centers for Medicare and Medicaid Services (USA) implemented coverage of intravenous L-carnitine administration for the treatment of erythropoietin-resistant anaemia and/or intradialytic hypotension in patients who exhibit low endogenous L-carnitine levels (<40 μmol/L).[8] It has been estimated that in the period of 1998–2003, 3.8–7.2% of all haemodialysis patients in the USA received at least one dose of L-carnitine, with 2.7–5.2% of patients receiving at least 3 months of supplementation.[9] In a recent survey conducted by our group, it was established that carnitine administration within the Australian nephrology community is virtually non-existent (unpublished data).

Until now, the debate over carnitine use in dialysis patients has not been brought to Australia. The aim of this review is to evaluate the existing findings associated with carnitine administration and discuss whether the use of L-carnitine supplementation is warranted within the Australian setting.

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CARNITINE HOMEOSTASIS

L-carnitine is a low-molecular-weight, polar molecule found in all mammalian species (Fig. 1).[1] The principal biological role of carnitine is in the transport of fatty acids across the inner mitochondrial membrane for fatty acid oxidation (Fig. 2).[2] It has also been established that carnitine has a role in regulating the cellular to mitochondrial ratio of free CoA to Acyl-CoA, in the transport of short and medium acyl groups from the peroxisome to the mitochondria, and in the removal of unwanted acyl groups from the body.[1,10]

Adequate levels of L-carnitine are largely achieved from dietary sources, in particular from red meat; however, it can

also be biosynthesized from lysine and methionine.[1] The enzymes required for the first four stages of the synthesis of L-carnitine are located within most tissues throughout the body; however, γ-butyrobetaine 2-oxoglutarate dioxygenase (required for the conversion of γ-butyrobetaine to L-carnitine) is present only in the kidney, liver and brain.[1] Consequently, these organs are important in the biosynthesis of L-carnitine. Plasma plays a critical role in the transport of L-carnitine from these sites of production to the muscles for storage and mediation of mitochondrial fatty acid uptake.[11,12]

The endogenous plasma levels of L-carnitine in the healthy human are 40–50 μmol/L with plasma total carnitine levels (L-carnitine + acylcarnitines) 50–60 μmol/L; acetyl-L-carnitine (the most abundant acylcarnitine) levels are usually 3–6 μmol/L.[5,13–16] Nonetheless, plasma carnitines only account for approximately 1% of the total body carnitine pool, with over 98% of carnitines present in the skeletal and cardiac muscles. A small amount is also present in the kidney, liver and brain.[11,12,17]

L-carnitine is not protein bound and is therefore extensively filtered at the glomerulus; however, extensive tubular reabsorption maintains adequate endogenous levels.[3,5] Loss of endogenous carnitine occurs primarily via renal excretion, with preferential excretion of the unwanted acylcarnitines, such that the ratio of acylcarnitine to L-carnitine in urine exceeds that in plasma.[4,5]

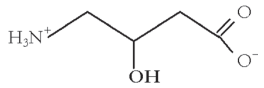


Fig. 1 Structure of carnitine, with the site of esterification illustrated in bold.

CARNITINE DEFICIENCY

It has been well established that alterations in carnitine homeostasis can have a detrimental effect on human health.[18,19] Disorders of carnitine deficiency may be categorized as either primary or secondary. These disorders have been reviewed extensively elsewhere;[18–20] in short, primary carnitine deficiency primarily results from an impairment in the OCTN2 carnitine transporter which restricts tissue uptake and potentiates renal loss; primary carnitine deficiency is not associated with any other illness which may result in the depletion of carnitine. Primary carnitine deficiency may be further categorized as either systemic carnitine deficiency or myopathic carnitine deficiency.[19] In the systemic form, there is a significant reduction in carnitine levels within all the carnitine compartments; whereas in the myopathic form, carnitine deficiency is restricted to the muscular compartment only. Patients with primary carnitine deficiency develop progressive cardiomyopathy, encephalopathy and muscle weakness, resulting in death from heart failure.[19,20] For these patients, L-carnitine supplementation is a life-saving treatment.

Secondary carnitine deficiency is less severe with respect to its short-term clinical impact; however, it is much more common. As opposed to primary carnitine deficiency, secondary carnitine deficiency is associated with, or caused by, another disorder, such as liver or kidney disease, a defect in fatty acid metabolism, or administration of certain pharmacological agents (such as pivampicillin or valproic acid).[18,19,21]

End-stage renal disease

Considering that the kidney has such a critical role in carnitine homeostasis, it is hardly surprising that alterations in carnitine pool composition occur in patients with ESRD. Numerous studies investigating these changes have established that as kidney function deteriorates, plasma concen-

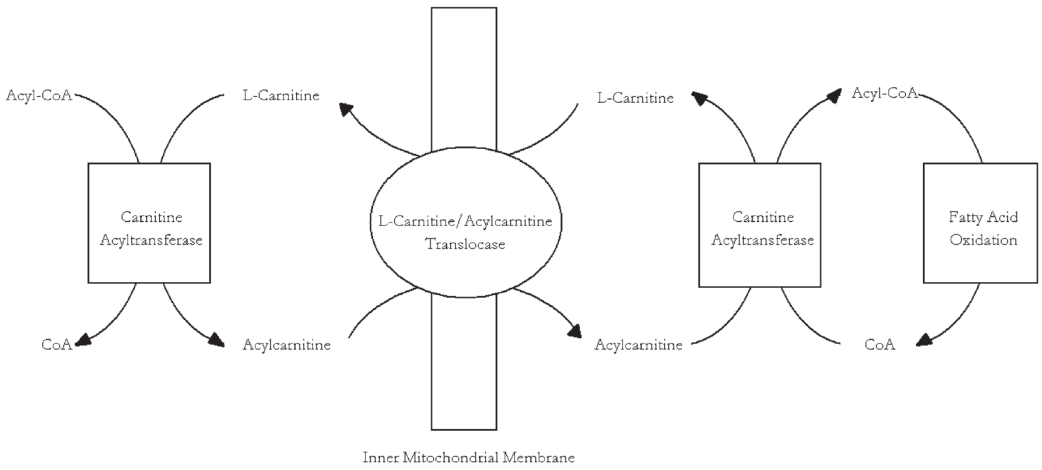


Fig. 2 Schematic diagram of the function of L-carnitine in the transport of fatty acids across the inner mitochondrial membrane.

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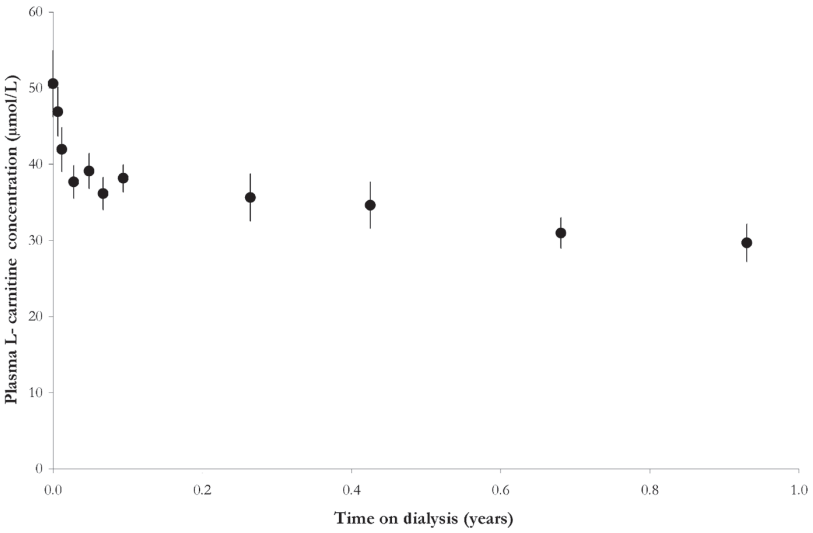


Fig. 3 Endogenous plasma L-carnitine levels during the first 12 months of haemodialysis. Endogenous predialysis plasma L-carnitine concentrations were examined in 21 ESRD patients throughout the first 12 months of dialysis treatment. Plasma L-carnitine concentrations significantly decreased within the first week if dialysis and continued to progressively decline during the first 12 months of treatment. ESRD, end-stage renal disease.

trations of endogenous L-carnitine and its acyl derivatives increase significantly.[11,12,22–25] Plasma L-carnitine concentrations have been shown to be up to twofold higher in the ESRD population than in healthy controls, while total acylcarnitine levels in this patient population have been observed at levels up to 4.6 times greater than in healthy subjects.[11,12,22–25]

Conversely, chronic haemodialysis treatment has been shown to be associated with a reduction in plasma and tissue L-carnitine concentrations and an accumulation of acylcarnitines.[11,12,17,22–35] It has been demonstrated that L-carnitine is extensively removed by the dialyser during a single dialysis session, due to the compound's low molecular weight and high hydrophilicity in combination with its lack of protein binding.[29,30] As a consequence, plasma L-carnitine levels in ESRD patients receiving haemodialysis treatment have been shown to be substantially lower than endogenous levels observed in healthy subjects,[6,24–26,31,34] with levels of 22.0 ± 5.4 μmol/L reported in long-term dialysis patients compared with an average plasma concentration of 43.3 ± 8.6 μmol/L in healthy adults.[6] Recent research has demonstrated a significant negative correlation between plasma endogenous L-carnitine levels and the duration of dialysis treatment (dialysis age), with the majority of the reduction occurring within the first few months of haemodialysis treatment (Fig. 3).[6] Long-term haemodialysis treatment (>12 months) has also been shown to be associated with a significant decrease in muscle carnitine stores, with a 38% reduction in L-carnitine levels compared with levels prior to the initiation of dialysis treatment.[6] Similar decreases in muscle total carnitine and acetyl-L-carnitine levels were also observed.

On the other hand, compared with the healthy population, acylcarnitine levels are significantly higher in ESRD patients on chronic haemodialysis therapy, particularly in those who have been receiving haemodialysis treatment for at least 12 months.[6] In fact, acylcarnitine levels make up approximately 50% of the total plasma carnitine pool in these patients (of which only half is accounted for by acetyl-L-carnitine), compared with 15% in healthy subjects (consisting primarily of acetyl-L-carnitine and negligible levels of the non-acetyl acylcarnitines) (Fig. 4).[6] The dialytic removal of acylcarnitines during a single dialysis session is significantly correlated to carbon-chain length of the acyl group, with no significant removal of the 18 carbon-chain length esters (Fig. 5).[7] Removal of these acylcarnitines is obviously impaired due to the increased molecular weight and lipophilicity that accompanies increasing chain length, in addition to protein binding which has been demonstrated for the longer-chain acylcarnitines.[36]

CARNITINE SUPPLEMENTATION IN CHRONIC HAEMODIALYSIS PATIENTS

Disturbances in carnitine homeostasis during long-term haemodialysis have been implicated as a contributing factor for certain clinical problems in ESRD patients, including skeletal myopathies, poor exercise performance, cardiomyopathy, and dialytic complications such as hypotension, cramps, weakness and fatigue.[37] Recent research has demonstrated that supplementation with L-carnitine is associated with a reduction in hospital use in the USA.[38,39]

A large number of studies have been conducted over the past 20 years to assess the efficacy of supplemental L-carnitine in treating certain dialysis-related clinical disorders. However, many of these studies have been small, retrospective trials, and few blinded, placebo-controlled, large-scale trials have been conducted. In addition, the prescription of L-carnitine (e.g. dose, route, duration of treatment) and patient population (e.g. dialysis age) differ greatly from one study to the next, making between-study comparisons difficult. However, perhaps the most important factor is in the definition of carnitine deficiency itself in

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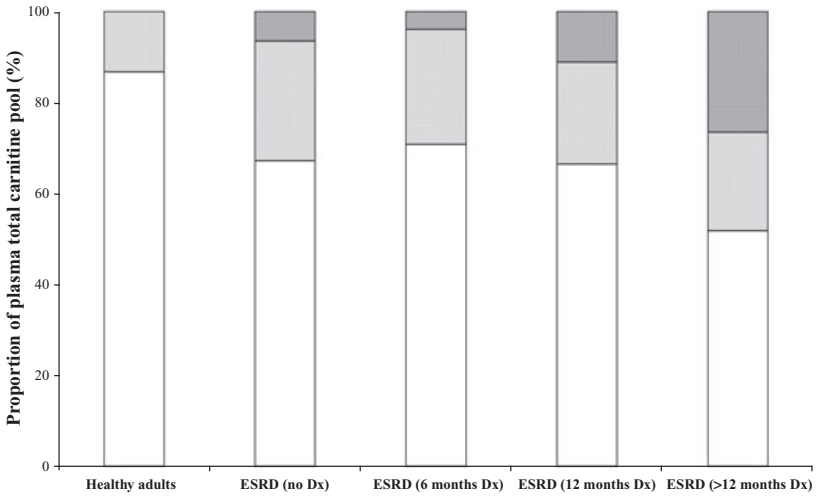


Fig. 4 Impact of haemodialysis on the composition of the plasma carnitine pool. The proportion of endogenous (□) L-carnitine, (▒) acetyl-L-carnitine and (■) non-acetyl acylcarnitines within the total carnitine pool was examined in healthy subjects, ESRD patients not receiving haemodialysis treatment, and ESRD patients undergoing haemodialysis treatment for various lengths of time. An increase in dialysis age is associated with a decrease in the proportion of L-carnitine and an increase in the proportion of non-acetyl acylcarnitines within the total carnitine pool. ESRD, end-stage renal disease.

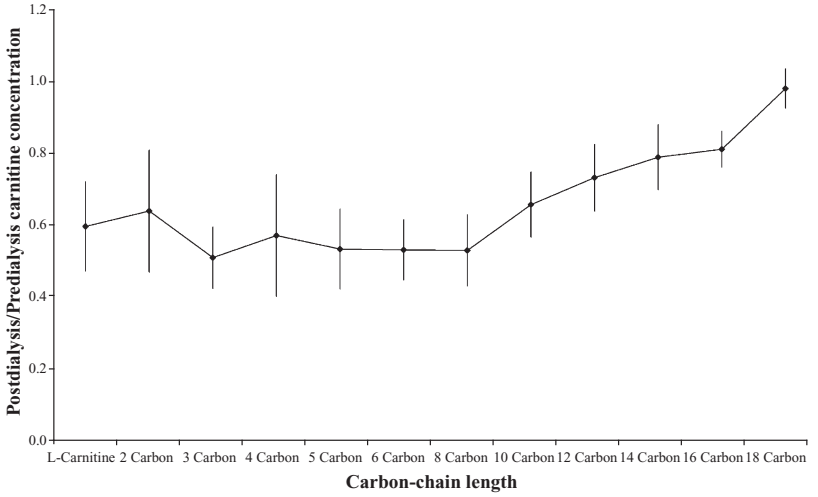


Fig. 5 Relationship between dialytic removal of carnitines and acyl group carbon-chain length. A significant correlation between the ratio of postdialysis to predialysis acylcarnitine concentrations (as an indicator of dialytic removal) and carbon-chain length of the acyl group has been demonstrated, indicating decreased dialytic removal with increasing chain length.

this patient population. Thus, in long-term haemodialysis patients, it may be more important to examine relationships between the clinical response to L-carnitine treatment and pretreatment L-carnitine (or acylcarnitine) levels as a proportion of the carnitine pool, rather than focusing on the pretreatment plasma L-carnitine levels (or change in L-carnitine levels after treatment) alone; no studies have investigated these more complex relationships.

Despite this, there appears to be an evidential basis for the use of L-carnitine in chronic haemodialysis patients; however, more controlled comprehensive trials are needed before any definitive conclusions can be made.

Pharmacokinetics and safety

L-carnitine has been administered to chronic haemodialysis patients according to a variety of different prescriptions for the treatment of dialysis-related carnitine deficiency. In most cases L-carnitine is given as an i.v. or oral (tablet or solution) dose; however, the pharmacokinetics of these two dose forms are vastly different, and therefore must be considered separately.

The i.v. form of L-carnitine is 100% bioavailable and undergoes negligible metabolism (with the exception of reversible acylation). A typical 1–2 g i.v. dose of L-carnitine to a healthy adult results in initial plasma carnitine concentrations up to 10-fold greater than the threshold for tubular reabsorption, and as a consequence, 70–90% is excreted unchanged in urine within 12–24 h post dose. Thus, in healthy people, a single dose of L-carnitine is not present in the body long enough for any significant amount to equilibrate into the body's carnitine pool (i.e. skeletal and cardiac muscle). On the other hand, as ESRD patients have no efficient removal process during the interdialysis period, the

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i.v. dose of L-carnitine remains in the bloodstream for sufficient time for it to be incorporated into the extravascular tissue compartments. In fact, in a study performed by our group, it was demonstrated that only 10% of a single i.v. dose of L-carnitine was recovered in dialysate during the next dialysis session, suggesting that approximately 90% of the dosed L-carnitine had moved into the tissues.[30] Chronic i.v. L-carnitine administration has been shown to result in a 60–200% increase in muscle L-carnitine levels.[17,31,40,41]

The usual, prescribed i.v. dose of L-carnitine is 20 mg/kg during the initiation of therapy, which represents approximately 5–10% of total body carnitine levels. It is thought that a dose of this magnitude may drive L-carnitine from the plasma into muscle stores; in any case, this dose level results in a steady increase in muscle carnitine levels over a period of weeks to months. Administration of a more physiologically relevant dose (e.g. 2 mg/kg) would obviously result in a much longer equilibration time.

Studies in healthy subjects have shown that oral L-carnitine is poorly bioavailable, with only 15% of a usual dose of 2 g being absorbed into the bloodstream, reducing to 5% bioavailability after a 6 g dose.[3,42,43] The bioavailability of oral L-carnitine in patients with renal disease is unknown. The unabsorbed portion of the oral dose of L-carnitine undergoes bacterial degradation in the gastrointestinal tract to form trimethylamine and γ-butyrobetaine. Trimethylamine is absorbed into the bloodstream and converted to trimethylamine-N-oxide. In healthy subjects, trimethylamine (which has the odour of rotting fish) is rapidly converted to trimethylamine-N-oxide and is excreted efficiently by the kidneys, and consequently these individuals experience no toxic effects. On the other hand, patients with kidney failure are unable to excrete trimethylamine-N-oxide effectively, and experience accumulation of trimethylamine-N-oxide and trimethylamine. A high level of these compounds might contribute to neurotoxicity and 'uraemic breath' in these patients.[44]

Previous studies have also demonstrated that orally administered L-carnitine undergoes a high degree of acetylation during gastrointestinal absorption in animals.[45,46] Although it is still unknown whether this is also true for humans, this deserves careful consideration as to whether high acetyl-L-carnitine levels are desirable in haemodialysis patients who already have high endogenous acylcarnitine levels.

