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Carnitine Profile Changes in Pediatric Hematopoietic Stem Cell Transplant: New Role for Carnitine?

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Summary: Carnitine is an essential cofactor for mitochondrial import and oxidation of fatty acids. High-dose chemotherapy and radiation, often required for hematopoietic stem cell transplant (HSCT), leads to tissue damage, mitochondrial dysfunction, and alterations in carnitine metabolism. The aim of this pilot cohort study was to describe plasma and urinary carnitine profiles during pediatric HSCT and their relationships with clinical outcomes. Plasma and urinary carnitine samples were collected from 22 pediatric patients before and through day 180 post-HSCT. Associations were observed between graft-versus-host disease and an elevated plasma total carnitine ($P = 0.019$), and also increased plasma acyl:free carnitine ratio with veno-occlusive disease ($P = 0.016$). Mortality was observed in those with their highest urinary total carnitine losses on day 0 ($P = 0.005$), and in those with an abnormal day 28 plasma ratio either above or below the reference range ($P = 0.007$). Changes in carnitine profiles were more reflective of metabolic stress and negative outcomes than of inadequate dietary intake. Associations observed direct larger studies to assess the validity of carnitine profiles as a prognostic indicator and also to assess whether prophylactic carnitine supplementation pre-HSCT could reduce mitochondrial injury and urinary losses and help mitigate inflammatory and metabolic comorbidities of HSCT.

Key Words: carnitine, stem cell transplant, graft-versus-host disease, mortality, chemotherapy

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Hematopoietic stem cell transplants (HSCTs) are routinely performed for several malignant and nonmalignant disorders, inherited metabolic disorders, and severe immunodeficiencies.[1] In HSCT, the toxicity of the required pretransplant preparative therapy can place children at high risk of morbidity and mortality in the posttransplant period,[2,3] especially in allogeneic HSCT where continued immune suppression to prevent graft-versus-host disease (GVHD) places the recipient at additional risk of complications.[3] In addition, the risk of hepatic injury caused by the development of hepatic veno-occlusive disease (VOD) is also increased because of postmyeloblative conditioning and can result in organ damage, multisystem organ failure, and death.[4–6]

Carnitine serves an intrinsic role as a cofactor in mitochondrial metabolism for the transport of long-chain fatty acids into the mitochondria for beta-oxidation, amino acid catabolism and detoxification of organic acids.[7] Carnitine measurements provide insight into the integrity of fatty acid oxidation processes. Total plasma carnitine, which is the sum of the free plus acylcarnitines, reflects total carnitine stores. The acylcarnitine to free carnitine ratio reveals the proportion of fatty acid conjugated acylcarnitine as a fraction of the unconjugated or "free" carnitine. This ratio is very sensitive to changes in mitochondrial metabolism as the mitochondrial relationship between acyl-CoA and free CoA is reflected in the acyl:free ratio.[8,9] Changes in carnitine ratios reflect mitochondrial disturbances and can indicate carnitine deficiency even in the presence of normal plasma carnitine levels.[9] A high ratio may indicate limited free carnitine when the conjugated acyl portion has markedly increased as occurs in disorders of organic or fatty acid metabolism when there is insufficient carnitine to remove organic acids and acyl residues out of the mitochondria.[9]

High doses of chemotherapy used in HSCT place recipients at risk of secondary carnitine deficiency[10] as sites of carnitine storage and metabolism such as the heart, liver, and kidneys[7,8,11] are most affected by chemotoxicity. Nephrotoxic effects of chemotherapy increase renal losses of carnitine and contribute to a secondary carnitine deficiency.[12–17] Similarly, gastrointestinal insults resulting from preparative regimens can also alter the absorption and metabolism of nutrients.[18,19] Other risk factors for carnitine deficiency include the metabolic stress of infections resulting in altered nutrient metabolism and the need for nutrition support such as parenteral nutrition (PN) which is usually carnitine free.[10] Total body irradiation (TBI), as part of some preparative regimens, can further disturb carnitine metabolism[20] through the excretion of metabolites resulting from increased beta-oxidation, DNA damage, and oxidative stress.[21]

There have been few published studies that have analyzed carnitine levels during HSCT. In one study, plasma carnitine levels in adults were found to be low posttransplant; however, levels were increased in those who developed GVHD.[10] This rise was thought to be due to an increased carnitine release from the muscle rather than an increase in production as GVHD can impair renal and liver function.[3,10,22] There have been studies that have examined urinary carnitine losses during chemotherapy in children[13,23,24]; however, as far as we are aware, there are no published studies on both plasma and urinary carnitine profiles during pediatric HSCT.