L-carnitine administration has shown to have an excellent safety profile. Early studies investigated the effects of D,L-carnitine administration and noted in a number of reports that this was associated with the development of myasthenia-like symptoms. It was later established that this was due to toxicity related to the D-isomer only. Transient nausea and vomiting have been associated with L-carnitine administration, and less frequently body odour, nausea, gastritis and seizures.[47]

Anaemia

Anaemia is a common complication associated with chronic haemodialysis treatment, characterized by both a decrease in erythrocyte precursor production and reduced survival of red blood cells; however, the most important cause of renal anaemia is a decrease in erythropoietin secretion by the kidney.[48] Recombinant human erythropoietin (rHuEPO) is the commonly used treatment for anaemia of ESRD patients; however, the rHuEPO dose required to maintain a target haemoglobin varies dramatically within the patient population.[49,50] In addition, the cost of this treatment is extremely high, with rHuEPO therapy accounting for up to 10% of the total treatment costs for chronic haemodialysis patients.[51] It is estimated that approximately A$100–150 million is spent on erythropoietin or darbepoietin treatment in Australia per year, with more than A$20 million spent under the Pharmaceutical Benefits Scheme.[52] However, it is important to note that the rationale for improving rHuEPO resistance in not merely economic; recent research by Zhang *et al.*[50] investigating the relationship between rHuEPO requirements and mortality has indicated that the highest mortality rate is observed in patients receiving high doses of rHuEPO but with low haematocrit levels (i.e. rHuEPO-resistant). Interestingly, patients with haematocrit levels in the desirable range (33–36%) receiving high doses of rHuEPO had a mortality rate twice that of those receiving a low dose of rHuEPO with the haematocrit levels in the same range, thereby suggesting that, wherever possible, the minimum rHuEPO dose necessary to achieve acceptable haematocrit levels should be prescribed.

L-carnitine has been proposed as a potential adjunct treatment to rHuEPO in the treatment of anaemia. From January 2003, the Centers for Medicare and Medicaid Services implemented coverage of intravenous L-carnitine administration for the treatment of erythropoietin-resistant anaemia in haemodialysis patients who have low endogenous levels of L-carnitine.[8] Numerous studies have investigated the potential use of L-carnitine for anaemia, with promising results.[53–68] These studies have been summarized in Table 1.

Studies have demonstrated that L-carnitine administration improved the response to rHuEPO in the haemodialysis population, with either an increase in haematocrit levels,[53–59] a decrease in the rHuEPO dose required,[60–65] or a decrease in erythropoietin resistance index (ERI; rHuEPO dose/week normalized for haematocrit or haemoglobin).[66–68] In a randomized, placebo-controlled trial, Trovato *et al.*[59] observed a significant increase in haematocrit levels in patients treated with 825 mg L-carnitine p.o. b.i.d. for 12 months; in contrast, haematocrit levels decreased by almost 10% in placebo-treated patients during the 12-month treatment period. Similarly, Labonia[62] noted a significant decrease in rHuEPO dose in patients treated with L-carnitine (1 g i.v. after dialysis for 6 months), compared with no change in placebo-treated subjects. Hurot *et al.*[69] performed a meta-analysis of 18 clinical trials investigating the effects of L-carnitine administration on anaemia, and noted that co-therapy with L-carnitine resulted in a significant improvement in haematocrit and/or rHuEPO requirements.

The underlying mechanism by which L-carnitine exerts its favourable effects on these anaemic indices in dialysis patients is still not yet fully understood. It has been

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Table 1 Summary of anaemia studies

Reference	Study design	Population	L-carnitine treatment		Findings
[53]	Two-way, parallel, open-label	12 HD patients 11 HD patients	1 g/day p.o. for 6 months 100 μmol/L dialysate for 6 months	↑ HCT	T_0 23.1 ± 5.2%; T_6 26.5 ± 4.4%
[54]	Case report	2 HD patients	2 mg/kg per Dx i.v. for 9 months	↑ HCT	T_0 22.3 & 20.9%; T_9 24.3 & 28.6%
[55]	One-way, open-label	10 HD patients	1 g b.i.d. p.o. for 2 months	↑ HCT	T_0 25.6 ± 2.4%; T_2 29.0 ± 2.6%
[56]	One-way, open-label	8 HD patients	1 g/Dx i.v. + 1 g/day p.o. for 2 months	↑ HCT	T_0 22.3 ± 3.6%; T_2 24.9 ± 2.7%
[57]	One-way, open-label	14 HD patients (EPO-resistant)	500 mg/day p.o. for 3 months	↑ HCT	T_0 24.0 ± 2.0%; T_3 26.1 ± 2.0%
[58]	One-way, open-label	15 HD patients	30 mg/kg per Dx i.v. for 3 months	↑ HCT	T_0 30.8 ± 1.9%; T_3 34.2 ± 2.4%
[59]	Two-way, parallel, double-blind	13 HD patients 11 HD patients	825 mg b.i.d. p.o. for 12 months Placebo for 12 months	↑ HCT	T_0 25.5 ± 1.4%; T_{12} 37.4 ± 2.2% T_0 24.0 ± 3.6%; T_{12} 21.8 ± 3.2%
[60]	One-way, open-label	14 HD patients	40–60 mg/kg per week i.v. for 3 months	↓ EPO	T_0 151 ± 21 U/kg per week; T_3 87 ± 32 U/kg per week
[61]	One-way, open-label	8 HD patients	2 g/Dx i.v. for 5 months	↓ EPO	T_0 50–60 U/kg per Dx; T_5 20–25 U/kg per Dx
[62]	Two-way, parallel, double-blind	13 HD patients 11 HD patients	1 g/Dx i.v. for 6 months Placebo i.v. for 6 months	↓ EPO	T_0 102.2 ± 52.6 U/kg pe week; T_6 63.3 ± 37.8 U/kg per week T_0 78.6 ± 32.1 U/kg per week; T_6 79.9 ± 46.7 U/kg per week
[63]	One-way, open-label	23 HD patients	1 g/Dx i.v. for 8 months	↓ EPO	T_0 7000 ± 3000 U; T_8 6000 ± 2000 U
[64]	Two-way, parallel, open-label	25 HD patients 35 HD patients	1 g/Dx i.v. & 1 g/non-Dx p.o. for 36 months No treatment for 36 months	↓ EPO	T_0 5976 ± 1732 U/week; T_{36} 3391 ± 659 U/week T_0 6100 ± 1587 U/week; T_{36} 5519 ± 1360 U/week
[65]	One-way, open-label	12 HD patients	15 mg/kg per Dx i.v. for 6 months	↓ EPO	T_0 5500 U/week; T_{36} 3500 U/week
[66]	Two-way, parallel, double-blind	10 HD patients (>65 years) 11 HD patients (>65 years)	1 g/Dx i.v. for 6 months Placebo i.v. for 6 months	↓ ERI	T_0 135 ± 79; T_6 118 ± 108 IU/kg per week per % T_0 136 ± 66; T_6 217 ± 204 IU/kg per week per %
[67]	Two-way, parallel, double-blind	14 HD patients 5 HD patients	5 mg/kg per Dx i.v. for 4 months 25 mg/kg per Dx i.v. for 4 months	↓ ERI	T_0 16.0 ± 11.0; T_4 13.6 ± 10.5 U/kg per week per g Hb
[68]	Two-way, parallel, double-blind	13 HD patients 14 HD patients	20 mg/kg per Dx i.v. for 6 months Placebo for 6 months	↓ ERI	Values not reported

Dx, dialysis session; EPO, rHuEPO dose; ERI, erythropoietin resistance index; HCT, haematocrit; HD, haemodialysis.

suggested that L-carnitine may act via stabilization of the red blood cell membrane, thereby increasing the lifespan of the erythrocyte;[59] this is in direct contrast to the described effects of long-chain acylcarnitines on red blood cells.[70–73] In one such study it was demonstrated that co-incubation of erythrocytes with L-carnitine can reverse the damaging detergent effects produced by palmitoyl-L-carnitine (a long-chain acylcarnitine).[73] Relationships between L-carnitine and erythrocyte osmotic fragility, deformability and membrane fluidity have been demonstrated.[58,73–75] Alternatively, the beneficial effects of L-carnitine on anaemia may be a result of a reduction in lipid peroxidation. Gallucci *et al.*[76] have demonstrated that patients with rHuEPO-resistant anaemia have significantly higher levels of malondialdehyde than responders to rHuEPO therapy and patients not receiving rHuEPO treatment. Following on from this, Veselá *et al.*[65] showed that L-carnitine administration for 6 months results in a significant reduction in malondialdehyde levels.

It should, however, be noted that, despite the fact that there is an overall improvement in anaemic parameters with L-carnitine supplementation, a number of studies have shown that the patient population appears to be separated into 'responders' and 'non-responders'.[62,66,67] Although in all of these studies, the division of patients into these subgroups was retrospective, the authors of one paper reported that 'a greater benefit was found in those patients with the higher increase in carnitine plasma levels'.[66] Kletzmayr *et al.*[67] noted that responders had higher baseline levels of all carnitines compared with non-responders, and therefore suggested that 'severely carnitine-depleted patients might

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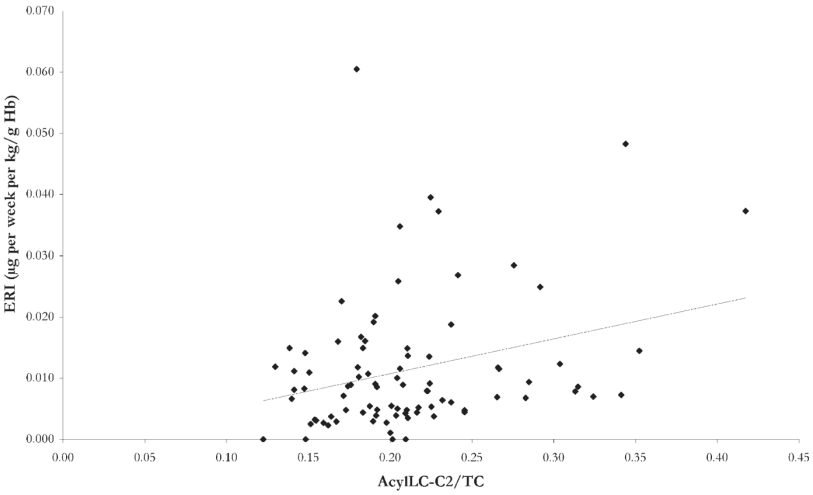


Fig. 6 Relationship between proportion of non-acetyl acylcarnitines with respect to the total carnitine pool (AcylLC-C2/TC) and erythropoietin resistance index (ERI). A significant relationship ($P = 0.006$) between AcylLC-C2/TC and ERI was demonstrated in 87 ESRD patients undergoing chronic haemodialysis treatment. ESRD, end-stage renal disease.

need a higher dosage or longer supplementation of carnitine in order to reduce the rHuEPO requirement'. The apparent subgrouping of patients as responders and non-responders needs to be investigated further.

Our group has recently demonstrated that there is a significant relationship between a patient's endogenous carnitine profile and ERI; specifically the proportion of non-acetyl acylcarnitines within the total carnitine pool is positively correlated with ERI, independent of dialysis age (Fig. 6, publication in preparation). In other words, a higher rHuEPO dose is required in patients exhibiting a higher proportion of medium- to long-chain carnitine esters in plasma. It is hypothesized that L-carnitine supplementation may be most beneficial in these patients. Only one study has investigated the effect of supplementation in a specific group of patients who were considered to be rHuEPO-resistant; however, this study was not controlled and used oral supplementation.[57] Further studies are needed to investigate the effect of L-carnitine administration in this specific population.

It is of our opinion that L-carnitine shows great promise as a potential adjunct treatment to rHuEPO for the treatment of anaemia in haemodialysis patients, at least in a subgroup of patients. However, other known factors, such as iron deficiency, should be first ruled out as a possible cause of hyporesponsiveness to rHuEPO in these patients. Further studies are needed to determine whether the effectiveness of L-carnitine for anaemia is constant in all haemodialysis patients or only a select population.

Cardiac function

Cardiac disease is a significant problem among dialysis patients, accounting for approximately half of all deaths in this patient population.[77] In addition, approximately three-quarters of ESRD patients commencing haemodialysis treatment suffer from some form of left ventricular dysfunction[78] as indicated by an impaired left ventricular ejection fraction (LVEF) which has been shown to be an independent predictor of congestive heart failure.[79] Intradialytic hypotension has been associated with a significant increased risk of mortality[80] and impaired myocardial contractile reserve.[81]

Several studies have investigated the effect of L-carnitine administration on cardiac function;[40,63,64,82–90] these are summarized in Table 2.

The relationship between L-carnitine and the incidence of hypotensive episodes in haemodialysis patients has been widely investigated. It has been noted that patients who experience hypotensive episodes have lower endogenous L-carnitine levels than their normotensive counterparts.[91] Administration of L-carnitine (990 mg/day p.o. for 2 months) has been shown to be associated with a significant reduction in the incidence of intradialytic hypotensive events when compared with placebo treatment.[82] The results of these studies are supported by the findings of Ahmad *et al.*[40] and Fujita *et al.*[83] Based on these studies, the Centers for Medicare and Medicaid Services have approved coverage of intravenous L-carnitine supplementation for the treatment of dialysis-related hypotension in haemodialysis patients with low endogenous levels of L-carnitine.[8]

The effect of L-carnitine on LVEF has also been described. A significant correlation between LVEF and endogenous L-carnitine levels has been reported, with these patients exhibiting considerable improvement in LVEF after administration of L-carnitine (1 g i.v. after dialysis) for 3 months, a result which was particularly pronounced in patients experiencing recurrent hypotensive episodes.[84] These symptomatic patients had a significantly lower ejection fraction than their asymptomatic counterparts, suggesting a significantly higher risk of mortality.[81] Additional studies have provided similar findings.[63,64,85,86] Although mounting evidence exists in favour of the role of L-carnitine in the treatment of left ventricular dysfunction, other studies have been performed with negative results;[87,88]

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Table 2 Summary of cardiac studies

Reference	study design	Population	L-carnitine treatment		Findings
[82]	Two-way, crossover, double-blind	9 HD patients	990 mg/day p.o. then placebo for 2 months each	↓ HT	Values not reported
		9 HD patients	Placebo then 990 mg/day p.o. for 2 months each		
[40]	Two-way, parallel, double-blind	38 HD patients	20 mg/kg per Dx i.v. for 6 months	↓ HT	T_0: 17 patients with HT; T_6: 7 patients with HT
		44 HD patients	Placebo for 6 months		T_0: 9 patients with HT; T_6: 11 patients with HT
[89]	Two-way, parallel, double-blind	15 HD patients 15 HD Patients	1–1.5 g/Dx i.v. for 2 months Placebo for 2 months	↓ HT	Values not reported
[83]	One-way, open-label	18 HD patients (>4 HT/week)	1200 mg/day p.o. for 3 months	↓ HT	Values not reported
[84]	One-way, open-label	13 HD patients	1 g/Dx i.v. for 3 months	↑ LVEF	T_0: 42.4 ± 19.4%; T_3: 48.6 ± 17.6%
[85]	One-way, open-label	6 HD patients (children)	15–30 mg/kg per day p.o. for 18 months	↑ LVEF	Values not presented
[86]	One-way, open-label	9 HD patients (impaired LVEF)	500 mg/day p.o. for 6 months	↑ LVEF	T_0: 44.9 ± 12.2%; T_6: 53.8 ± 13.8%
[63]	One-way, open-label	11 HD patients	1 g/Dx i.v. for 8 months	↑ LVEF	T_0: 32.0%; T_8: 41.8%
[64]	Two-way, parallel, open-label	25 HD patients	1 g/Dx i.v. & 1 g/Non-Dx p.o. for 36 months	↑ LVEF	Values not reported
		35 HD patients	No treatment for 36 months		
[87]	Two-way, parallel, double-blind	14 HD patients	2 g/Dx i.v. for 6 weeks	↔ ECG	No difference in echocardiogram measurements
		14 HD patients	Placebo for 6 weeks		
[88]	One-way, open-label	11 HD patients	1 g/day p.o. then 0.5 g/day p.o. for 1 month each	↔ ECG	No difference in echocardiogram measurements
[90]	One-way, open-label	17 HD patients	2 g/Dx p.o. for 1–2 months	↓ ARR	Values not reported
[40]	Two-way, parallel, double-blind	38 HD patients	20 mg/kg per Dx i.v. for 6 months	↔ ARR	No difference in incidence of arrhythmias
		44 HD patients	Placebo for 6 months		

ARR, arrhythmias; Dx, dialysis session; ECG, echocardiogram; HD, haemodialysis; HT, hypotensive episodes; LVEF, left ventricular ejection fraction.

however, the results of these studies should be interpreted with caution, as the patients in these studies had normal baseline ejection fractions.

The use of L-carnitine therapy for the treatment of cardiac arrhythmias remains a contentious subject. Suzuki *et al.*[90] reported that the administration of L-carnitine (2 g p.o. prior to dialysis) for 4–8 weeks resulted in a significant reduction in the incidence and severity of arrhythmias in the patient population. Conversely, no change in the frequency of arrhythmias was reported after administration of 20 mg/kg i.v. L-carnitine after dialysis for 6 months when compared with a placebo-treated group, although the authors of this study did note that very few dialysis-related arrhythmias were reported in both the carnitine- and placebo-treated groups.[40]

The findings of these studies indicate that L-carnitine shows a great deal of potential for use in the treatment of cardiac dysfunction, in particular in the treatment of dialysis-related hypotension. However, the relationship between L-carnitine and cardiac arrhythmias is less compelling, and additional well-designed trials are needed to investigate this further.

Plasma lipids

Approximately 40% of all chronic haemodialysis patients experience some form of dyslipidaemia.[92] The majority of patients present with Type IV hyperlipidaemia, with a reduction in plasma high-density lipoprotein (HDL) choles-

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terol levels.[92] As a consequence, these dyslipidaemic haemodialysis patients may be at a high risk of developing atherosclerotic cardiovascular disease.[93,94] In addition, recent research has found that traditional treatment of dyslipidaemia with statins is associated with no clinical benefit in cardiovascular end-points and an increase in the incidence of fatal strokes in some haemodialysis patients.[95] Consequently, treatment with statins is not recommended in these patients, and the investigation of alternative agents is warranted.[96]

Although the aetiology of dyslipidaemia in these patients is complex, carnitine deficiency has been implicated as a possible contributing factor in uraemic hyperlipidaemia. It has been hypothesized that carnitine supplementation may improve dyslipidaemia in these patients by stimulating oxidation of long-chain fatty acids, thereby decreasing their availability for esterification to triglycerides.[92] Based on this theory, numerous studies have investigated the effects of L-carnitine administration on plasma lipids in chronic haemodialysis patients, with varying results.[31,34,89,92,97–109] The findings of these studies are presented in Table 3.

A number of studies have demonstrated that long-term L-carnitine administration results in a significant reduction in triglyceride levels,[97–99,101] whereas others report that supplementation is associated with a significant increase in HDL cholesterol.[92,102,103] In a study by Vacha *et al.*[104] administration of L-carnitine for at least 12 months resulted in both a decrease in triglycerides and an increase in HDL cholesterol, with levels returning to baseline after cessation of L-carnitine treatment. Reintroduction of L-carnitine treatment produced the same improvement in plasma lipid levels. Trivelli *et al.*[105] reported comparable findings. Although numerous studies have demonstrated the beneficial effects of L-carnitine supplementation on dyslipidaemia in chronic haemodialysis patients, many studies have failed to show any significant changes in plasma lipid profiles,[31,89,106–108] with some studies reporting a negative effect on levels.[34,109] A meta-analysis of these studies indicated that L-carnitine supplementation was not associated with any significant effect on lipid parameters.[69] The authors suggested that additional adequately sized trials should be performed.

The results of these studies indicate that the potential use of L-carnitine for the treatment of dyslipidaemia is a complex issue. Additional studies have suggested that the haemodialysis population can be divided into groups of 'responders' and 'non-responders', although there appears to be no clear distinction between these two groups. Elisaf *et al.*[98] noted that patients with hypertriglyceridaemia (triglycerides > 200 mg/dL) exhibited significant reductions in triglyceride levels with L-carnitine treatment; however, normotriglyceridaemic patients showed no significant changes. A significant correlation between response to carnitine supplementation and baseline triglyceride levels was described. Similarly, Vacha *et al.*[110] observed that patients with low HDL cholesterol (<400 mg/L) responded significantly to L-carnitine treatment (20 mg/kg per dialysis session i.v.) with a decrease in triglyceride levels and an increase in HDL cholesterol; whereas patients with high HDL cholesterol (>400 mg/L) had no changes in plasma lipid profiles. However, administration of a higher dose of L-carnitine (60 mg/kg per dialysis session i.v.) to hyporesponsive patients resulted in a significant reduction in cholesterol and triglyceride levels. Other studies have noted that responders exhibited significantly higher baseline short- and long-chain acylcarnitine levels than non-responders, and responders had significantly higher L-carnitine levels with administration than non-responders.[111,112]

The potential use of L-carnitine for the treatment of dyslipidaemia in chronic haemodialysis patients is not a clear-cut case, as evidenced by the discrepancy in the findings in the above-mentioned studies. Limited research in this area has been conducted over the past few years investigating this, which is surprising considering the recent significant increases in the incidence of dyslipidaemia in our society. Additional, appropriately designed studies are needed to examine this potential role for L-carnitine, in particular in specific dyslipidaemic populations, such as patients with high triglyceride levels and low HDL cholesterol levels.

Dialytic symptoms

Muscle weakness is a common complaint experienced by haemodialysis patients, as are other symptoms of altered muscle function, such as cramps. Carnitine deficiency has been implicated as a contributing factor to these conditions.

In a study investigating the effect of L-carnitine supplementation on haemodialysis patients, both with and without muscular symptoms such as cramps and asthenia, it was noted that significantly lower endogenous plasma carnitine levels were observed in the symptomatic patients than their non-symptomatic counterparts.[113] Of the 30 symptomatic patients in this study, approximately two-thirds had some improvement in their symptoms with L-carnitine treatment, which in most cases was classified as a mild or moderate improvement. Comparable results were described by Bellinghieri *et al.*,[28] in which L-carnitine treatment resulted in a significant improvement in symptom scores for muscle cramps, postdialysis asthenia and interdialysis asthenia in haemodialysis patients. These symptoms worsened during the washout phase of the trial when L-carnitine treatment was ceased. Conflicting results were reported in another study, in which no significant changes in muscle cramps or fatigue were observed after L-carnitine treatment.[41] However, it is worth noting that these patients were only administered L-carnitine treatment for 6 weeks and these patients exhibited normal muscle carnitine levels at baseline.

The benefit of L-carnitine therapy on measurements of muscle function does, however, appear to be limited, as evidenced by the findings of Rogerson *et al.*[107] and Siami *et al.*[114] It was noted in the latter study that, although no overall significant effects on muscle activity were found, four of the seven patients who received carnitine treatment showed an improvement of 2 or more grades in the assessment of muscle activity. The authors postulated that there is

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Table 3 Summary of lipid studies

Reference	Study design	Population	L-carnitine treatment		Findings
97	One-way, open-label	10 HD patients	50 mg/kg per Dx i.v. for 2 months (D,L-carnitine)	↓ TRIG	T_0: 550 ± 115 mg/mL; T_2: 357 ± 75 mg/mL
98	One-way, open-label	28 HD patients	5 mg/kg per Dx i.v. for 6 months	↓ TRIG	T_0: 225 ± 76 mg/dL; T_6: 201 ± 79 mg/dL
99	Two-way, parallel, open-label	8 HD patients	0.5 g/Dx i.v. for 2 months then 1.0 g/Dx i.v. for 1.5 months	↓ TRIG	T_0: 336 ± 56 mg/dL; $T_{3.5}$: 244 ± 82 mg/dL
		8 HD patients	Placebo for 3.5 months		T_0: 329 ± 72 mg/dL; $T_{3.5}$: 444 ± 269 mg/dL
100	One-way, open-label	8 HD patients	900 mg/day p.o. for 2 months	↓ TRIG	T_0: 287 ± 74 mg/dL; T_2: 188 ± 22 mg/dL
101	One-way, open-label	25 HD patients	5 mg/kg b.i.d. p.o. for 3 weeks	↓ TRIG	Values not reported
102	Two-way, crossover, double-blind	13 HD patients	1 g/Dx i.v. then placebo for 40 days each	↑ HDL	Values not reported
		14 HD patients	Placebo then 1 g/Dx i.v. for 40 days each		
92	Two-way, parallel, single-blind	9 HD patients	660 mg/day p.o. for 1 month	↑ HDL	T_0: 42.4 ± 5.9 mg/100 mL; T_1: 52.7 ± 5.4 mg/100 mL
		6 HD patients	Placebo for 6 months		Values not reported
103	One-way, open-label	20 HD patients	2 g/Dx i.v. & 2 g/Non-Dx p.o. for 3 months	↑ HDL/LDL	Values not reported
104	Two-way, parallel, open-label	11 HD patients	1 g/Dx i.v. for 1 month then 2 g/Dx dialysate for 3 months	↓ TRIG & ↑ HDL	Values not reported
		11 HD patients	1 g/Dx i.v. for 1 month then 4 g/Dx dialysate for 3 months		
105	Two-way, parallel, open-label	6 HD patients (Type IV HL)	2 g/Dx i.v. for 3 months	↓ TRIG & ↑ HDL	Values not reported
		6 HD patients (Type IV HL)	Placebo for 3 months		
106	Two-way, crossover, double-blind	9 HD patients	1 g t.i.d. p.o. then placebo for 5 weeks each	↔ LIPID	No difference in plasma lipid measurements
		9 HD patients	Placebo then 1 g t.i.d. p.o. for 5 weeks each		
31	Two-way, parallel, double-blind	38 HD patients	20 mg/kg per Dx i.v. for 6 months	↔ LIPID	No difference in plasma lipid measurements
		44 HD patients	Placebo for 6 months		
107	One-way, open-label	6 HD patients (asthenia)	2 g/day p.o. for 6 weeks	↔ LIPID	No difference in plasma lipid measurements
89	Two-way, parallel, double-blind	15 HD patients	1–1.5 g/Dx i.v. for 2 months	↔ LIPID	No difference in plasma lipid measurements
		15 HD patients	Placebo for 2 months		
108	Two-way, parallel, double-blind	21 HD patients	100 µmol/L dialysate for 6 months Placebo for 6 months	↔ LIPID	No difference in plasma lipid measurements
109	Two-way, parallel, open-label	6 HD patients	990 mg t.i.d. p.o. for 1 month	↑ TRIG	T_0: 180 ± 66 mg%; T_1: 219 ± 88 mg%
		4 HD patients	Placebo for 1 month		T_0: 222 ± 35 mg%; T_1: 222 ± 35 mg%
34	One-way, open-label	23 HD patients	1 g/Dx i.v. for 1 month	↑ TRIG	T_0: 185 ± 22 mg/dL; T_1: 273 ± 23 mg/dL

Dx, dialysis session; HD, haemodialysis; HDL, high-density lipoprotein; HL, hyperlipidaemia; LDL, low-density lipoprotein; TRIG, triglycerides.

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a subset of patients in whom L-carnitine administration appears to improve muscle function.

The benefit of L-carnitine treatment on muscle function and weakness is not established. These dialytic symptoms are life-altering and, in many cases, debilitating for the numerous haemodialysis patients who experience them regularly. Consequently, L-carnitine treatment may be justified, in some cases, for the potential improvement of these symptoms.

CONCLUSION

Although large clinical trials demonstrating the positive effect of L-carnitine on rHuEPO-resistant anaemia in haemodialysis patients are limited, the collective data suggest that L-carnitine treatment is beneficial in the treatment of this condition. This is supported by a formal meta-analysis which demonstrated that L-carnitine co-therapy was associated with a significant improvement in haematocrit and/or rHuEPO requirements. Although the direct link between an improvement in anaemic parameters as a result of L-carnitine supplementation and an improvement in a patient's clinical status has not been established, studies have demonstrated that L-carnitine supplementation is associated with a reduction in hospital use in the USA.[38,39]

There is by no means definitive evidence for the role of L-carnitine supplementation in the treatment of cardiac function, dyslipidaemia or dialytic symptoms in long-term haemodialysis patients; however, there are mounting data which indicate that L-carnitine may be a useful adjunct to traditional therapy in the treatment of these conditions. Interestingly, Steiber *et al.*[68,115] recently reported a significant correlation between the acylcarnitine/L-carnitine ratio and results of the physical component of the Short Form-36 in male individuals, and a significant improvement in these scores from baseline with L-carnitine treatment. This is in keeping with the findings of Sloan *et al.*,[116] in which it was demonstrated that L-carnitine supplementation had a positive effect on measures of quality of life.

Although numerous studies have demonstrated that L-carnitine is beneficial for the treatment of dialysis-related disorders, little is known about the specific patient groups for which L-carnitine supplementation provides the most benefit. Studies have suggested that response to L-carnitine treatment may be related to endogenous L-carnitine levels or changes in levels from baseline; however, this has not been investigated fully. Additional studies investigating the response to treatment and the relationship to more complex indices, such as the proportion of L-carnitine within the total carnitine pool or the accumulation of specific acylcarnitines, are needed.

Although it should never be a case of 'monkey see, monkey do', that L-carnitine treatment is covered within the US system is a fact that we should not ignore. Due to the excellent safety profile associated with this treatment, it is of our opinion that supplementation with L-carnitine may be warranted for the treatment of dialysis-related carnitine deficiency, particularly in those patients with rHuEPO-resistant anaemia, cardiac dysfunction, intradialytic hypotension and dialytic symptoms. Nevertheless, further well-designed studies evaluating the relationship between these conditions and L-carnitine levels are needed, in particular investigations into the phenomenon of 'responders' and 'non-responders', only then will we have measures to predict those patients for whom L-carnitine administration will be beneficial.

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ORIGINAL PUBLICATION

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Determination of the reference range of endogenous plasma carnitines in healthy adults

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Abstract

Background: L-carnitine is an endogenous substance, vital in the transport of fatty acids across the inner mitochondrial membrane for oxidation. Disturbances in carnitine homeostasis can have a significant impact on human health; therefore, it is critical to define normal endogenous concentrations for L-carnitine and its esters to facilitate the diagnosis of carnitine deficiency disorders. This study was conducted to determine the normal concentrations of a number of carnitines in healthy adults using three analytical methods. The impact of age and gender on carnitine concentrations was also examined.
Methods: Blood samples were collected from 60 healthy subjects of both genders and various ages. Plasma samples were analysed for endogenous carnitine concentrations by radioenzymatic assay, high-performance liquid chromatography and electrospray tandem mass spectrometry.
Results: Precision and accuracy of results obtained for each assay were within acceptable limits. Average endogenous concentrations obtained from the three analytical methods in this study were in the range of 38–44, 6–7 and 49–50 μmol/L for L-carnitine, acetyl-L-carnitine and total carnitine, respectively. Comparison of results between the genders indicated that males had significantly higher endogenous plasma L-carnitine and total carnitine concentrations than females. Age was found to have no impact on plasma carnitine concentrations.
Conclusion: These results are useful in the evaluation of biochemical or metabolic disturbances and in the diagnosis and treatment of patients with carnitine deficiency.

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Introduction

L-carnitine (3-hydroxy-4-*N*-trimethylammonium butyrate) is an endogenous substance present in all mammalian species. A wide range of biological activities of L-carnitine have been described, including buffering of the ratio of free coenzyme A (CoA) to Acyl CoA and as a scavenger of unwanted acyl groups; however, the most important role of L-carnitine is in the transport of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation.[1] Mitochondrial fatty acid oxidation is a fundamental source of cellular energy, particularly in cardiac and skeletal muscle; therefore it is not surprising that approximately 98% of the total carnitine concentrations within the body are located in these tissues.[2,3] Despite the fact that plasma comprises only approximately 1% of the total body carnitine pool, it is commonly used in studies as an indication of the total body carnitine status in patients and subjects.[4,5]

Disturbances in carnitine homeostasis have been shown to impact greatly on human health. Metabolic disorders resulting in low plasma L-carnitine concentrations are associated with cardiomyopathy, encephalopathy, muscle weakness and even early death if left untreated.[6,7] The less severe, but more common, secondary carnitine deficiency may be the result of impaired renal or hepatic function, or may be induced by administration of certain therapeutic agents, such as pivampicillin and valproic acid.[6,8,9] It is therefore critical to properly define normal endogenous values for L-carnitine and its esters so that these carnitine deficiency disorders may be correctly and easily diagnosed.

Analytical methods used for the measurement of plasma carnitine concentrations can be categorized as enzymatic, chromatographic and mass spectrometric assays. Enzymatic assays represent the more traditional approach and most published papers of L-carnitine pharmacology have utilized this type of assay. High-performance liquid chromatography (HPLC) potentially has a wider application as it allows for the quantification of specific short-chain acylcarnitines, in addition to L-carnitine, which may be used as markers of metabolic diseases. Mass spectrometry

methods are established in the diagnosis of metabolic disorders and have the added advantage of allowing the measurement of L-carnitine and an entire range of its esters in one analytical run. These assays have been reviewed in more detail elsewhere.[10]

While a large number of studies have reported a range of plasma L-carnitine concentrations in healthy adults (Table 1), most of these published reports have used modifications of early enzymatic assays.[11–14] With the advent of newer HPLC and mass spectrometry methods, it is not known whether utilization of these methods has affected the interpretation of normal values. Consequently, the aim of this study was to determine normal concentrations and reference intervals for a number of endogenous carnitines in the healthy adult population using three analytical approaches – radioenzymatic assay (REA), HPLC and electrospray ionisation-tandem mass spectrometry (ESI MS/MS). In addition, the impact of gender and age on endogenous carnitine concentrations was examined.

Materials and methods

This study was reviewed and approved by the Human Research Ethics Committee of the University of South Australia. Subjects were completely 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).

Sixty healthy adult subjects were recruited for study participation and were divided into four categories according to gender and age, as outlined in Table 2. A single blood sample (20 mL) was collected via venepuncture from each subject and transferred into heparinized collection tubes. Samples were stored on ice and centrifuged within 30 minutes of collection for the separation of plasma. Plasma was divided into three aliquots for sample analysis, one each for analysis by REA, HPLC and ESI MS/MS. An

Table 1 Summary of previously reported healthy subject endogenous carnitine concentrations

			Analyte concentration (μmol/L)			
Reference	Population	Analytical method	L-Carnitine	Acetyl-L-carnitine	Total acylcarnitine	Total carnitine
Debska-Slizien *et al*.[4]	29 Adults (19 males, 10 females)	REA	49 ± 11		8.3 ± 4.6	53 ± 13
Savica *et al*.,[5] Bellinghieri *et al*.[18]	14 adults (10 males, four females)	REA	46.82 ± 1.72	5.43 ± 0.42		
Bene *et al*.[19]	35 adults (22 males, 13 females)	MS/MS	30.029 ± 1.902	10.227 ± 0.368	12.166 ± 0.978	
Borum[20]	446 males	REA				51.7 ± 10.8
	444 Females					43.8 ± 11.3
Furst and Gloggler[21]	Four adults	REA	23.9 ± 5.0			30.9 ± 6.4
Golper *et al*.[22]	? adults	REA	48 ± 2.5			57 ± 2.3
Harper *et al*.[23]	29 adults	REA	38.8 ± 7.3		5.9 ± 4.1	44.6 ± 8.6
	13 males		41.3 ± 4.9		5.7 ± 4.1	47.0 ± 5.4
	16 females		36.7 ± 8.3		6.0 ± 4.2	42.7 ± 10.3
Jones *et al*.[24]	? adults	REA	36.6 ± 9.5		6.5 ± 3.7	43.1 ± 9.8
Leschke *et al*.[25]	43 adults	REA	49.8 ± 2.0			
Lohninger *et al*.[26]	12 males	?	38.7 ± 8.7			49.3 ± 2.3
Lombard *et al*.[27]	15 males	?	39.9 ± 4.9			49.4 ± 7.3
	15 females		32.5 ± 5.3			43.3 ± 5.5
Maccari and Hülsmann[28]	14 adults	REA	20.70 ± 0.97	0.84 ± 0.20		26.55 ± 1.18
Mayer *et al*.[29]	18 adults	REA	38 ± 8.7			50 ± 12
Niu *et al*.[30]	? males	HPLC	41.2 ± 6.1	6.2 ± 0.6		53.1 ± 8.5
	? females		35.2 ± 4.9	5.7 ± 0.7		45.4 ± 5.6
Penn and Schmidt-Sommerfeld[31]	Six adults	REA	40.1 ± 8.5		8.4 ± 4.0	48.5 ± 9.1
Rodriguez-Segade *et al*.[32,33]	49 adults	REA	47.1 ± 7.7		6.2 ± 2.4	53.3 ± 8.4
	19 males		51.4 ± 7.0		5.9 ± 3.0	57.3 ± 7.4
	30 females		44.3 ± 6.0		6.5 ± 2.0	50.8 ± 8.1
Segre *et al*.[34]	15 males	REA	51.0 ± 13.6			
Van Es *et al*.[35]	40 adults	REA	40.2 ± 7.7			50.9 ± 9.7
Vinci *et al*.[36]	30 adults (15 males, 15 females)	REA	41.9 ± 8.4			55.1 ± 9.7
Wanner *et al*.[37,38]	20 adults (10 males, 10 females)	REA	37.4 ± 1.3			46.0 ± 2.5
Wanner *et al*.[39]	20 adults	REA	39.8 ± 2.1			49.7 ± 2.4

Data are expressed as mean ± SD. REA, radioenzymatic assay; MS/MS, mass spectrometry; HPLC, high-performance liquid chromatography; ?, data not reported

Table 2 Demographic data for each subject group

	Subject group			
Parameter	**Females 18–40**	**Females >40**	**Males 18–40**	**Males >40**
No. of subjects	15	15	15	15
Gender	Female	Female	Male	Male
Age: Group (years)	18–40	>40	18–40	>40
Age: mean ± SD (years)	25.9 ± 3.87	50.8 ± 8.13	28.2 ± 7.08	52.0 ± 6.71

additional aliquot was taken from each plasma sample and combined to form a pooled plasma sample for use as a quality control.

All plasma samples were stored at −20°C until sample analysis. Previous preliminary studies have demonstrated that L-carnitine and its esters are stable for four weeks under the collection and storage conditions that were used.

All analytical laboratories were blinded to sample identification codes.