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Determining whether HSCT in children can affect carnitine profiles and whether there are any associations with clinical outcomes warranted study. Therefore, the primary objective of this study was to describe changes in concentrations of plasma total carnitine (PTC), urinary total carnitine (UTC), plasma acyl:free carnitine ratio (PCR) and urinary acyl:free carnitine ratio (UCR) in pediatric patients before receiving HSCT preparative therapy and through to the first 180 days post-HSCT.

The secondary objective was to describe associations between PTC, UTC, PCR, and UCR post-HSCT with adverse clinical outcomes such as GVHD, VOD, severe sepsis and death, and other potential confounding factors including age, sex, days on PN, chemotherapy, and TBI.

MATERIALS AND METHODS

Twenty-two children (ages 1 to 18 y) receiving an HSCT at the Alberta Children's Hospital from December 2009 to February 2012 were invited to participate in this institutional review board–approved study. A signed consent form as per the University of Calgary Conjoint Research Ethics Board guidelines was obtained before enrollment in the study. The protocols for HSCT began with preparative chemotherapy with or without TBI as indicated by diagnosis. Stem cells were infused on day 0. Plasma and urinary carnitine samples were collected on each patient 1 week before the scheduled start of the preparative therapy and on days 0, 7, 14, 21, 28, 60, 90, and 180 post-HSCT or until families withdrew from the study or if the patients died before day 180. All samples were stored at −70°C before analysis in the Biochemical Genetics Laboratory at the Alberta Children's Hospital. Carnitine profiles were measured using liquid chromatography coupled tandem mass spectrometry as adapted from previous methods.[25,26] Reference ranges used by the Biochemical Genetics Laboratory were 32.5 to 73.6 μM for PTC, 20.4 to 46.6 μmol/mmol creatinine for UTC, and 0.12 to 0.58 for PCR and 0.7 to 3.4 for UCR.

Clinical data that were considered potential predictor variables and/or confounding variables for carnitine values during treatment included age, sex, diagnosis, type of transplant, preparative regimen for transplant, development of acute kidney injury (AKI)[27] and use of PN. Each patient was carefully reviewed (using AKI diagnosis according to The Kidney Disease: Improving Global Outcomes [KDIGO] guidelines[27] to examine whether AKI coincided with the measurement of UTC levels. Adverse clinical outcome variables considered were death, severe sepsis,[28] VOD and GVHD.

Statistical Analysis

Patient characteristics were described using the median and 25th to 75th percentiles for continuous variables and percentages for categorical variables. Box plots were used to describe the carnitine values before preconditioning for HSCT until 6 months following the HSCT, stratified by clincial outcomes including mortality or GVHD.

To analyze the relationships between carnitine values measured before and for the first 4 weeks after HSCT with clinical outcomes, we used a 2-stage process as suggested by Matthews et al[29] for the analysis of serial measurements of the outcome variable taken on individuals. The potential relationships between the baseline variables (sex, type of bone marrow transplant, and radiation) and outcomes (GVHD, severe sepsis, VOD, and death) were described using the effect size for PTC, PCR, and UTC recognizing this is a pilot study of a small size. We considered *P*-values <0.01 to be statistically significant.

RESULTS

Twenty-two patients were enrolled in the study. All 22 patients received myeloblative conditioning with the majority (15/22) receiving an allogeneic HSCT with fludarabine/busulfan/antithymocyte globulin ± TBI as preparative therapy (Table 1). Of the 15 allogeneic HSCT patients, 9 received TBI with a dose of 400 cGy in 7 patients and 1200 cGy in the other 2. A total of 10 patients died; 2 died before day 100 attributed to severe sepsis, 1 was lost to follow-up after day 60, and the remaining 8 died after day 180, leaving 19 subjects available for day 180 analysis. All 22 patients had PTC levels available pre-HSCT and up to day 14; however, UTC levels were not available before bone marrow transplant for 8 patients. Sixteen of 22 patients had complete urine data pre-HSCT and on day 0. No differences were noted in both pre-HSCT PTC and UTC levels between oncology patients who had received prior chemotherapy versus nononcology patients who had not received prior chemotherapy ($P = 1.00$ and 0.94, respectively).