Quality control samples

Identical quality control samples were prepared for each of the three analytical methods and submitted for analysis with the subject samples. In order to test precision, 10 aliquots of a pooled plasma sample (QCP) were included in the sample set for each method. For assessment of accuracy, quality control samples with known concentrations were prepared with dialysed plasma and designated as QCA (L-carnitine 7.50 μmol/L, acetyl-L-carnitine 1.50 μmol/L, total carnitine 9.75 μmol/L), QCB (L-carnitine 30.0 μmol/L, acetyl-L-carnitine 6.00 μmol/L, total carnitine 39.0 μmol/L) and QCC (L-carnitine 150 μmol/L, acetyl-L-carnitine 30.0 μmol/L, total carnitine 195 μmol/L). Five aliquots of each of these quality control samples were included for analysis by each analytical method.

Radioenzymatic assay

Plasma samples were analysed for L-carnitine (free carnitine) concentrations by the Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, South Australia. Samples were analysed by REA based on the method of Cederblad and Lindstedt,[15] using the reaction:

$$\text{L} - \text{carnitine} + \text{acetyl} - \text{CoA} \leftrightarrow \text{acetyl} - \text{L} - \text{carnitine} + \text{CoASH}$$

Briefly, the analytical method comprised four steps: sample preparation, incubation, separation and quantification of the product. Samples were prepared by mixing 10 μL of plasma, 90 μL of water and 20 μL of carnitine acetyltransferase (1.25 mg/mL, F Hoffman La Roche Ltd, Basel, Switzerland). To this, 70 μL of a reagent solution was added, which consisted of ^{14}C-acetyl-CoA (0.25 mCi/2.5 mL, NEN Life Science Products Inc., Boston, MS, USA) and acetyl-CoA (Sigma-Aldrich Pty Ltd.) in a combined concentration of 43 μmol/L and 0.025 mCi/3.5 mL in a potassium phosphate buffer (0.3 mmol/L, pH 7.6) containing 1.4 mmol/L *N*-ethyl maleimide (Sigma-Aldrich Pty Ltd., St Louis, MO, USA).

Each sample was incubated for 60 minutes at room temperature and then separated using an ion-exchange resin (BioRad AG2-X8, 20–50 mesh chloride form, BioRad Laboratories, Hercules, CA, USA). Radiolabelled product was quantified by scintillation counting. Standard curve samples were prepared using an L-carnitine chloride solution (Sigma-Aldrich Pty Ltd) over the range of 0–200 μmol/L and were processed according to the same procedure as the subject plasma samples.

High-performance liquid chromatography

Plasma samples were quantified for L-carnitine, acetyl-L-carnitine and total carnitine concentrations by the Centre for Pharmaceutical Research, University of South Australia, Adelaide, South Australia. Sample analysis was carried out using the fluorometric detection method described by Longo *et al.*[16]

In short, samples were purified by solid-phase extraction (Bond Elut SAX-Isolute cartridges 100 mg/mL, Varian, Palo Alto, CA, USA) followed by derivatisation with 1-aminoanthracene (Sigma-Aldrich Chemie GmbH) with 1--3-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich Chemical Company Inc., Milwaukee, WI, USA) present as a catalyst. Chromatography was carried out using a Shimadzu pump (LC-10AD), automatic injector (SIL-10AD), system controller (SCL-10A) and spectrofluorometric detector (RF-10AXL; Shimadzu Corporation, Kyoto, Japan) together with a Varian Res Elut-BD C18 analytical column (4.6 mm × 250 mm, with spherical particles of 5 μmol/L; Varian) and an Alltech column heater (Model 530, 220 V; Alltech Associates Inc., Deerfield, IL, USA). The mobile phase was run at a flow rate of 1 mL/min and consisted of 35% acetonitrile (HiPerSolv for HPLC, BDH Laboratory Supplies, Leicestershire, UK) and 65% ammonium acetate buffer (0.1 mol/L, pH 3.5). The detector wavelengths were 248 nm (excitation) and 418 nm (emission). The sample injection volume was 20 μL and run time was 20 minutes. Integration was carried out using CLASS-VP software (Version 6.10, Shimadzu Corporation).

The standard curve was constructed using samples of dialysed human plasma spiked with aqueous solutions of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine. Calibration ranges for each of the analytes were: 2.5–160 μmol/L for L-carnitine, 0.5–32 μmol/L for acetyl-L-carnitine and 3.25–208 μmol/L for total carnitine (L-carnitine+acetyl-L-carnitine+propionyl-L-carnitine).

Electrospray tandem mass spectrometry

Subject plasma samples were analysed for L-carnitine, total carnitine and an array of 31 acylcarnitines (including

acetyl-L-carnitine) by Pediatrix Screening, Bridgeville, PA, USA, based on the method described by Chace *et al*.[17] (with modifications for plasma). Four 25 μL aliquots of each plasma sample were pipetted onto the designated target areas on purpose-prepared filter paper (S&S grade 903, Schleicher & Schuell Bioscience Inc., Keene, NH, USA) and allowed to dry for two to three hours at room temperature. Once dry, the filter papers were sealed inside individual glassine envelopes (Westvaco number 10 stamp envelopes, MeadWestvaco Corporation, Glen Allen, VA, USA) and then placed in plastic-covered foil zipper pouches containing desiccant pillows. The filter papers were then shipped to Pediatrix Screening for analysis.

The analysis involved extraction of plasma from a 3/16 in. punch of filter paper using 300 μL methanol containing stable isotopically labelled internal standards over a period of 30 minutes. Samples were then derivatized with 50 μL acidified butanol for 15 minutes at 65°C to form butyl-esters of the carnitines. The derivatized carnitines were then analysed by flow injection ESI MS/MS (API-Sciex 3000, Foster City, CA, USA) with a throughput of two minutes per sample. The modified method utilized multiple deuterated internal standards, allowing for correction for any free carnitine produced during the derivatization with acidified butanol.

Calibration ranges for each of the analytes were: 0–80 μmol/L for L-carnitine, 0–50 μmol/L for acetyl-L-carnitine and 0–10 μmol/L for non-acetyl acylcarnitines.

Total acylcarnitine concentrations were determined as the sum of all 31 of the quantified acylcarnitines. Total carnitine concentrations were calculated as the sum of L-carnitine and total acylcarnitines.

Data analysis

Statistical comparisons were performed on Ln-transformed data using an analysis of variance with *post hoc* analysis (contrasts) if significance was detected. Statistical examination of the relationship between carnitine concentrations and age was conducted using linear regression. 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.

Reference interval was defined as the range of values which included 95% of the healthy population. As the distribution of carnitine concentrations within the population is slightly skewed, the lower and upper limits of the reference intervals for each analyte were determined as the 2.5 and 97.5 percentiles of the data.

Results

Sixty healthy adult subjects participated in the study and were partitioned into groups according to gender and age. The demographic details of each study group are outlined in Table 2.

The mean precision and accuracy values for L-carnitine, acetyl-L-carnitine and total carnitine were within 9% for all the analyses; the accuracy for total acylcarnitines was slightly higher for QCA (23.0%). Precision and accuracy results for each of the analytes by each analytical method are outlined in Table 3. It should be noted that the concentrations of each analyte quantified by ESI MS/MS for the high quality control samples (QCC) were outside the standard range and consequently these results were excluded in the calculation of accuracy values.

Mean ± standard deviation (SD) and 95% confidence interval for L-carnitine, acetyl-L-carnitine, total acylcarnitine (defined as the sum of all acylcarnitines, including acetyl-L-carnitine) and total carnitine concentrations for each analytical method are detailed in Table 4. L-carnitine concentrations quantified by both HPLC and ESI MS/MS were significantly higher than those measured by REA. Both the L-carnitine and acetyl-L-carnitine concentrations measured by HPLC were slightly, but significantly, higher than the concentrations quantified by ESI MS/MS. No significant differences were found between total carnitine concentrations quantified by HPLC and ESI MS/MS.

The effects of gender on carnitine concentrations were examined using the three analytical methods (Table 5). L-carnitine concentrations for the male subjects were shown to be significantly higher than those for females by all three analyses. Similarly, total carnitine concentrations were significantly higher in the males when compared with female subjects for both the HPLC and ESI MS/MS analyses, whereas no differences in acetyl-L-carnitine and acylcarnitine concentrations were found between the two genders. Average endogenous concentrations of L-carnitine

Table 3 Precision (data expressed as %CV) and accuracy (data expressed as mean ± SD) results for L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations quantified by radioenzymatic assay (REA), high-performance liquid chromatography (HPLC) and electrospray ionisation-tandem mass spectrometry (ESI MS/MS)

	Analytical method		
Parameter	**REA**	**HPLC**	**ESI MS/MS**
Precision			
L-Carnitine	2.90	5.00	8.58
Acetyl-L-carnitine	NA	3.97	7.56
Total acylcarnitine	NA	NA	6.59
Total carnitine	NA	3.19	8.12
Accuracy			
L-Carnitine			
QCA	−1.33 ± 7.30	7.21 ± 3.77	−6.39 ± 6.32
QCB	3.33 ± 4.71	8.15 ± 5.18	−8.47 ± 4.34
QCC	0.133 ± 7.47	4.44 ± 4.99	NA
Acetyl-L-carnitine			
QCA	NA	7.10 ± 8.33	6.91 ± 9.18
QCB	NA	4.21 ± 4.47	3.54 ± 5.11
QCC	NA	2.46 ± 4.89	NA
Total acylcarnitine			
QCA	NA	NA	23.0 ± 8.07
QCB	NA	NA	5.75 ± 3.75
QCC	NA	NA	NA
Total carnitine			
QCA	NA	3.19 ± 12.2	0.390 ± 6.57
QCB	NA	3.96 ± 5.18	−5.19 ± 4.04
QCC	NA	9.00 ± 7.37	NA

NA, not analysed; Precision: 100 × SD of 10 measured concentrations of QCP/mean of 10 measured concentrations of QCP. Accuracy: 100 × (measured concentration − actual concentration)/actual concentration

Table 4 Comparison of analytical methods: endogenous plasma L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations obtained by radioenzymatic assay (REA), high-performance liquid chromatography (HPLC) and electrospray ionisation tandem mass spectrometry (ESI MS/MS) (n = 60)

	Analytical method		
Parameter	**REA**	**HPLC**	**ESI MS/MS**
L-carnitine	38 ± 8.0 (36–40)	43.3 ± 8.57 (41.1–45.5)*	41.2 ± 10.1 (38.5–43.8)*†
Acetyl-L-carnitine	NA	6.65 ± 2.11 (6.10–7.19)	6.43 ± 2.01 (5.92–6.95)†
Total acylcarnitine	NA	NA	8.67 ± 2.38 (8.07–9.31)
Total carnitine	NA	49.2 ± 9.33 (46.8–51.6)	49.9 ± 11.0 (47.0–52.7)

NA, not analysed. Data are expressed as mean ± SD (95% confidence interval)
*$P < 0.05$, compared with REA
†$P < 0.05$, compared with HPLC

Table 5 Effect of gender: endogenous plasma L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations for females (n = 30) and males (n = 30) obtained by radioenzymatic assay (REA), high-performance liquid chromatography (HPLC) and electrospray ionisation-tandem mass spectrometry (ESI MS/MS)

	Gender	
Parameter	**Female**	**Male**
Radioenzymatic assay		
L-Carnitine	35 ± 7.0 (32.6–37.8)	41 ± 7.9 (38.4–44.3)*
High-performance liquid chromatography		
L-Carnitine	40.0 ± 7.70 (37.2–42.9)	46.6 ± 8.22 (43.5–49.7)*
Acetyl-L-carnitine	6.29 ± 2.21 (5.47–7.12)	7.00 ± 1.97 (6.27–7.74)
Total carnitine	45.9 ± 8.81 (42.6–49.2)	52.5 ± 8.79 (49.2–55.7)*
Electrospray ionisation-tandem mass spectrometry		
L-Carnitine	38.2 ± 8.01 (35.2–41.2)	44.1 ± 11.3 (39.9–48.3)*
Acetyl-L-carnitine	6.29 ± 2.31 (5.43–7.16)	6.58 ± 1.67 (5.96–7.20)
Total acylcarnitine	8.21 ± 2.62 (7.41–9.47)	9.15 ± 2.03 (8.20–9.68)
Total carnitine	46.7 ± 9.45 (43.1–50.2)	53.1 ± 11.7 (48.7–57.4)*

*$P < 0.05$, compared with female group

and total carnitine were in the range of 35–40 μmol/L and 45–47 μmol/L for females and 41–47 μmol/L and 52–53 μmol/L for males.

Differences in carnitine concentrations between the two different age groups (18–40 and >40 years) were examined (Table 6) and age was found to have no impact on carnitine concentrations quantified by any of the analytical methods. No significant differences between the age groups were found within each gender group. Similarly, statistical correlations between age and carnitine concentrations resulted in no significant results (data not presented).

The reference intervals for L-carnitine (stratified into females and males), acetyl-L-carnitine, total acylcarnitine and total carnitine (stratified into females and males) for each analytical method are presented in Figure 1. The reference intervals show significant overlap between the analytical methods and are comparable in width for each analyte.

Discussion

Numerous studies have reported plasma carnitine concentrations in healthy subjects with varying results [4,5,18–39] (Table 1). Mean ± SD values for L-carnitine in healthy adults have been reported as low as 20.7 ± 0.97 μmol/L[28] and as high as 49.8 ± 2.0 μmol/L.[25] Similar variations have been observed in the reporting of total carnitine (26.6 ± 1.18 to 57 ± 2.3 μmol/L), acetyl-L-carnitine (0.84 ± 0.20 to 10.2 ± 0.368 μmol/L) and other carnitine concentrations.[19,22,28] The variability appears to be independent of the assay used, with the majority of studies reporting values obtained from REA analysis. These inconsistencies present complexities in defining normal endogenous concentrations for carnitines in healthy adults and consequently the diagnosis of carnitine deficiency disorders becomes difficult. This study has therefore been designed to determine normal concentrations and reference intervals for endogenous L-carnitine, total carnitine and acetyl-L-carnitine in healthy adults using three different analytical methods.

The analytical methods employed in this study are validated techniques and are commonly used for diagnostic and/or research purposes. The precision and accuracy results obtained in this study for each of the assays were well within acceptable limits.

Comparison of carnitine concentrations obtained from each analytical method indicated that there were significant differences in the concentrations of L-carnitine and acetyl-L-carnitine quantified. However, examination of the data reveals that the average plasma concentrations are similar and in the range of 38–44 μmol/L. Analysis of samples by HPLC and ESI MS/MS resulted in average plasma concentrations which differed by only 4.8%, whereas

Table 6 Effect of age: endogenous plasma L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations for subjects aged 18–40 years (n = 30) and >40 years (n = 30) obtained by radioenzymatic assay (REA), high-performance liquid chromatography (HPLC) and electrospray ionization-tandem mass spectrometry (ESI MS/MS)

	Age group	
Parameter	**18–40**	**>40**
Radioenzymatic assay		
L-carnitine		
All subjects	39 ± 7.3 (36–42)	38 ± 8.7 (34–41)
Female subjects	36 ± 6.8 (32–39)	35 ± 7.4 (31–39)
Male subjects	42 ± 6.2 (39–46)	40 ± 9.3 (35–45)
High-performance liquid chromatography		
L-carnitine		
All subjects	43.7 ± 8.29 (40.6–46.8)	43.0 ± 8.96 (39.6–46.3)
Female subjects	40.3 ± 8.08 (35.8–44.8)	39.8 ± 7.57 (35.6–44.0)
Male subjects	47.1 ± 7.24 (43.1–51.1)	46.1 ± 9.34 (41.0–51.3)
Acetyl-L-carnitine		
All subjects	6.55 ± 2.14 (5.76–7.35)	6.74 ± 2.11 (5.95–7.53)
Female subjects	6.22 ± 1.80 (5.23–7.22)	6.36 ± 2.62 (4.91–7.81)
Male subjects	6.88 ± 2.45 (5.52–8.23)	7.12 ± 1.42 (6.33–7.91)
Total carnitine		
All subjects	48.5 ± 8.91 (45.2–51.8)	49.9 ± 9.83 (46.2–53.5)
Female subjects	45.7 ± 8.41 (41.0–50.3)	46.1 ± 9.48 (40.9–51.4)
Male subjects	51.3 ± 8.75 (46.5–56.2)	53.6 ± 8.98 (48.6–58.5)
Electrospray ionisation-tandem mass spectrometry		
L-carnitine		
All subjects	42.1 ± 9.91 (38.4–45.8)	40.2 ± 10.4 (36.3–44.1)
Female subjects	38.8 ± 8.37 (34.1–43.4)	37.6 ± 7.88 (33.3–42.0)
Male subjects	45.4 ± 10.5 (39.6–51.2)	42.8 ± 12.2 (36.1–49.6)
Acetyl-L-carnitine		
All subjects	6.30 ± 1.81 (5.62–6.98)	6.57 ± 2.21 (5.75–7.40)
Female subjects	6.11 ± 1.72 (5.15–7.06)	6.47 ± 2.83 (4.91–8.04)
Male subjects	6.49 ± 1.94 (5.42–7.56)	6.66 ± 1.42 (5.88–7.45)
Total acylcarnitine		
All subjects	8.47 ± 2.16 (7.66–9.28)	8.90 ± 2.64 (7.92–9.89)
Female subjects	8.22 ± 2.14 (7.03–9.40)	8.66 ± 3.34 (6.81–10.5)
Male subjects	8.72 ± 2.21 (7.50–9.95)	9.16 ± 1.77 (8.18–10.1)
Total carnitine		
All subjects	50.6 ± 10.4 (46.7–54.4)	49.1 ± 11.7 (44.8–53.5)
Female subjects	47.0 ± 9.47 (41.8–52.2)	46.3 ± 9.76 (40.9–51.7)
Male subjects	54.1 ± 10.4 (48.4–59.9)	52.0 ± 13.2 (44.7–59.3)

Data are expressed as mean ± standard deviation (95% confidence interval)

assay by REA produced lower average concentrations than the other analytical methods (7.8% lower than ESI MS/MS and 12.2% lower than HPLC). Endogenous acetyl-L-carnitine concentrations quantified by HPLC and ESI MS/MS were approximately 6–7 μmol/L, with 3.3% difference in average concentrations between the assays. There were no significant differences in total carnitine concentrations between the methods (49–50 μmol/L, 1.4% difference). The concentrations of carnitines obtained in the present study are comparable with those previously reported in healthy adult subjects in several studies.[5,18,23,26,29–31,35,36]

Examination of the effect of gender on plasma carnitine concentrations indicated that concentrations of L-carnitine and total carnitine are significantly higher in adult males than females in each of the analytical methods utilized. In this study, average L-carnitine concentrations were in the range of 35–40 μmol/L for females and 41–47 μmol/L for males, whereas total carnitine concentrations were in the range of 45–47 μmol/L for females and 52–54 μmol/L for males. Similarly, Lombard *et al.*[27] has previously demonstrated significantly higher plasma L-carnitine and total carnitine concentrations in males than in females with L-carnitine concentrations of 39.9 ± 4.9 μmol/L and 32.5 ± 5.3 μmol/L and total carnitine concentrations of 49.4 ± 7.3 μmol/L and 43.3 ± 5.5 μmol/L in males and females, respectively. Niu *et al.*[30] also reported significantly higher concentrations of L-carnitine and total carnitine in plasma of healthy male subjects than females; however, it was also noted that acetyl-L-carnitine concentrations were significantly different between genders, a result which was not demonstrated in the present study. Differences between the genders in plasma total carnitine concentrations were also noted by Borum *et al.*[20] Harper *et al.*[23] also examined the effect of gender on plasma carnitine concentrations and although a significant difference was observed in L-carnitine concentrations, no differences

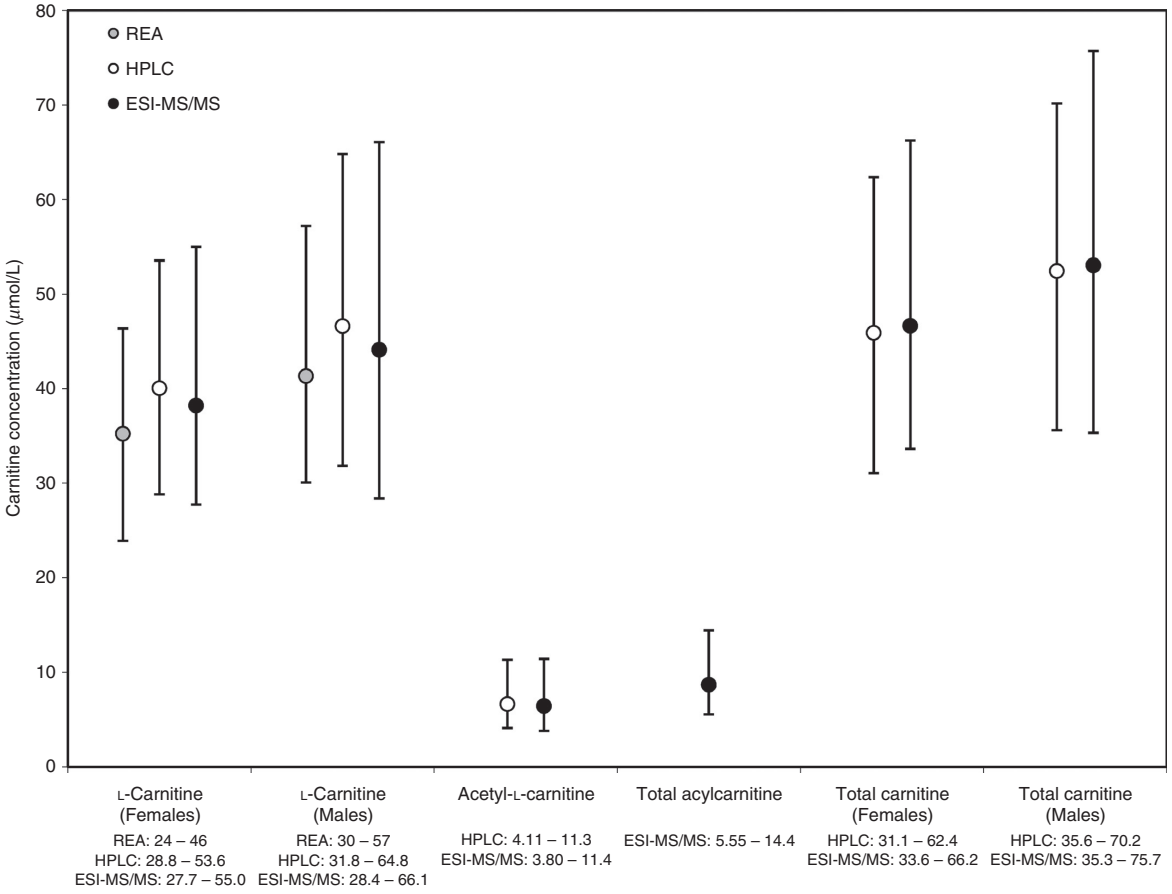


Figure 1 Reference intervals for endogenous L-carnitine, acetyl-L-carnitine, total acylcarnitine and total carnitine concentrations obtained by radioenzymatic assay, high-performance liquid chromatography and electrospray ionisation-tandem mass spectrometry. Data are expressed as mean within reference interval

between males and females were noted for total carnitine. The average plasma carnitine concentrations for males and females calculated in the present study are similar to those reported in the above studies.

A limited number of studies have examined the effect of age on plasma carnitine concentrations in healthy subjects. Age was found to have no impact on any of the analytes quantified in the present study, neither with significant differences between the two age groups examined, nor differences between the age groups within the genders. Similarly, no significant correlations between age and carnitine concentrations were observed. Borum *et al.*[20] previously demonstrated a significant correlation between age and endogenous plasma total carnitine concentrations in healthy females (but not males), a result which could not be replicated in the present study.

The reference intervals calculated from the three analytical methods in this study are very similar, with significant overlap in the intervals for each of the analytes and similar interval widths (Figure 1). However, there were slight differences in the upper and lower limits of the reference intervals obtained in this study, illustrating the importance of the development of laboratory-specific reference intervals for use in the diagnosis of primary and secondary carnitine deficiencies. In doing so, gender should be taken into consideration in the determination of reference intervals for L-carnitine and total carnitine; however, age is shown to have no impact. Laboratory-specific reference intervals are expected to be similar to those obtained from our laboratories.

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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 μg/kg/week/gHb) exhibited subnormal L-carnitine levels (<30 μM); conversely, patients with normal L-carnitine levels (>30 μM) displayed low ERI values (<0.02 μg/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

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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:

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

Study patients were prospectively divided into pre-defined Low ERI and High ERI groups based on a cut-off ERI value of 0.02 μg/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 μg/kg/week) are recommended for L-carnitine therapy [28]. An endogenous L-carnitine concentration of <30 μM was defined as subnormal 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 ± standard deviation (SD) values for each carnitine parameter for all of the study patients are summarized in Table 3, along with mean ± 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 μM). Similarly, all patients with normal L-carnitine levels had ERI values of <0.02 μg/kg/week/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 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 ± 5.13 μM c.f. 15.0 ± 4.28 μM, $P = 0.0116$) 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 ± 9.50 μM and 0.200 ± 0.0534, respectively, compared with values of 22.9 ± 4.62 μM 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.

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

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

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

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

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

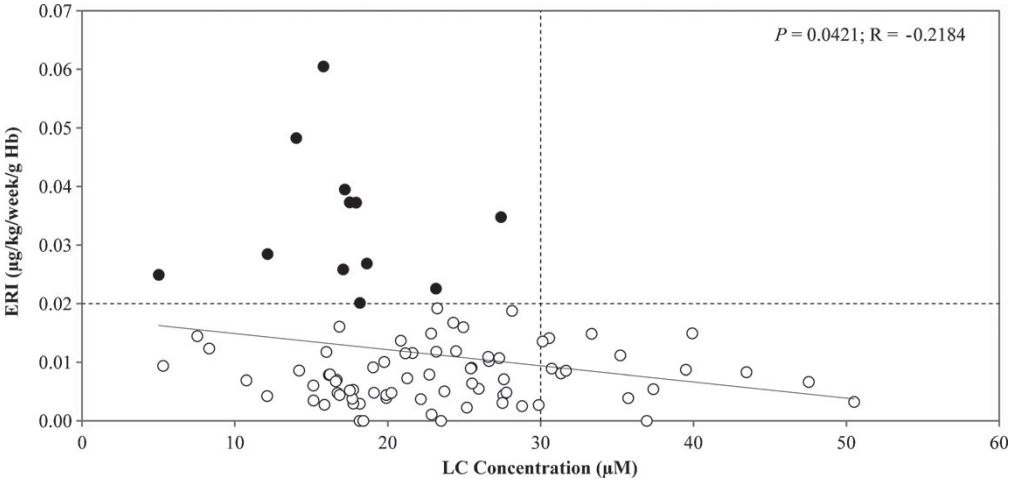


Fig. 1. Relationship between endogenous plasma L-carnitine concentrations and erythropoietin resistance index (ERI) (○ Low ERI patients; ● High ERI patients).

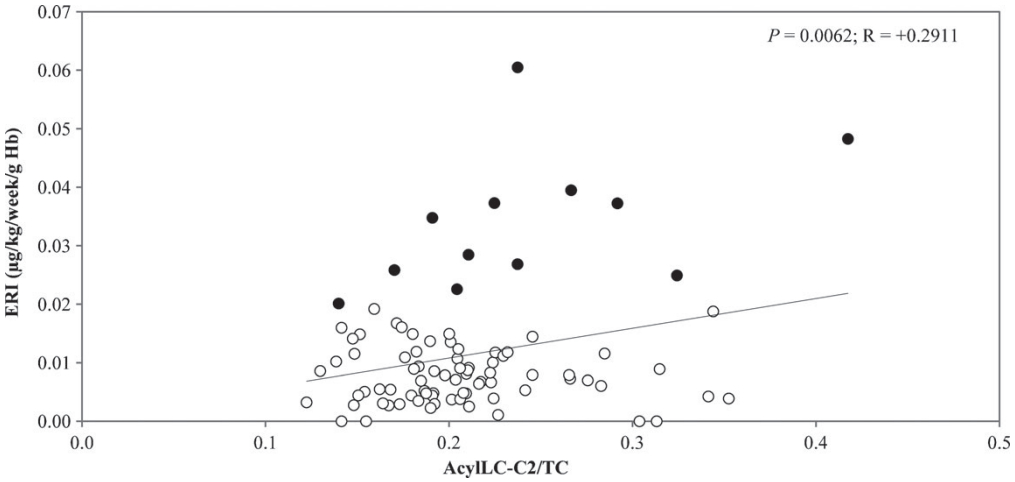


Fig. 2. Relationship between the proportion of non-acetyl acylcarnitines within the total endogenous plasma carnitine pool (AcylLC-C2/TC) and erythropoietin resistance index (ERI) (○ Low ERI patients; ● High ERI patients).

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, Kletzmayr *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. ≥ 0.02 μg/kg/week/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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Reliability and validity of indices of hand-grip strength and endurance

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***Background/aim*:** *Grip strength is useful in clinical practice for the assessment of disease and/or rehabilitation progression. Brief maximal gripping is seldom required in everyday occupations, with repeated or sustained gripping at sub-maximal power more commonly involved. It has been proposed that assessment of both maximal hand-grip force and endurance is utilised. While the suitability of maximal contraction measures has been clearly established, the reliability and validity of other hand-grip indices have not been investigated. This study examined the reliability of various hand-grip indices and their validity in relation to distance walked during the six-minute walk test, a standardised exercise capacity test.*

***Methods*:** *Subjects undertook static sub-maximal (50%) and maximal force contraction hand-grip testing from which various indices were derived, and six-minute walk testing from which distance walked was determined. Testing was repeated on three separate occasions for determination of test–retest reliability.*

***Results*:** *Pre- and post-fatigue maximal contraction measurements demonstrated excellent test–retest reliability and validity. Conversely, other hand-grip indices were shown to be unreliable and exhibited no relationship with distance walked and hence concurrent validity could not be established.*

***Conclusions*:** *Based on the results of this study, it is recommended that pre- and post-fatigue maximal contraction may be utilised for the assessment of client ability and progression due to their established validity and test–retest reliability. However, previously proposed measures of fatigue such as endurance (duration of sustained contraction), Strength Decrement Index and work performed (function of endurance and force of contraction) are unreliable and invalid and may have limited use in clinical practice.*

KEY WORDS *fatigue, functional assessment, outcome measures, rehabilitation services.*

Introduction

There is an abundance of daily activities for which some degree of hand-grip strength is necessary for a successful outcome. Hand strength is often affected by injury or disease and therefore measurement of grip strength is critical for occupational therapists and other health professionals for assessment of disease progress and progression in rehabilitation (Boissy, Bourbonnais, Carlotti, Gravel & Arsenault, 1999; Innes, 1999). Hand-grip dynamometry is a useful assessment tool for multiple purposes in clinical practice, including 'the assessment of upper limb impairment; in evaluating work capacity for those with hand injuries and other work-related injuries; the evaluation of people with other impairments and disabilities such as rheumatoid arthritis, chronic fatigue syndrome, developmental disabilities, muscular dystrophy and stroke; determining the efficacy of different treatments for people with a range of disabilities; part of an overall fitness assessment; and in determining the level of effort exerted' (Innes). In addition, assessments of grip strength have been shown to be strongly related to functional status and other health conditions such as osteoporosis in a range of patient groups (Dourado *et al*., 2006; Gosselink, Troosters & Decramer, 1996; Hasegawa, Schneider & Reiners, 2001; Rantanen *et al*., 1999; Stalenhoef, Diederiks, Knottnerus, Kester & Crebolder, 2002).

In light of the importance of grip ability for the monitoring of patient outcomes in practice, it is critical that the reliability and validity of these test protocols are established. Research has clearly established that the assessment of maximal contraction by hand-grip dynamometry is appropriate for use in healthy subjects (Boissy *et al*.,

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1999; Massy-Westropp, Rankin, Ahern, Krishnan & Hearn, 2004) and in those with neurological conditions (Bertrand, Mercier, Bourbonnais, Desrosiers & Gravel, 2007; Boissy *et al*.; Videler, Beelen, Aufdemkampe, de Groot & Van Leemputte, 2002). The assessment of the fatigue of maximal hand-grip has also proven reliable in healthy study participants (Yamaji, Demura, Nagasawa, Nakada & Kitabayashi, 2002). However, the majority of occupations require the use of sustained or repeated gripping at less than maximal forces and thus it has been proposed that both maximal hand-grip force and endurance are utilised in clinical practice for the assessment of patient progress (Nicolay & Walker, 2005). This therefore raises the need for reliable measurement protocols and normative data. Past studies have assessed hand-grip endurance utilising a range of protocols and indices. A majority of sub-maximal (20–80%) static hand-grip endurance tests previously conducted have relied on the duration of contraction as an indicator of hand-grip fatigability (Aniansson, Grimby, Hedberg, Rungren & Sperling, 1978; Nwuga, 1975; Petrofsky & Lind, 1975; Smolander *et al*., 1998). Similarly, duration has been used in sub-maximal tests of other muscle groups (Larsson & Karlsson, 1978; Smolander *et al*.), as well as in maximal hand-grip tests (Nicolay & Walker; Nwuga). Previous studies have also suggested that Strength Decrement Index (SDI), calculated as the deterioration of strength as a proportion of initial values, is a useful assessment of muscle fatigue (Clarke, Shay & Mathews, 1955). Despite the wide use of the duration of contraction and SDI as indicators of fatigue, the reliability of these measures has not been reported. The aim of this study was to examine the test–retest reliability of indices of sub-maximal, static hand-grip testing in healthy adult subjects.

Given that grip strength is not only a direct measure of hand skeletal muscle strength but also an index of overall muscle strength, endurance and ability, an association between peripheral strength and exercise capacity has been proposed (Dourado *et al*., 2006; Enright *et al*., 2003; Gosselink *et al*., 1996). The six-minute walk test (6MWT) is a standardised test for the assessment of exercise capacity. It was primarily developed for the assessment of functionality in patients with cardiac or respiratory disease; however, its use has extended to other patient groups (Enright *et al*.). It has been established as superior to subjective patient self-reporting tools and safer, easier to administer and better tolerated than other exercise capacity tests (Enright *et al*.; Enright & Sherrill, 1998). Distance walked during the 6MWT has been shown to strongly correlate with exercise capacity determined by maximal cardiopulmonary exercise testing and is strongly related to patient mortality in patients with primary pulmonary hypertension (Miyamoto *et al*., 2000). Studies have also demonstrated that peripheral muscle strength, including maximal hand-grip strength, is an important predictor of distance walked during the 6MWT. Enright *et al.* have demonstrated a significant ($P < 0.003$) correlation between maximal grip strength and distance walked during the 6MWT in 2281 adults aged ≥ 68 years. Similar associations between peripheral strength and results of the 6MWT have been reported in patients with chronic obstructive pulmonary disease (Dourado *et al*.; Gosselink *et al*.). Although the concurrent validity of maximal grip strength and distance walked during the 6MWT has been previously established, this has not been examined for other indices of hand-grip strength and endurance. The secondary aim of this study was to determine the concurrent validity of sub-maximal, static hand-grip testing in healthy adult subjects in relation to results of the 6MWT.

Methods

This study was reviewed and approved by the Human Research Ethics Committee of the University of South Australia. Participants were fully informed of the study procedures and provided written informed consent prior to study initiation. The study was conducted in accordance with the study protocol, the Declaration of Helsinki and the National Statement of Ethical Conduct in Human Research issued by the National Health and Medical Research Council (Australia).

Healthy adult subjects underwent hand-grip testing and the 6MWT according to the protocols outlined below. Testing was performed on three separate occasions (separated by at least 24 hours) at a similar time of the day, by the same assessor for determination of test–retest reliability. To reduce bias and possible effects on motivation, subjects were not informed of their progress during any of the testing procedures.

Hand-grip test

Hand-grip was assessed using a Smedley Hand Dynamometer (Stoelting Co., Wood Dale, IL, USA) modified with grip cushioning. Subjects were seated in an upright position with their shoulder adducted and elbow flexed at 90°, the forearm allowed to rest lightly on the arm of the chair or on the subject's thigh. For each subject, maximal voluntary contraction was determined as the highest of three contractions (INITIAL MAX) (Lagerstrom & Nordgren, 1998). After a one-minute rest period (Trossman & Li, 1989), subjects were instructed to squeeze the dynamometer in a static contraction at a level of 50% maximal for as long as possible, the duration of which was recorded (DURATION). The level of static contraction was monitored by the assessor who provided verbal feedback to ensure that a level of 50% maximal was maintained. Subjects were then required to perform an additional post-fatigue maximal contraction within 10 seconds of completion of the 50% maximal static contraction (FINAL MAX). Hand-grip testing was performed on both the dominant and non-dominant hands for each subject.

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Six-minute walk test

The 6MWT was conducted according to the American Thoracic Society (ATS) Guidelines for the 6MWT (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories, 2002). Briefly, subjects were instructed to walk as far as possible for six minutes on a flat, non-undulating surface. Participants were directed to walk in a circuit between two markers spaced 30 m apart. The total distance walked during the six minute testing period was recorded (DISTANCE).

Statistical methods

Indices of hand-grip testing were calculated for each subject, on each testing day, for both the dominant and non-dominant hands. Indices included:

- Pre-fatigue maximal contraction (INITIAL MAX).
- Post-fatigue maximal contraction (FINAL MAX).
- Duration of 50% maximal static contraction (DURATION).
- Work performed (WORK = DURATION × 50% INITIAL MAX).
- Strength Decrement Index (SDI = 100% × (INITIAL MAX − FINAL MAX)/INITIAL MAX).

Reliability of indices was calculated as the single measures intra-class correlation coefficient (ICC) of Day 1–3 data for which an ICC value of ≥ 0.9 is classified as 'excellent'; ≥ 0.7 is 'acceptable'; and ≤ 0.7 indicates that the measurement system is inadequate. To determine the presence of a training effect, an analysis of variance (ANOVA) was used to compare test results on each of the assessment days. Concurrent validity was investigated as the relationship between hand-grip indices and distance walked during the 6MWT using linear regression.