Baseline and most treatment variables (specifically sex, days on PN, and type of HSCT) were not associated with changes in carnitine levels (Table 2). The change of PCR was associated with radiation treatment ($P = 0.047$, Table 2).

Carnitine Profiles and Mortality

Carnitine profiles for plasma and urine, stratified by child health status (ie, the child lived or died), are shown in Figure 1. At baseline, the median PTC was within the reference interval for those who survived, while the baseline median for those who died was at the low end of the interval (Fig. 1A). Both groups, regardless of whether they lived or

TABLE 1. Descriptive data for Baseline and Outcome Variables

Patient Profile	N = 22
Sex (male/female)	10/12
Age, median (IQR) (y)	4.8 (1.9-9.4)
Total body irradiation (7 to 400 and 2 to 1200 cGy)	9
Parenteral nutrition days, median (range)	18 (0-174)
Hematopoietic stem cell transplantation type	
Autologous/allogeneic	7/15
Chemotherapy groups	
Fludarabine/busulfan/ATG ± TBI	12
Cyclophosphamide, etoposide, melfalan	3
Cyclophosphamide/cyclophosphamide	2
Busulfan melfelan/melfelan thiotepa	3
Busulfan cyclophosphamide ± VP16	2
Outcomes	
Graft-versus-host disease (n = 5/15 allogeneic)	
Acute	5
Chronic	3
Acute and chronic	3
Veno-occlusive disease	3
Death	10
Relapse	3
Severe sepsis[28]	5

ATG indicates antithymocyte globulin; IQR, interquartile range; TBI, total body irradiation.

TABLE 2. Results of the 4-Week Analysis Indicating *P*-values and Effect Sizes for Plasma and Urinary Carnitine

	Plasma Carnitine				Urinary Carnitine	
	Total		Ratio		Total	
Baseline Variables	***P***	**ES**	***P***	**ES**	***P***	**ES**
Age	0.79	0.06	0.21	−0.28	0.056	−1.14
Days on PN	0.78	−0.10	0.43	−0.08	0.89	0.08
Sex	0.96	0.02	0.09	0.76	1.00	0.09
Type of HSCT	0.96	0.02	0.85	−0.09	1.00	−0.10
Radiation	0.97	−0.02	0.047	0.97	0.10	0.50
Outcomes						
VOD	0.35	0.60	0.016	−1.64	1.00	−0.19
Death	0.47	0.32	0.33	−0.44	0.005	1.00
Sepsis	0.83	−0.11	0.47	−0.37	1.00	0.14
Chronic GVHD	0.019	−1.60	0.99	−0.01	0.50	0.36
Acute GVHD	0.25	−0.66	0.77	0.16	1.00	0.22

Potential relationships between baseline and outcome variables and 4-week carnitine patterns, described as *P*-values and the effect size of the relationship between the variable and the 4-week summary statistic (slope of decrease in carnitine values for plasma and peak for urine). In general, variables with effect sizes >0.5 are more likely to be detected in larger studies, whereas variables with small effect sizes (<0.2) would be unlikely to be detected even if the sample size was much larger to have any clinical significance ($P<0.10$).

ES indicates effect size; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplant; PN, parenteral nutrition; VOD, veno-occlusive disease.

died, had a drop in median PTC levels on day 0. However, the children who died had more variability in PTC levels post-HSCT compared with those who lived; the median for the patients who died did not reach the normal reference interval until day 60, whereas the median for the patients who lived reached the normal reference interval on day 28. Median PTC levels for both groups were within the normal range on day 180.

Median PCR levels were within the reference interval whether they lived or died (Fig. 1B). However, for the 18 patients that we had day 28 PCR levels on, all 5 patients with an abnormal PCR either above (n = 3) or below (n = 2) the reference range on day 28 died, while 10/13 patients with a normal day 28 PCR lived (Fig. 2, $P=0.007$).

Analysis of urinary carnitine indicated that the baseline UTC median placement relative to the reference interval of the surviving participants and their response to chemotherapy differed from those who died. The pre-HSCT UTC median of those who survived was near the upper end of the reference interval, while the pre-HSCT UTC median for those who died approximated the lower end of the interval (Fig. 1C). An elevation in UTC on day 0 was associated with the occurrence of death ($P=0.005$, Table 2). Among those with the highest urinary losses on day 0 who died, the day 0 median was 6 times the pre-HSCT median or 2.4 times the upper reference interval limit. Three of these patients died from relapse and 2 from complications of chronic GVHD. The other relationship between the baseline or outcome variables with an elevated UTC at day 0 was with age (Table 2). Patients with elevated UTC losses on day 0 were older with a mean age of 11.5 ± 5.8 versus 4.6 ± 3.6 years for patients with lower losses ($P=0.056$). Median UTC levels returned to the normal range by day 60 in those that survived and day 180+ in those who died.

Regarding the examination of whether AKI coincided with the measurement of UTC levels, there was no AKI on day 0 in the 5 patients with the highest UTC losses on day 0. Serum creatinine measurements were normal for the patient that had the highest peak UTC losses on day 0.

At baseline, the pattern of UCR levels was opposite to the PTC with respect to mortality: the median UCR was at the low end of the reference interval in those who survived, while the baseline median for those who died was at the upper end of the interval (Fig. 1D). The UCR was also more varied among those who survived compared with those who died, especially for days 0, 21, 60, and 180 (Fig. 1D).

Clinical Outcomes

Ten of the 22 patients died: 3 died from complications of chronic GVHD, 4 died from relapse and 3 died from sepsis. Overall, 5 developed acute GVHD grade 2, stage III of the skin, and 1 of these categorized as the late onset of acute GVHD of the gut grade 3 and 3 developed severe chronic GVHD (1 of the lung, 1 of the liver, and 1 of skin). All 3 passed away from complications resulting from chronic GVHD. Three patients experienced VOD and 5 severe sepsis (Table 1). Associations between changes in carnitine fractions with baseline and outcome variables are listed in Table 2. Mean time of death post-HSCT was 17 months.

As with the children who died, the children who received an allogeneic HSCT and developed GVHD had more variability in PTC and UTC levels post-HSCT than those without GVHD (Figs. 3A, C). The highest median urinary losses also occurred on day 0 in those that developed GVHD. The slope of the line for PTC was significantly related to chronic GVHD ($P=0.019$; Table 2). The mean slope of the line for PTC in the 4 patients with chronic GVHD was 3.1 (95% confidence interval [CI]: −2.8, 9.0) and in the 10 patients without GVHD it was −0.06 (95% CI: −0.64, 0.51). There were no associations noted with PCR or UCR and GVHD (Figs. 3B, D).

There was evidence of a relationship between a change in the PCR and VOD ($P=0.016$, Table 2). The 3 patients who developed VOD had a mean slope of 0.011 (95% CI: −0.008, 0.031) in PTC and the 19 patients who did not develop VOD had a mean slope of 0.0045 (95% CI: 0.0029, 0.0062). Two of the 3 patients who developed VOD had an elevated PCR on day 28. An elevated UCR was also observed during the days when diagnosed with VOD; however, the findings were not significant ($P=1.00$; Table 2).

DISCUSSION

Our pilot study identified that changes in both plasma and urine carnitine profiles before HSCT preparative therapy and up to day 180 post-HSCT were associated with GVHD, VOD, and mortality, with the majority of the carnitine changes occurring within the first month of HSCT.