WinNonlin Professional Version 5.2 (Pharsight Corporation, Mountain View, CA, USA) and SPSS Version 16.0 (SPSS Inc., Chicago, IL, USA) were used for performing statistical analyses. Significance was set at an α-level of 0.05.

Results

Twenty-one healthy adult subjects (12 men, nine women) were recruited for study participation, of whom 16 underwent both testing protocols on three separate occasions for determination of test–retest reliability. The mean ± standard deviation (SD) age of the subjects was 29.8 ± 6.9 years. All subjects were free of significant illnesses, which would impact on the study results.

Mean ± SD INITIAL MAX values were 34 ± 8.2 and 33 ± 10 kg for the dominant and non-dominant hands respectively. Comparably, FINAL MAX values were 26 ± 7.2 kg after sub-maximal static contraction for 93 ± 32 seconds using the dominant hand and 24 ± 8.6 kg after contraction for 85 ± 25 seconds using the non-dominant hand. Mean ± SD DISTANCE values were 651 ± 77.5 m.

TABLE 1: *Results of the test–retest reliability (intra-class correlation coefficient of Day 1–3 data) and the determination of a training effect (ANOVA of Day 1–3 data) for each parameter measured, as well as the examination of the relationship between hand-grip indices and distance walked during the six-minute walk test (linear regression)*

	Reliability	Training effect	Regression (DISTANCE vs. Index)	
Index	ICC	*P*-value	*P*-value	R^2
Hand-grip test (dominant hand)				
INITIAL MAX	0.924	0.928	0.017	0.265
FINAL MAX	0.924	0.942	0.003	0.374
DURATION	0.582	0.992	0.455	0.030
WORK	0.593	0.823	0.117	0.124
SDI	0.465	0.839	0.114	0.126
Hand-grip test (non-dominant hand)				
INITIAL MAX	0.967	0.983	0.016	0.271
FINAL MAX	0.947	0.919	0.007	0.328
DURATION	0.572	0.473	0.972	0.0001
WORK	0.753	0.644	0.143	0.110
SDI	0.408	0.683	0.196	0.087
Six-minute walk test				
DISTANCE	0.819	0.567	—	—

ICC, intra-class correlation coefficient; SDI, Strength Decrement Index.

Test–retest reliability values for each parameter are presented in Table 1. Values for INITIAL MAX and FINAL MAX data were excellent (ICC > 0.9), and were comparable with that obtained for the distance walked during the 6MWT (ICC = 0.819). Test–retest reliability values for the other indices measured were poorer, particularly for SDI and DURATION (Table 1).

Test parameters obtained on each study day were statistically compared in order to determine the presence of a training effect. No significant between-day differences were observed in any of the hand-grip indices or in the results of the 6MWT. Results of the ANOVA are presented in Table 1.

In the statistical correlation of calculated indices of hand-grip and results of the 6MWT, there was a highly significant correlation between DISTANCE and INITIAL MAX for both the dominant and non-dominant hands ($P = 0.017$ and $P = 0.016$, respectively), as well as DISTANCE and FINAL MAX for the dominant and non-dominant hands ($P = 0.003$ and $P = 0.007$, respectively) (Fig. 1). No significant correlations were found for the other hand-grip indices. Linear regression results for each index are presented in Table 1.

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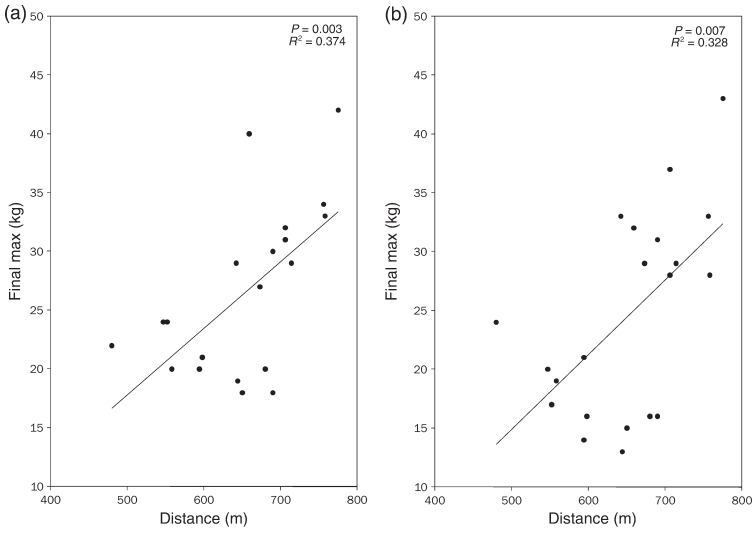


FIGURE 1: *Relationship between post-fatigue maximal hand-grip contraction (*FINAL MAX*) for the dominant (a) and non-dominant (b) hands and distance walked during the six-minute walk test (*DISTANCE*).*

Discussion

The use of hand-grip dynamometry has extended beyond the assessment of maximal voluntary contraction alone and has also been used for the assessment of grip endurance to gain a better overall picture of patient functionality (Aniansson *et al*., 1978; Nicolay & Walker, 2005; Nwuga, 1975; Petrofsky & Lind, 1975; Smolander *et al*., 1998). While the suitability of maximal grip strength measurements has been clearly established (Bertrand *et al*., 2007; Boissy *et al*., 1999; Massy-Westropp *et al*., 2004; Videler *et al*., 2002; Yamaji *et al*., 2002), the reliability and validity of these additional hand-grip assessments have not been reported. Consequently, this study aimed to establish the reliability of various indices of hand-grip strength and endurance and their concurrent validity in relation to distance walked during the 6MWT, a standardised exercise capacity test.

Maximal hand-grip contraction (INITIAL MAX) values and results of the 6MWT obtained in this study were comparable with age- and gender-specific normative values reported previously for healthy adults (Enright & Sherrill, 1998; Massy-Westropp *et al*., 2004; Miyamoto *et al*., 2000). These parameters showed excellent test–retest reliability, which is in keeping with the results of previous research in healthy volunteers (Boissy *et al*., 1999; Massy-Westropp *et al*.; Yamaji *et al*., 2002) and patients with neurological conditions (Bertrand *et al*., 2007; Boissy *et al*.; Videler *et al*., 2002). A highly statistically significant correlation between the distance walked during the 6MWT (DISTANCE) and maximal force contraction (INITIAL MAX) was observed for both the dominant and non-dominant hands, consistent with the findings of previous studies in chronic obstructive pulmonary disease patients (Dourado *et al*., 2006; Gosselink *et al*., 1996) and in elderly adults (Enright *et al*., 2003). This is also consistent with previous studies, which have demonstrated that maximal hand-grip strength is strongly related, even predictive of other physical characteristics of concern to occupational therapists and health professionals, for example, disability and frailty in clients aged above 65 years (Rantanen *et al*., 1999), bone mineral density (Hasegawa *et al*., 2001), the risk of falls (Stalenhoef *et al*., 2002) and nutritional status (Guo, Zhang, Ma, Zhang & Huang, 1996). These findings support the use of maximal hand-grip force in clinical practice and research as an indicator of patient status and progression.

For the first time, this study has also investigated the reliability and validity of other measures of hand-grip strength and endurance. Static contraction at a sub-maximal level (50%) until fatigue was found to result in an approximate 25% reduction in maximal grip force (FINAL MAX). Similar to pre-fatigue maximal contraction, these post-fatigue maximal contraction values were shown to have excellent test–retest reliability (ICC > 0.9) and correlated well with results of the 6MWT ($P < 0.01$) indicating its suitability for monitoring patient outcomes in practice and a research setting. In addition, statistical comparisons of FINAL MAX data on each of the assessment days indicated the lack of a learning effect, suggesting that this parameter may be used for both single point evaluations as well as in longitudinal assessments.

Conversely, other previously proposed indices of hand-grip fatigability such as DURATION, WORK and SDI demonstrated poor test–retest reliability and concurrent validity of these measures against the 6MWT was unable

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to be established. As a result, these parameters cannot be recommended for clinical use. Furthermore, previous research has commonly utilised these measures as indicators of fatigability (Aniansson *et al*., 1978; Larsson & Karlsson, 1978; Nicolay & Walker, 2005; Nwuga, 1975; Petrofsky & Lind, 1975; Smolander *et al*., 1998), particularly duration of contraction, and thus results of these studies may need to be interpreted with caution.

Based on the results of this study, it is recommended that pre- and post-fatigue maximal contraction may be utilised individually or in combination for the assessment of client ability and progression because of their established validity and test–retest reliability. However, previously proposed measures of fatigue such as endurance (duration of sustained contraction), SDI and work performed (function of endurance and force of contraction) have been shown to be unreliable and invalid and therefore may have limited use in clinical practice and research.

In addition, the highly significant correlations between pre- and post-fatigue maximal contraction and distance walked during the 6MWT observed in this study suggest that these indices of grip strength are predictive of exercise capacity. The use of these hand-grip measurements as an indicator of exercise capacity may be particularly useful for the assessment of patient groups for whom performing 6MWT or traditional treadmill or bicycle-based tests is not feasible because of limited mobility or lack of a suitable test environment; however, this warrants further investigation.

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ORIGINAL PUBLICATION

Reuter SE, Evans AM. Long-chain acylcarnitine deficiency in chronic fatigue syndrome patients. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med*; in press.

Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity

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Abstract. Reuter SE, Evans AM (School of Pharmacy & Medical Sciences, University of South Australia, Adelaide, SA, Australia; Sansom Institute for Health Research, University of South Australia, Adelaide, SA, Australia). Long-chain acylcarnitine deficiency in patients with chronic fatigue syndrome. Potential involvement of altered carnitine palmitoyltransferase-I activity. *J Intern Med* 2010; doi: 10.1111/j.1365-2796.2010.02341.x.

Objective. The underlying aetiology of chronic fatigue syndrome is currently unknown; however, in the light of carnitine's critical role in mitochondrial energy production, it has been suggested that chronic fatigue syndrome may be associated with altered carnitine homeostasis. This study was conducted to comparatively examine full endogenous carnitine profiles in patients with chronic fatigue syndrome and healthy controls.

Design. A cross-sectional, observational study.

Setting and subjects. Forty-four patients with chronic fatigue syndrome and 49 age- and gender-matched healthy controls were recruited from the community and studied at the School of Pharmacy & Medical Sciences, University of South Australia.

Main outcome measures. All participants completed a fatigue severity scale questionnaire and had a single fasting blood sample collected which was analysed for L-carnitine and 35 individual acylcarnitine concentrations in plasma by LC-MS/MS.

Results. Patients with chronic fatigue syndrome exhibited significantly altered concentrations of C8:1, C12DC, C14, C16:1, C18, C18:1, C18:2 and C18:1-OH acylcarnitines; of particular note, oleyl-L-carnitine (C18:1) and linoleyl-L-carnitine (C18:2) were, on average, 30–40% lower in patients than controls ($P < 00001$). Significant correlations between acylcarnitine concentrations and clinical symptomology were also demonstrated.

Conclusions. It is proposed that this disturbance in carnitine homeostasis is reflective of a reduction in carnitine palmitoyltransferase-I (CPT-I) activity, possibly a result of the accumulation of omega-6 fatty acids previously observed in this patient population. It is hypothesized that the administration of omega-3 fatty acids in combination with L-carnitine would increase CPT-I activity and improve chronic fatigue syndrome symptomology.

Keywords: acylcarnitine, carnitine, carnitine palmitoyltransferase, chronic fatigue syndrome, fatty acid.

Introduction

Chronic fatigue syndrome (also known as myalgic encephalomyelitis) is a term used to describe a heterogeneous condition that is primarily characterized by persistent debilitating fatigue that cannot be attributed to any alternative condition [1–4]. Despite being widely recognized, it remains one of the most perplexing conditions in medical science as there is little understanding of its pathogenesis and aetiology. Consequently, there remains no universally effective treatment option and diagnosis is based on the presence of a number of poorly understood signs and symptoms. Additional research to ascertain the underlying pathology of chronic fatigue syndrome is vital to establish appropriate diagnostic criteria as well as effective treatment protocols.

It is estimated that the prevalence of chronic fatigue syndrome is approximately 0.2–0.7% in the community and 0.5–2.5% in primary care [4] with the condition predominantly affecting young adults

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(onset 20–40 years) with a higher incidence in women (female–male ratio 3 : 1) [1–4]. Chronic fatigue syndrome has been associated with a significant loss in productivity [5] and a high total annual cost burden [4, 6, 7]. However, it is important to note that the impact of chronic fatigue syndrome is not merely economic with reports that sufferers experience a heavy psychosocial burden and a substantially reduced quality of life [8].

A variety of factors have been attributed to the pathophysiology of chronic fatigue syndrome, including immunologic, biochemical, psychiatric and neurological [2, 3]; however, at this time, no firm associations can be drawn. Numerous studies have emphasized a viral cause of chronic fatigue syndrome [2, 3] and recently a link between chronic fatigue syndrome and infection with certain retroviruses has been reported [9], although this result remains controversial [10–14].

L-carnitine is an endogenous compound, found in all mammalian species [15]. Adequate levels of L-carnitine are achieved from dietary sources, in particular from red meat, as well as via biosynthesis in the kidneys, liver and to some extent in the brain [15]. The principal biological role of carnitine is in the transport of fatty acids across the inner mitochondrial membrane for fatty acid oxidation via the reversible binding of acyl groups from Coenzyme A (CoA) (Fig. 1) [16]. It has also been established that carnitine has a role in regulating the cellular to mitochondrial ratio of free CoA to AcylCoA, in the transport of short and medium acyl groups from the peroxisome to the mitochondria and in the removal of unwanted acyl groups from the body [15, 17]. Alterations in carnitine homeostasis can have a detrimental effect on human health [18, 19]. In its severest form, carnitine deficiency is associated with progressive cardiomyopathy, encephalopathy and muscle weakness, resulting in death from heart failure [19, 20].

In the light of carnitine's critical role in fatty acid oxidation, it has been suggested that chronic fatigue syndrome may be associated with alterations in L-carnitine and acylcarnitine homeostasis [21]. Previous studies have reported a reduction in endogenous plasma L-carnitine (free carnitine), total carnitine and/or acylcarnitine levels in patients with chronic fatigue syndrome [22–26]; however, these results have not been replicated in other studies [27, 28]. Whilst these studies have provided useful information on the composition of the endogenous carnitine pool in chronic fatigue syndrome, they have only examined free carnitine and total acylcarnitine levels (i.e. the sum of all individual acylcarnitines) and consequently alterations in the levels of some individual acylcarnitines may be effectively 'cancelled out' by relatively normal levels of other acylcarnitines in these patients. Recently, tandem mass spectrometry methods have been developed which are capable of quantifying individual acylcarnitine levels in human plasma [29, 30] and therefore provide a more adequate representation of the full carnitine profile.

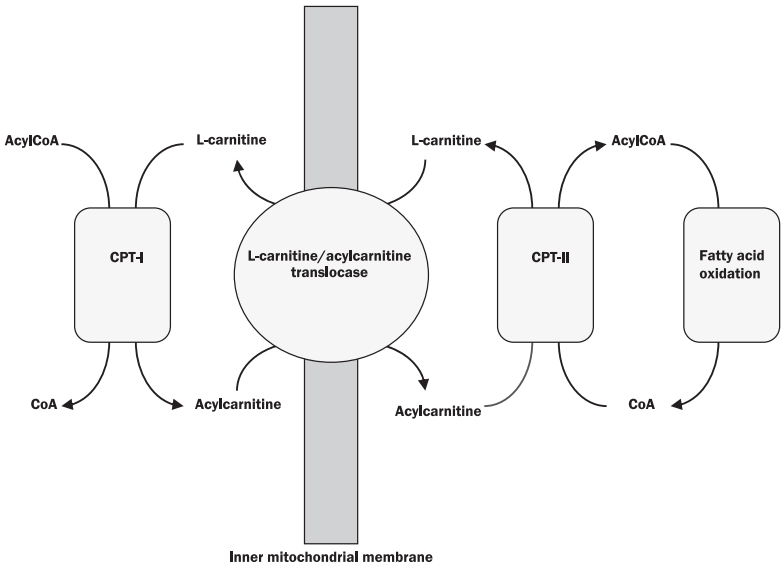


Fig. 1 *The role of L-carnitine in fatty acid oxidation. CoA, Coenzyme A; CPT, Carnitine Palmitoyltransferase.*

One previous study examined individual plasma acylcarnitine concentrations in patients with chronic fatigue syndrome [31] in which the authors reported no significant differences in acylcarnitine levels between 25 patients and 25 healthy controls. However, it should be noted that in this study, only 20 individual acylcarnitines were quantified, and consequently, the individual acylcarnitines reported accounted for less than two-thirds of the reported total acylcarnitine levels. In addition, the lower limit of quantification of the assay used was well above the levels reported for all of the medium- and long-chain acylcarnitines quantified. Consequently, no firm conclusions regarding carnitine pool composition in patients with chronic fatigue syndrome can be drawn from this study.

The present study was conducted to examine the levels of endogenous plasma L-carnitine and 35 individual acylcarnitines in patients with chronic fatigue syndrome compared with age- and gender-matched healthy controls.

Materials and methods

Study design

This study was reviewed and approved by the Human Research Ethics Committee of the University of South Australia. Participants 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).

Patients with chronic fatigue syndrome were recruited via the Chronic Fatigue Syndrome Society of South Australia [ME/CFS Society (SA) Inc]. Patients had been previously diagnosed with chronic fatigue syndrome by a physician according to the standard diagnostic criteria [4]. Age- and gender-matched healthy subjects, with no significant illnesses, were recruited from the general population via advertising. Neither patients nor subjects had received any carnitine supplementation within 2 months of assessment.

On the study assessment day, each participant completed a fatigue severity scale questionnaire and had a single fasting blood sample collected via venepuncture for carnitine profiling.

Fatigue severity scale

The fatigue severity scale is a validated functional measure which comprises nine items that are rated according to a Likert-type rating scale from 1 to 7, with '1' indicating no impairment and '7' indicating severe impairment (Table 1) [32]. The fatigue severity scale has been shown to be an appropriate and accurate measure of fatigue severity and symptomology and is able to distinguish between individuals with chronic fatigue syndrome-like symptomology and those individuals with no or varying levels of general fatigue [33].

Carnitine profiling

A single fasting blood sample was collected from each study participant for analysis of plasma L-carnitine and individual acylcarnitine concentrations. Analysis was conducted by the Department of Genetic Medicine, Women's and Children's Hospital (Adelaide, SA, Australia) using a 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

Table 1 *Fatigue severity scale [32]*

Indicate your agreement/disagreement with the following statements on a scale of 1 to 7, with a score of 1 indicating strongly disagree and a score of 7 indicating strongly agree							
My motivation is lower when I am fatigued	1	2	3	4	5	6	7
Exercise brings on my fatigue	1	2	3	4	5	6	7
I am easily fatigued	1	2	3	4	5	6	7
Fatigue interferes with my physical functioning	1	2	3	4	5	6	7
Fatigue causes frequent problems for me	1	2	3	4	5	6	7
My fatigue prevents sustained physical functioning	1	2	3	4	5	6	7
Fatigue interferes with me carrying out certain duties and responsibilities	1	2	3	4	5	6	7
Fatigue is among my three most disabling symptoms	1	2	3	4	5	6	7
Fatigue interferes with my work, family or social life	1	2	3	4	5	6	7

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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-minute 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 precursor scan of 85.1 amu. The levels of acylcarnitines were determined against the respective deuterated stable isotope using Analyst® software (Applied Biosystems Inc).

Individual carnitines analysed are given in Table 2. Total acylcarnitine levels were taken as the sum of all individual acylcarnitine concentrations, and total carnitine levels were calculated as the sum of L-carnitine and total acylcarnitine concentrations. It should be noted that, because of the nature of the tandem mass spectrometry method, some assay results represent the sum of 2 or 3 carnitine esters; for example, C4 represents the sum of 2 structural isomers with a four carbon-chain acyl group (butyryl-L-carnitine and isobutyryl-L-carnitine).

Statistical analysis

Unless otherwise indicated, data are expressed as mean ± standard deviation. Carnitine concentrations and demographic characteristics (i.e. age and fatigue severity scale results) obtained from patients with chronic fatigue syndrome were statistically compared to those obtained from healthy subjects using an analysis of variance (ANOVA). Statistical examination of the relationship between carnitine pool composition and fatigue severity was conducted using linear regression of fatigue severity scale results versus endogenous carnitine levels. Gender distribution between the groups was compared using Pearson's Chi-Squared (χ^2) cross-tabulation analysis.

Significance was set at an α-level of 0.05. WinNonlin® Professional Version 5.3 (Pharsight Corporation, Mountain View, CA, USA) was used for ANOVA analysis. SPSS for Windows Version 16.0 (SPSS Inc, Chicago, IL, USA) was used for conduct of the linear regression and Pearson's Chi-Squared cross-tabulation analyses.

Results

Forty-four patients with chronic fatigue syndrome (17 men; 27 women), with an average age of 49.9 ± 15.0 years, participated in the study. In addition, 49 healthy subjects (20 men; 29 women), aged 45.6 ± 11.6 years, were recruited to serve as controls. Average fatigue severity scale scores for the chronic fatigue syndrome group were 6.22 ± 0.660, compared with scores of 3.04 ± 1.23 for the healthy control group ($P < 0.0001$). There were no significant differences in age or gender distribution between the groups.

Endogenous plasma L-carnitine, total carnitine, total acylcarnitine and individual acylcarnitine concentrations for the chronic fatigue syndrome and healthy control groups are presented in Table 2. There were no significant differences in L-carnitine, total carnitine or total acylcarnitine levels between the groups, whereas patients with chronic fatigue syndrome had significantly lower C8:1, C14, C16:1, C18, C18:1 and C18:2 concentrations and significantly higher C12DC and C18:1-OH levels than healthy subjects. In most of these cases, endogenous acylcarnitine levels differed by approximately 20% between the groups; however, of particular note, C18:1 and C18:2 were 30–40% lower in patients with chronic fatigue syndrome than in healthy controls (Fig. 2) ($P < 0.0001$).

Significant negative correlations between the results of the fatigue severity scale and endogenous plasma concentrations of C8:1, C14, C16:1, C18:1 and C18:2 were observed. Importantly, there were highly significant associations between fatigue severity and C18:1 ($P = 0.0009$, $R = -0.3547$) and C18:2 ($P < 0.0001$, $R = -0.4191$) levels. Significant positive correlations between fatigue severity and concentrations of C12DC, C16 and C18:1-OH were demonstrated; however, it should be noted that the data for C12DC was skewed because of two patients with chronic fatigue syndrome who exhibited highly elevated levels.

Discussion

On account of carnitine's critical role in energy metabolism, a number of previous studies have investigated endogenous plasma carnitine levels in patients with chronic fatigue syndrome, with equivocal results. Some studies have suggested that chronic

Table 2 *Endogenous plasma carnitine concentrations (μmol L^{-1}) for patients with chronic fatigue syndrome (n = 44) and healthy controls (n = 49)*

Endogenous Plasma Carnitine		Healthy Controls	Chronic Fatigue Syndrome Patients	
LC	L-Carnitine	45.2 ± 9.79	45.0 ± 11.3	
TC	Total Carnitine	59.5 ± 12.9	58.8 ± 13.6	
AcylLC	Total Acylcarnitines	14.3 ± 4.13	13.8 ± 3.45	
C2	Acetyl-L-Carnitine	10.6 ± 3.37	10.2 ± 2.72	
C3	Propionyl-L-Carnitine	0.502 ± 0.153	0.489 ± 0.199	
C3DC	Malonyl-L-Carnitine	0.0447 ± 0.0217	0.0416 ± 0.0166	
C4	Butyryl-L-Carnitine	0.255 ± 0.0926	0.250 ± 0.107	
C4-OH	Hydroxy-Butyryl-L-Carnitine	0.0239 ± 0.0117	0.0252 ± 0.0130	
C4DC	Succinyl-L-Carnitine	0.0750 ± 0.0135	0.104 ± 0.135	
C5	Isovaleryl-L-Carnitine	0.111 ± 0.0408	0.103 ± 0.0424	
C5:1	Tiglyl-L-Carnitine	0.0276 ± 0.00787	0.0285 ± 0.0110	
C5-OH	Hydroxy-Isovaleryl-L-Carnitine	0.0352 ± 0.00789	0.0392 ± 0.0103	
C5DC	Glutaryl-L-Carnitine	0.129 ± 0.0419	0.132 ± 0.0575	
C6	Hexanoyl-L-Carnitine	0.0602 ± 0.0249	0.0605 ± 0.0213	
C6:1	Hexenoyl-L-Carnitine	0.0211 ± 0.00818	0.0222 ± 0.00988	
C6DC	Adipyl-L-Carnitine	0.0947 ± 0.0121	0.0971 ± 0.0277	
C8	Octanoyl-L-Carnitine	0.0994 ± 0.0655	0.0953 ± 0.0553	
C8:1	Octenoyl-L-Carnitine	0.178 ± 0.117	0.132 ± 0.0752 *	$P = 0.0201$
C8DC	Suberyl-L-Carnitine	0.0549 ± 0.00773	0.0601 ± 0.0177	
C10	Decanoyl-L-Carnitine	0.161 ± 0.113	0.154 ± 0.106	
C10:1	Decenoyl-L-Carnitine	0.105 ± 0.0520	0.109 ± 0.0536	
C10:2	Decadienoyl-L-Carnitine	0.0374 ± 0.0184	0.0395 ± 0.0216	
C10DC	Sebacyl-L-Carnitine	0.0970 ± 0.0118	0.0999 ± 0.0162	
C12	Lauroyl-L-Carnitine	0.0668 ± 0.0364	0.0633 ± 0.0276	
C12:1	Dodecenoyl-L-Carnitine	0.0681 ± 0.0424	0.0707 ± 0.0437	
C12DC	Dodecanedioyl-L-Carnitine	0.0782 ± 0.0124	0.108 ± 0.0489*	$P < 0.0001$
C14	Myristoyl-L–Carnitine	0.0751 ± 0.0248	0.0612 ± 0.0269*	$P = 0.0023$
C14:1	Myristoleyl-L-Carnitine	0.0694 ± 0.0435	0.0651 ± 0.0324	
C14:2	Tetradecadienoyl-L-Carnitine	0.0390 ± 0.0164	0.0398 ± 0.0177	
C14-OH	Hydroxy-Myristoyl-L-Carnitine	0.0149 ± 0.00667	0.0157 ± 0.00605	
C16	Palmitoyl-L–Carnitine	0.352 ± 0.148	0.404 ± 0.220	
C16:1	Palmitoleyl-L-Carnitine	0.0586 ± 0.0276	0.0472 ± 0.0212*	$P = 0.0383$
C16-OH	Hydroxyl-Palmitoyl-L-Carnitine	0.0104 ± 0.00361	0.0114 ± 0.00570	
C16:1-OH	Hydroxy-Palmitoleyl-L-Carnitine	0.0193 ± 0.00764	0.0211 ± 0.0111	
C18	Stearoyl-L-Carnitine	0.103 ± 0.0350	0.0874 ± 0.0326*	$P = 0.0104$
C18:1	Oleyl-L-Carnitine	0.401 ± 0.170	0.279 ± 0.159*	$P < 0.0001$
C18:2	Linoleyl-L-Carnitine	0.232 ± 0.111	0.146 ± 0.0911*	$P < 0.0001$
C18:1-OH	Hydroxy-Oleyl-L-Carnitine	0.0178 ± 0.00858	0.0228 ± 0.0116*	$P = 0.0191$

Data are expressed as mean ± standard deviation; *$P < 0.05$.

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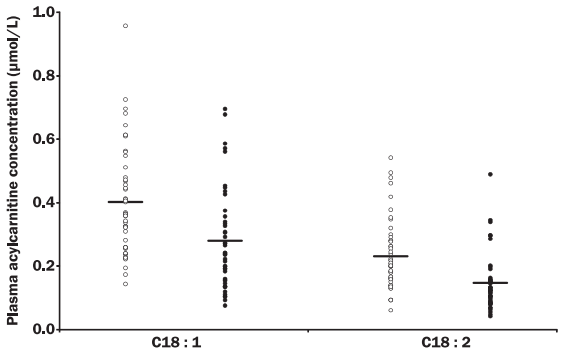


Fig. 2 *Endogenous plasma oleyl-L-carnitine (C18:1) and linoleyl-L-carnitine (C18:2) concentrations (µmol L^{-1}) in patients with chronic fatigue syndrome (●) and healthy controls (○).*

fatigue syndrome is associated with a reduction in endogenous plasma L-carnitine [22, 25, 26] and total carnitine levels [26]; however, these findings have not been replicated in other studies [23, 24, 27, 28, 31]. Similarly, chronic fatigue syndrome has also been associated with a reduction in endogenous plasma (total) acylcarnitine concentrations [22–24, 26], whilst other studies have reported no differences in levels between patients with chronic fatigue syndrome and healthy controls [27, 28, 31]. For instance, Plioplys & Plioplys [26] reported significantly lower endogenous levels of L-carnitine, total carnitine and total acylcarnitines in 35 patients with chronic fatigue syndrome compared with normative data obtained from the Mayo Clinic, whereas in a series of experiments conducted by Kuratsune *et al.* [22–24], the authors reported no differences in L-carnitine and total carnitine levels between patients with chronic fatigue syndrome and healthy controls in their earlier studies whilst their later study noted a significant reduction in L-carnitine levels in the patient population. Significantly lower total acylcarnitine levels in patients with chronic fatigue syndrome were reported in all three studies conducted by Kuratsune *et al.* [22–24]. In contrast, Jones *et al.* [27] reported no significant differences in L-carnitine, total carnitine and total acylcarnitine levels between 31 patients with chronic fatigue syndrome and 31 healthy controls.

Previous studies have predominantly used enzymatic assays for the quantification of L-carnitine, total carnitine and total acylcarnitine levels. This analysis, whilst useful, does not provide information on individual acylcarnitine levels; it is therefore possible that chronic fatigue syndrome is associated with alterations in the levels of only some acylcarnitines and consequently potential differences may be missed in the examination of total acylcarnitine levels in these studies. Only one previous study has investigated individual acylcarnitine levels in patients with chronic fatigue syndrome [31]. The study reported no significant differences in individual acylcarnitine levels between 25 female patients with chronic fatigue syndrome and 25 female healthy controls; however, only a limited number of individual acylcarnitines were quantified and no firm conclusions can be made. The present study was therefore conducted to examine the levels of endogenous plasma L-carnitine and 35 individual acylcarnitines in patients with chronic fatigue syndrome compared with age- and gender-matched healthy controls.

The present study confirmed that chronic fatigue syndrome is not associated with alterations in L-carnitine, total carnitine or total acylcarnitine levels; however, significant differences in levels between patients and healthy subjects were demonstrated for eight of the 35 of individual acylcarnitines quantified. Of particular note was the substantial reduction in oleyl-L-carnitine (C18:1) and linoleyl-L-carnitine (C18:2) levels in chronic fatigue syndrome, a result that has not been previously demonstrated. Soetekouw *et al.* [31] failed to find a significant difference in levels between patients and controls for five of the eight acylcarnitines which were found to be altered in this study (the remaining 3/8 were not analysed); this may be contributed to a lack of power. For example, whilst the levels reported for C8:1 are similar to those observed in this study, Soetekouw *et al.* [31] examined carnitine profiles in just 25 patients and 25 controls and, based on these subject numbers, we estimate that their study only had 45% power to determine a difference between the groups.

This study has also demonstrated significant relationships between severity of fatigue and endogenous plasma acylcarnitine levels. Importantly, highly significant associations were demonstrated for oleyl-L-carnitine and linoleyl-L-carnitine, with lower levels associated with greater fatigue severity as estimated by the fatigue severity scale. The relationship between fatigue severity and individual acylcarnitine levels in chronic fatigue syndrome has not been previously investigated. Plioplys & Plioplys [26] demonstrated significant negative relationships between L-carnitine and total carnitine concentrations and fatigue severity, but not total acylcarnitine levels. In keeping with the findings of our study, Kuratsune *et al.* [21] reported that an improvement in the condition of

chronic fatigue syndrome patients was associated with a concurrent increase in endogenous plasma acylcarnitine levels, indicating the importance of carnitine homeostasis in the symptomology of chronic fatigue syndrome.

Based on the L-carnitine/acylcarnitine pathway in fatty acid oxidation, it is conceivable that the observed deficiency in long-chain acylcarnitines in chronic fatigue syndrome may be reflective of either (i) an increase in the activity of L-carnitine/acylcarnitine translocase or (ii) a reduction in the activity of carnitine palmitoyltransferase-I (CPT-I) (Fig. 1). An increase in L-carnitine/acylcarnitine translocase activity would result in enhanced long-chain acylcarnitine transfer across the inner mitochondrial membrane and hence an increase in substrate availability for muscle β-oxidation; a scenario that is improbable given the symptomology of chronic fatigue syndrome. More likely is that the deficiency in long-chain acylcarnitines in these patients is suggestive of reduced formation via CPT-I; in principle, this would result in less acylcarnitines available for transport across the inner mitochondrial membrane. Consequently, there would be a reduction in the amount of acylcarnitines within the mitochondria available for reverse transesterification by carnitine palmitoyltransferase-II (CPT-II) and hence a reduction in long-chain fatty acid oxidation (Fig. 1). In keeping with this, previous research has established that mitochondrial long-chain fatty acid oxidation is reduced in patients with chronic fatigue syndrome [34, 35]. In further support, patients diagnosed with CPT-II deficiency (in which less substrate is available for β-oxidation within the mitochondria) exhibit remarkably similar symptoms to chronic fatigue syndrome patients including myalgia, cramps, muscle stiffness, painful muscles and muscle weakness [19]. As long-chain fatty acids are the most energy-rich substrates for β-oxidation, it is plausible that small changes in acylcarnitine levels would have a significant impact on energy production, leading to fatigue.

The findings of a previous study by Maes *et al.* [36] further support our hypothesis. In their study, endogenous levels of fatty acids were examined in 22 patients with chronic fatigue syndrome and 12 healthy controls, and it was demonstrated that chronic fatigue syndrome was accompanied by increased levels of omega-6 poly-unsaturated fatty acids and mono-unsaturated fatty acids. Interestingly, of the fatty acids measured by Maes *et al.* [36] for which the corresponding acylcarnitine was quantified in the present study (C14, C16, C16:1, C18, C18:1, C18:2), for five of the six cases there was a significant reduction in acylcarnitine levels and an increase in corresponding free fatty acid levels (C14, C16:1, C18, C18:1 & C18:2). In fact, when our findings are linked with those of Maes *et al.* [36], it can be speculated that the ratio of free fatty acid to acylcarnitine for these acyl groups is approximately 2- to 3-fold higher in patients with chronic fatigue syndrome than in healthy controls, indicating a substantial disruption in fatty acid/carnitine homeostasis in these patients. This may be because of either (i) a reduction in the activity of AcylCoA synthase required for the conversion of free fatty acid to AcylCoA or (ii) reduced activity of CPT-I. As CPT-I is the rate-controlling enzyme in mitochondrial fatty acid oxidation [37], it is hypothesized, based on these findings, that a reduction in CPT-I activity contributes to the symptomology of chronic fatigue syndrome.

In keeping with this, high levels of omega-6 fatty acids (such as C18:2 seen in this patient group) have been shown to inhibit CPT-I activity [38] in rats, whereas an increase in the ratio of omega-3 to omega-6s has been shown to increase CPT-I activity in both rats [39] and healthy volunteers [40, 41]. As L-carnitine is also known to increase CPT-I activity [42], it is proposed that administration of omega-3 fatty acids in combination with L-carnitine would improve CPT-I activity in chronic fatigue syndrome, thereby decreasing the ratio of free fatty acid to acylcarnitine and theoretically normalizing mitochondrial fatty acid oxidation in these patients.

No studies have been conducted to investigate the impact of administration of L-carnitine in combination with essential fatty acids on chronic fatigue syndrome symptomology. Preliminary exploratory studies have determined the impact of carnitine supplementation (alone) on chronic fatigue syndrome severity; however, no double-blind, placebo-controlled studies have been conducted. In an open-label study, Plioplys & Plioplys [43] demonstrated that L-carnitine supplementation (1 g t.i.d p.o) resulted in significant improvements in fatigue severity after 2 months of supplementation. Similarly, in an open-label, randomized study, investigating the effect of administration of acetyl-L-carnitine and/or propionyl-L-carnitine in chronic fatigue syndrome, Vermeulen & Scholte [44] noted that supplementation with acetyl-L-carnitine (2 g daily p.o. for 24 weeks) resulted in significant improvements in mental fatigue and attention concentration, whilst administration of propionyl-L-carnitine (2 g daily p.o.

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for 24 weeks) resulted in significant improvements in general and physical fatigue. Although the present study has demonstrated that endogenous levels of these carnitines (i.e. L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine) are not altered in chronic fatigue syndrome, we propose that their administration alters the equilibrium balance between L-carnitine and acylcarnitines via CPT-I, thereby impacting positively on the levels of acylcarnitines which have been shown to be depleted in this patient population. Previous studies have also demonstrated that administration of essential fatty acids (alone) results in a significant improvement in chronic fatigue syndrome symptomology [45–47].

Whilst the cause of this proposed impairment of mitochondrial fatty acid oxidation in patients with chronic fatigue syndrome is unknown, it is possible that this may be linked to the recent finding that chronic fatigue syndrome is associated with an increase in the incidence of viral infection [2, 3, 9]. Previous research has indicated that viral infection may result in altered fatty acid oxidation [48], and therefore, it is feasible that the condition may be triggered by a viral infection which leads to a disruption in free fatty acid and carnitine homeostasis, thereby impacting on the clinical condition of these patients. This hypothesis warrants further investigation.

Conflict of interest

The authors declare that no conflict of interest exists.

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ORIGINAL PUBLICATION

Reuter SE, Faull RJ, Evans AM. Patient functionality in chronic haemodialysis patients: The importance of carnitine pool composition. *Nephrol Dial Transplant*; submitted.