Chemotherapy-induced mitochondrial injury is one of the earliest signs of intestinal and renal dysfunction.[30] The majority of patients in our study (19/22) experienced high UTC losses during HSCT with 9 having elevated losses on day 0. However, our results indicated that only peak urinary loss on day 0 rather than pre-HSCT or day 7+ was associated with patient mortality. The renal injury did not likely account for these high losses as indicated by the absence of AKI during the day 0 losses in these patients. The significant UTC losses are reflective of susceptibility to the toxic effects of the conditioning chemotherapy whether due to genetic

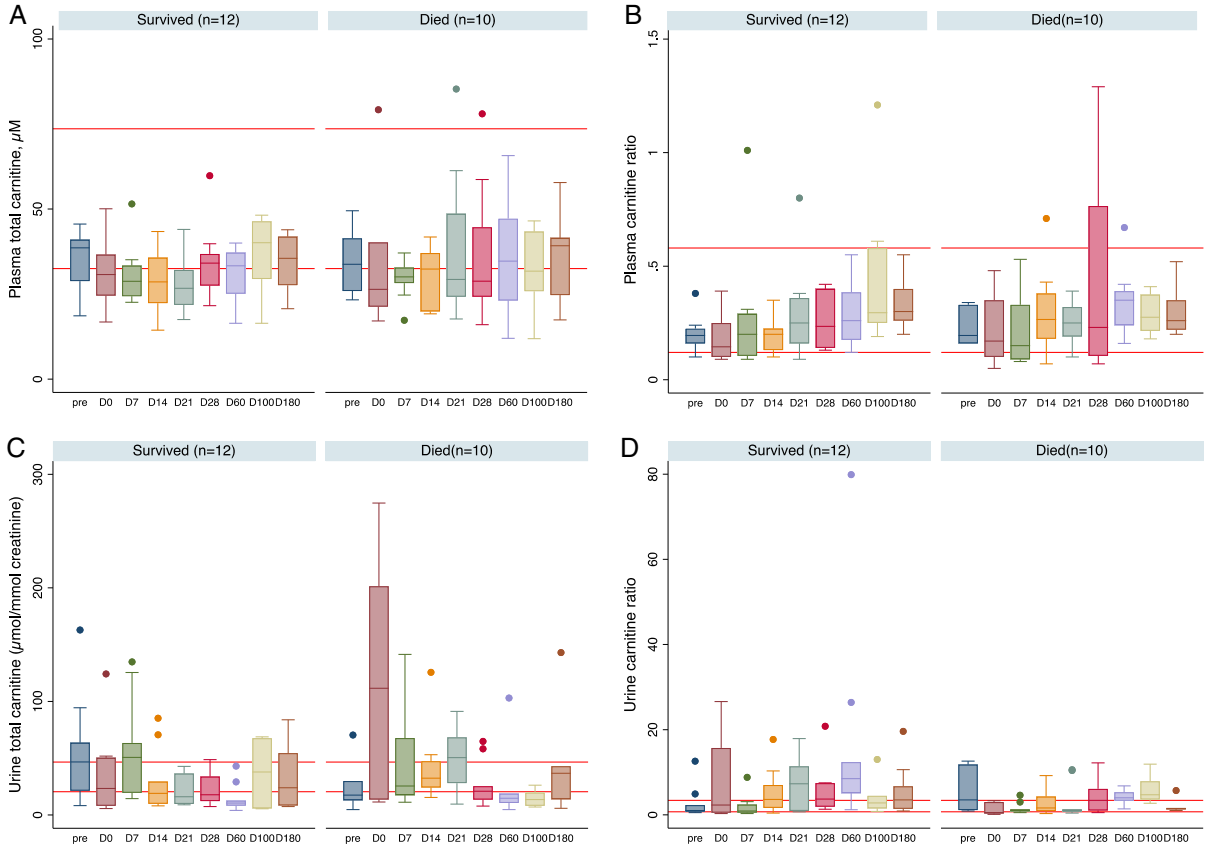

FIGURE 1. Carnitine profiles categorized by whether the children survived or died. Plasma total carnitine (A), plasma carnitine ratio (B), urine total carnitine (C), and urine carnitine ratio (D). The red horizontal lines represent the local reference intervals. Reference ranges: plasma total carnitine: 32.5 to 73.6 μM; plasma carnitine ratio: 0.12 to 0.58; urinary total carnitine: 20.4 to 46.6 μmol/mmol/creatinine; urinary carnitine ratio: 0.7 to 3.4. full color online

polymorphisms or metabolic stressors which alter the metabolic response to chemotherapy and may prognosticate poor outcome. Urinary carnitine losses are proportional to the magnitude of the injury, and the catabolic response is directly related to the degree of carnitine loss.[21] The 5 patients that died and had the highest losses on day 0 all had UTC levels within the normal range pre-HSCT, while those patients that had high UTC levels pre-HSCT lived. Urinary carnitine is also involved in the removal of toxic metabolites and organic acids[31]; thus, the significant increase in urinary losses on day 0 may reflect a greater accumulation of these toxins arising from a more extensive degree of HSCT conditioning-related toxicity and tissue damage. In addition, patients with the greatest UTC losses on day 0 were also older, suggesting an additional age-related effect, possibly influenced by hormone levels and variations in drug metabolism.[32] In view of these results, further exploration of these patterns of UTC losses pre-HSCT and weekly post-HSCT is warranted in a larger study to determine if a peak in UTC losses occurring on day 0 is a reliable noninvasive prognostic indicator of negative clinical outcomes post-HSCT.

The development of GVHD and VOD are among the most common toxicities leading to serious complications that compromise outcomes following allogeneic HSCT.[22,33] Our findings of an association between PTC status and GVHD is consistent with the same finding in adults in which the PTC levels were increased and in proportion to the severity of the GVHD.[10] The release of carnitine into the bloodstream may be associated with rapid tissue release or leakage arising from chemotherapy-induced cellular damage[10,13] to replace renal carnitine losses induced by chemotherapy damage.[13,14] We noted similar findings of elevated plasma carnitine ratio levels in our study during the time period of VOD development which is also reflective of cellular damage and toxicity.[5]

Tissue damage to the gastrointestinal tract from both chemotherapy and radiation has been found to play a critical role in the initiation of GVHD through its effect on the mucosal barrier and subsequent release of proinflammatory cytokines such as tumor necrosis factor, interleukins 1 and 6.[33–35] Damage to the intestinal mucosa allows bacterial translocation of the endotoxin lipopolysaccharide into the circulation, signaling the inflammatory response through toll receptor ligands and resulting in the release of tumor necrosis factor α and the cytokine storm leading to GVHD.[22,34–38] Increased levels of acylcarnitines, indicative of alterations in carnitine metabolism,[9] have the potential to further activate proinflammatory pathways through possible interference with membrane functions and signaling.[39,40] The altered carnitine metabolism, as indicated by our results, may have promoted incomplete fatty acid oxidation and accumulation of acylcarnitines thereby further contributing to proinflammatory toxicity.[41,42] This disruption in oxidative phosphorylation and

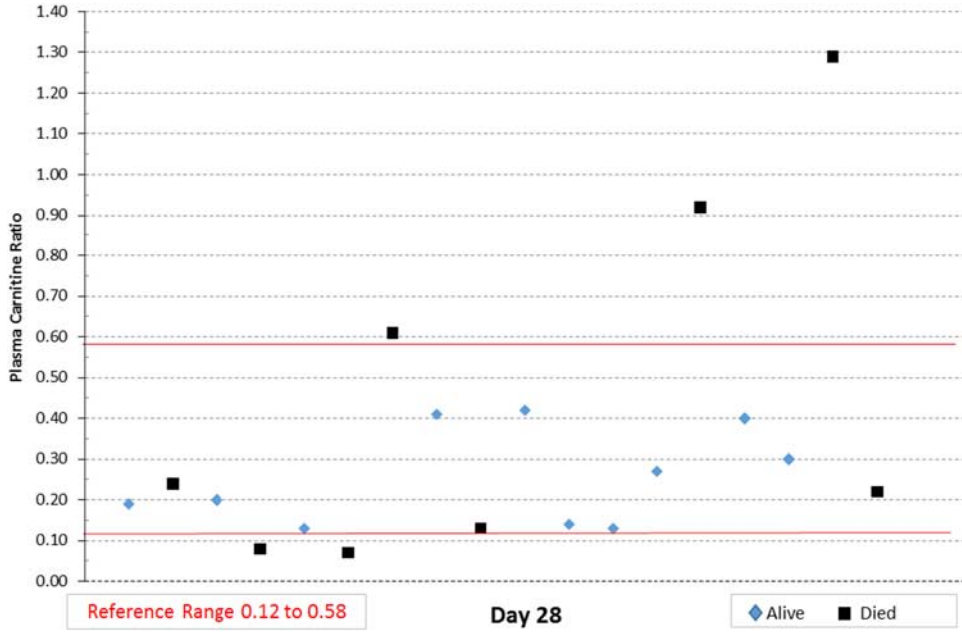

FIGURE 2. Day 28+ distribution of plasma carnitine ratio levels along the reference interval (horizontal lines) indicating the profiles of those who died versus those who survived. All those who had plasma carnitine ratio levels outside the reference range died. Reference range: 0.12 to 0.58. full color online