Not available.

INVITED REVIEW

Reuter SE, Evans AM. Kinetics of carnitine and acylcarnitines. *Curr Drug Metab*; prepared for submission.

Not available.

EDITORIAL COMMENT

Reuter SE, Evans AM. What is the value of L-carnitine level determination for carnitine supplementation in haemodialysis patients? *Kidney Int*; prepared for submission.

Not available.

CONFERENCE PRESENTATION

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of a single haemodialysis session on plasma acylcarnitine levels in end-stage renal disease. Clinical Pharmacology and Therapeutics (CPT) Conference, August 2004: Brisbane, Australia.

Abstract

L-carnitine is an endogenous substance that plays a critical role in transporting fatty acids across the mitochondrial membrane for β-oxidation. Plasma L-carnitine levels are regulated by the healthy kidney, with active tubular reabsorption essential in maintaining homeostasis. Patients undergoing chronic haemodialysis treatment tend to have low plasma L-carnitine levels, mainly due to the loss of endogenous L-carnitine via the dialysate. The effect of a single dialysis session on plasma endogenous acylcarnitines has not been investigated. Pre- and post-dialysis blood samples were collected from 50 end-stage renal disease patients receiving haemodialysis. Plasma samples were analysed for a range of acylcarnitines using a validated electrospray tandem mass spectrometry method. Acylcarnitines were grouped according to carbon-chain length as short- (C2-C6), medium- (C8-C12) and long-chain (>C14) carnitines. Results were statistically compared using a paired student's t-test, with significance set at an α-level of 0.05. Mean ± SD pre-dialysis short-, medium- and long-chain acylcarnitine levels were 13.59 ± 6.17 μmol/L, 2.54 ± 0.87 μmol/L and 1.93 ± 0.79 μmol/L, respectively. Post-dialysis levels were 4.73 ± 3.49 μmol/L, 1.17 ± 0.61 μmol/L and 1.55 ± 0.80 μmol/L for the short-, medium- and long-chain esters, respectively. Statistical analysis of the data showed that all of the esters were significantly removed during dialysis, with the exception of 3-hydroxy-palmitoyl-L-carnitine and the 18 carbon-chain esters. The clinical implications of the inefficient removal of these acylcarnitines by haemodialysis are yet to be determined.

CONFERENCE PRESENTATION

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Effect of haemodialysis on endogenous plasma acylcarnitine levels in end-stage renal disease patients. Clinical Pharmacology and Therapeutics Conference (CPT), August 2004: Brisbane, Australia.

Abstract

L-carnitine is an endogenous substance that plays a critical role in transporting fatty acids across the mitochondrial membrane for β-oxidation. It has been established that patients with end-stage renal disease undergoing chronic haemodialysis have low endogenous plasma L-carnitine levels, however the effect of haemodialysis on plasma acylcarnitines has not been investigated. Blood samples were collected from 60 healthy volunteers and 50 end-stage renal disease patients receiving chronic haemodialysis. Plasma samples were analysed for a range of acylcarnitines using a validated electrospray tandem mass spectrometry method. Acylcarnitines were grouped according to carbon-chain length as short- (C2-C6), medium- (C8-C12) and long-chain (>C14) carnitines. Results were statistically compared using an unpaired student's t-test, with significance set at an α-level of 0.05. Mean ± SD short-, medium- and long-chain acylcarnitine levels in the healthy population were 7.59 ± 2.12 µmol/L, 0.67 ± 0.30 µmol/L and 0.43 ± 0.12 µmol/L, respectively. Significantly higher levels were observed in end-stage renal disease patients with values of 13.59 ± 6.17 µmol/LM, 2.54 ± 0.87 µmol/L and 1.93 ± 0.79 µmol/L for the short-, medium- and long-chain esters, respectively. C8:1 concentrations (tentatively identified as octenoyl-L-carnitine) were significantly higher in end-stage renal disease patients than in the healthy population. Similarly, the 16 and 18 carbon-chain esters were much higher in end-stage renal disease patients with concentrations up to 7.3 times higher than those in the healthy population. The clinical implications of these elevated acylcarnitines in end-stage renal disease patients receiving chronic haemodialysis are yet to be determined.

CONFERENCE PRESENTATION

Reuter SE, Evans AM, Faull RJ, Chace DH, Fornasini G. Impact of haemodialysis on individual endogenous plasma acylcarnitine levels in end-stage renal disease. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2004: Melbourne Australia.

Abstract

L-carnitine is an endogenous substance required for the transfer of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation. The healthy kidney plays a critical role in the homeostatic control of the body's carnitine pool by extensive reabsorption of L-carnitine and preferential excretion of acylcarnitines. Patients with end-stage renal disease undergoing haemodialysis have low endogenous L-carnitine levels and elevated acylcarnitines. Until recently, it has not been possible to quantify these acylcarnitines and measurements have given values for the sum of all carnitine esters. This study evaluated endogenous concentrations of an array of acylcarnitines in healthy individuals and end-stage renal disease patients receiving haemodialysis, and examined the impact of a single haemodialysis session on acylcarnitine levels. Blood samples were collected from 60 healthy subjects and 50 end-stage renal disease patients undergoing haemodialysis (pre- and post-dialysis samples). Samples were analysed for plasma L-carnitine and individual acylcarnitine levels using a validated electrospray tandem mass spectrometry assay. Twenty-nine of the 31 acylcarnitines measured were significantly higher in end-stage renal disease patients when compared to healthy controls ($p<0.05$); in particular, C5 and C8:1 concentrations were substantially elevated. In most cases, plasma acylcarnitine levels significantly decreased over the course of a single dialysis session; however, post-dialysis concentrations invariably remained significantly higher than those in healthy subjects. Dialytic removal of acylcarnitines diminished once the acyl-chain length exceeded 8 carbons. The clinical implications of the accumulation of these acylcarnitines and their impaired clearance have not yet been determined; however, these results give us a basis on which future studies can be designed.

CONFERENCE PRESENTATION

Reuter SE, Evans AM. Development of secondary carnitine deficiency in chronic haemodialysis patients. Adelaide Pharmacology Group (APG) Annual Meeting, November 2005: Adelaide, Australia.

Abstract

Not available.

CONFERENCE PRESENTATION

Reuter SE, Evans AM. Possible impact of L-carnitine and acylcarnitines on the treatment of anaemia in end-stage renal disease patients undergoing haemodialysis. Joint Australasian Pharmaceutical Science Association (APSA) and Australian Society for Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) Annual Conference, December 2005: Melbourne, Australia.

Awarded 'Outstanding Presentation in Pharmaceutical Science'.

Abstract

Anaemia is a common complication associated with haemodialysis, usually managed by treatment with erythropoietin (rHuEPO); however, the rHuEPO dose required to maintain a target haemoglobin level varies considerably within the population. In addition, the cost of treatment is extremely high with rHuEPO therapy accounting for up to 10% of the total treatment costs of haemodialysis patients. L-carnitine has been proposed as a potential adjunct therapy to rHuEPO in the treatment of rHuEPO-resistant anaemia. Numerous studies have investigated the effect of L-carnitine administration on rHuEPO dose and/or haematocrit, with varying results; however, no definitive conclusions have been made. We have demonstrated that haemodialysis causes distortions in the endogenous carnitine pool and this study investigated the relationship between endogenous L-carnitine levels and rHuEPO requirements in end-stage renal disease patients undergoing chronic long-term haemodialysis treatment. Pre-dialysis blood samples were collected from 71 patients and assessed for plasma L-carnitine, acylcarnitine and total carnitine levels by high performance liquid chromatography or electrospray tandem mass spectrometry. Erythropoietin resistance index (ERI) was calculated as rHuEPO dose/kg/week, normalised for haemoglobin levels. A significant negative correlation between endogenous plasma L-carnitine levels and ERI was found ($p = 0.01$). Patients categorised as high ERI (>0.02) all exhibited sub-normal endogenous L-carnitine levels (<30 µmol/L); conversely, patients with normal L-carnitine levels (>30 µmol/L) displayed low ERI values (<0.02). The ratio of acylcarnitine/total carnitine was significantly positively correlated with ERI ($p = 0.04$). This data illustrates the relationship between carnitine levels and the response to rHuEPO treatment in chronic haemodialysis patients, in particular the importance of the proportion of acylcarnitine to total carnitine levels within the plasma carnitine pool. This proportion may be more indicative of the response to L-carnitine supplementation than absolute carnitine levels alone. This hypothesis needs to be investigated more extensively with a randomised, placebo-controlled trial.

CONFERENCE PRESENTATION

Reuter SE, Evans AM. Assessment of muscle function and relationship to carnitine levels in chronic haemodialysis patients. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2006: Adelaide, Australia.

Abstract

The six-minute walk test is a standardised test commonly used for the assessment of muscle function and fatigue. However, the logistics of conducting the six-minute walk test in some patient populations is not practical, in particular in patients who have limited walking capacity or in whom walking poses a significant health risk. Muscle fatigue is a common complaint experienced by haemodialysis patients and carnitine deficiency has been implicated as a contributing factor towards this. The aim of this study was to develop an alternative assessment of muscle fatigability and to compare this new method to the previously validated six-minute walk test. Subsequently, the test was used to investigate the relationship between muscle fatigability and endogenous carnitine levels in chronic haemodialysis patients. Healthy subjects (n = 12) were recruited for validation of a new hand grip test of muscle fatigue. Subjects were instructed to grasp a hand-grip dynamometer with their non-dominant hand at a level of 50% of their maximum contraction for as long as possible after which a post-fatigue maximum contraction reading was recorded. Hand-grip fatigue results were compared to results of the six-minute walk test. The hand-grip fatigue test was performed on haemodialysis patients (n = 10) and compared to endogenous levels of L-carnitine and acylcarnitines. Carnitine levels were assayed using a validated electrospray tandem mass spectrometry method. A highly significant correlation between hang-grip fatigue and results of the six-minute walk test was demonstrated in the healthy subject group ($p = 1.8 \times 10^{-6}$). A significant correlation between hand-grip fatigue and the proportion of non-acetyl acylcarnitines within the total plasma carnitine pool was demonstrated in the haemodialysis population ($p = 0.0040$). This study demonstrated that the hand-grip test is an acceptable method for the measurement of muscle fatigue without the need for extensive mobility. Use of this test in the haemodialysis population illustrated that the disturbed carnitine profile (commonly observed in these patients) may contribute, at least in part, to the asthenia frequently experienced with long-term dialysis treatment. The effect of L-carnitine supplementation on muscle fatigue in haemodialysis patients needs to be investigated further.

CONFERENCE PRESENTATION

Reuter SE, Evans AM. Possible impact of L-carnitine and acylcarnitines on the treatment of anaemia in end-stage renal disease patients undergoing haemodialysis. Australian Society for Medical Research (ASMR) Scientific Meeting, June 2007: Adelaide, Australia.

Abstract

Anaemia is a common complication associated with haemodialysis, usually managed by treatment with erythropoietin (rHuEPO). 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 rHuEPO treatment (despite adequate iron stores) and consequently receive a particularly high dose of rHuEPO to achieve acceptable (but usually sub-optimal) haemoglobin levels. The cost of treatment is extremely high with rHuEPO therapy accounting for up to 10% of the total treatment costs for haemodialysis patients. L-carnitine has been proposed as a potential adjunct therapy to rHuEPO in the treatment of rHuEPO-resistant anaemia. Numerous studies have investigated the effect of L-carnitine administration on rHuEPO dose and/or haematocrit, with varying results and no definitive conclusions have been made. We have demonstrated that haemodialysis causes alterations to the composition of the endogenous carnitine pool and this study investigated the relationship between endogenous L-carnitine levels and rHuEPO requirements in renal disease patients undergoing long-term haemodialysis treatment. Pre-dialysis blood samples were collected from 87 patients and assessed for plasma L-carnitine, individual acylcarnitine and total carnitine levels by electrospray tandem mass spectrometry. Erythropoietin resistance index (ERI) was calculated as rHuEPO dose/kg/week, normalised for haemoglobin levels. A significant negative correlation between endogenous plasma L-carnitine levels and ERI was found ($p = 0.042$). Patients categorised as high ERI (>0.02) all exhibited sub-normal endogenous L-carnitine levels (<30 µmol/L); conversely, patients with normal L-carnitine levels (>30 µmol/L) displayed low ERI values (<0.02). More importantly, the ratio of non-acetyl acylcarnitines/total carnitine was significantly positively correlated with ERI ($p = 0.006$). This data illustrates the relationship between carnitine levels and the response to rHuEPO treatment in chronic 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 carnitine levels alone.

CONFERENCE PRESENTATION

Reuter SE, Faull RJ, Evans AM. Clinical impact of alterations in endogenous carnitine pool composition in chronic haemodialysis patients. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2007: Sydney, Australia.

Awarded 'Outstanding Presentation in Pharmaceutical Science'.

Abstract

L-carnitine deficiency in haemodialysis patients has been implicated as a contributing factor for many dialysis-related disorders due to its vital role in the utilisation of fatty acids and the safe removal of acyl groups. This study was conducted in order to investigate the relationship between endogenous carnitine profiles in long-term haemodialysis patients and the incidence of dialysis-related symptoms including renal anaemia, cardiac dysfunction and dialytic symptoms such as muscle cramps, fatigue and intradialytic hypotension. Forty-nine end-stage renal disease patients receiving long-term haemodialysis treatment were recruited for study participation. Patients were assessed for responsiveness to erythropoietin therapy (as indicated by erythropoietin resistance index, ERI); cardiac dysfunction (according to New York Heart Association (NYHA) classification); skeletal muscle fatigue (assessed using hand-grip dynamometry); and the incidence of muscle cramps and intradialytic hypotension. Blood samples were also collected for quantification of plasma L-carnitine, total carnitine and individual acylcarnitine levels by electrospray tandem mass spectrometry. This study demonstrated that patients who are hyporesponsive to erythropoietin treatment (ERI>0.020 μg/kg/week/gHb) have a significantly higher proportion of non-acetyl acylcarnitines within the total carnitine pool (AcylLC-C2/TC) compared with those patients who are more responsive to erythropoietin treatment ($p = 0.0158$). Similarly, AcylLC-C2/TC values were also significantly higher in patients experiencing muscle cramps than those who did not ($p = 0.0447$) and in patients with higher skeletal muscle fatigability than their less fatigable counterparts ($p = 0.0193$). In addition, it was noted that patients who experienced intradialytic hypotension had a significantly lower proportion of L-carnitine within the total carnitine pool (LC/TC) than patients who did not experience intradialytic hypotension ($p = 0.0382$). Likewise, patients with a high NYHA rating (Class II - IV) had significantly lower LC/TC values than patients who did not have any symptomatic cardiac dysfunction ($p = 0.0362$). This study demonstrated an association between alterations in carnitine pool composition and clinical conditions commonly experienced by haemodialysis patients. It is proposed that L-carnitine supplementation would result in an improvement in these dialysis-related conditions by the improvement in carnitine pool composition.

CONFERENCE PRESENTATION

Reuter SE, Faull RJ, Evans AM. Endogenous plasma carnitine pool composition and response to erythropoietin treatment in chronic haemodialysis patients. XIV International Congress of Nutrition and Metabolism in Renal Disease (ICNMRD) June 2008: Marseille, France.

Abstract

Although the aetiology of renal anaemia is complex, the alteration in the composition of the endogenous carnitine pool associated with chronic haemodialysis has been implicated as a contributing factor to rHuEPO hyporesponsiveness in some patients. Studies have demonstrated that supplemental L-carnitine is beneficial for the treatment of rHuEPO-resistant anaemia; however, the extent of this benefit varies significantly between patients. Currently, the basis for this phenomenon of 'responders' and 'non-responders' is unknown. In order to investigate this further, this study examined the relationship between the composition of the endogenous plasma carnitine pool and rHuEPO requirements in chronic haemodialysis patients. Pre-dialysis blood samples were collected from 87 patients and analysed for plasma L-carnitine and individual acylcarnitine concentrations by electrospray tandem mass spectrometry. As an indication of rHuEPO responsiveness, erythropoietin resistance index (ERI) was calculated as rHuEPO dose/kg/week normalised for haemoglobin levels. A marginally significant negative correlation between L-carnitine levels and ERI was observed ($p = 0.0421$). More importantly, there was a highly significant positive correlation between the ratio of non-acetyl acylcarnitines/total carnitine (AcylLC-C2/TC) and ERI ($p = 0.0062$); this relationship was independent of dialysis age. This study demonstrated a clear relationship between AcylLC-C2/TC and rHuEPO requirements in chronic haemodialysis patients. This ratio is likely to be more indicative of carnitine pool status as it accounts for both the accumulation of non-acetyl acylcarnitines and the reduction in L-carnitine levels which are associated with haemodialysis treatment. Based on these results, it is hypothesised that patients with a more disturbed carnitine profile (i.e. high AcylLC-C2/TC) will be more likely to 'respond' to administration of L-carnitine for renal anaemia; this warrants further investigation.

CONFERENCE PRESENTATION

Reuter SE, Evans AM. Chronic fatigue syndrome: Disruption in carnitine and free fatty acid homeostasis. Australasian Pharmaceutical Science Association (APSA) Annual Conference, December 2009: Hobart, Australia.

Abstract

Chronic fatigue syndrome is a term used to describe a heterogeneous condition which is primarily characterised by persistent debilitating fatigue that cannot be attributed to any alternative condition. The underlying aetiology of chronic fatigue syndrome is unknown and no diagnostic test exists; consequently, diagnosis of the condition is based on symptomology. In light of their role in mitochondrial energy production, it has been suggested that chronic fatigue syndrome may be associated with alterations in carnitine and/or free fatty acid homeostasis. Previous research has demonstrated that patients with chronic fatigue syndrome exhibit higher levels of certain omega-6 free fatty acids; however, no firm conclusions regarding carnitine pool composition in chronic fatigue syndrome have been made. For the first time, this project has investigated the levels of endogenous plasma L-carnitine and 35 individual acylcarnitines in chronic fatigue syndrome patients compared with healthy controls. Forty-four chronic fatigue syndrome patients and 49 age- and gender-matched healthy controls participated in the study. All subjects completed an assessment of fatigue severity and had blood samples collected for plasma carnitine profiling. chronic fatigue syndrome patients exhibited significantly altered levels of 8/35 acylcarnitines quantified when compared to healthy subjects. Of particular note, C18:1 and C18:2 were 30-40% lower in patients than controls ($p<0.0001$). Significant correlations between acylcarnitine concentrations and fatigue severity were also demonstrated. This study has demonstrated that chronic fatigue syndrome is associated with disturbances in carnitine homeostasis, in particular significant reductions in long-chain acylcarnitine levels. It is hypothesised that this is a result of a reduction in carnitine palmitoyltransferase-I activity which is required for the conversion of acyl-CoA and L-carnitine to free CoA and acylcarnitine. This reduction in carnitine palmitoyltransferase-I activity may be the result of the accumulation of omega-6 free fatty acids seen in this patient group. It is proposed that administration of omega-3 free fatty acids in combination with L-carnitine would increase carnitine palmitoyltransferase-I activity and improve chronic fatigue syndrome symptomology.