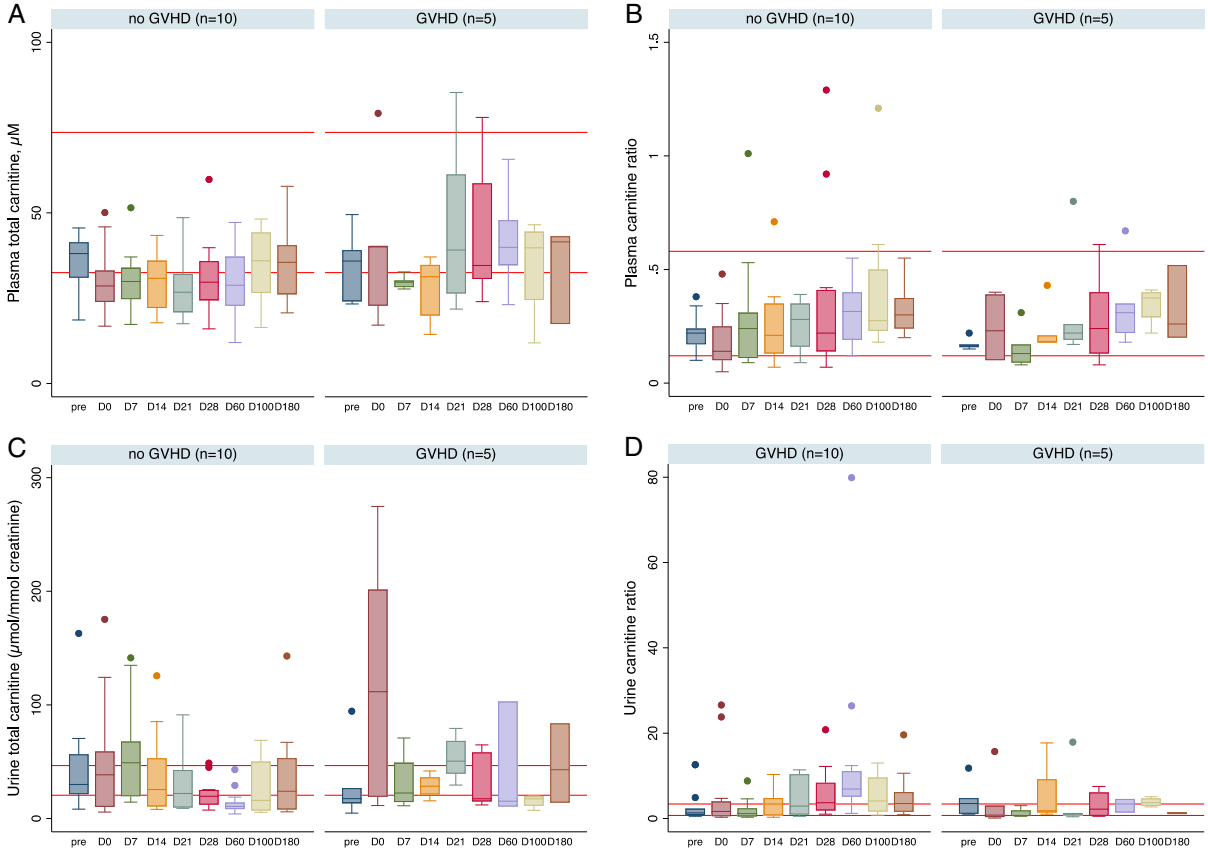

FIGURE 3. Carnitine profiles categorized by whether the children who had received an allogeneic transplant among those who did or did not develop graft-versus-host disease (GVHD) for: plasma total carnitine (A), plasma carnitine ratio (B), urine total carnitine (C), and urine carnitine ratio (D). The red horizontal lines represent the local reference intervals. Reference ranges: plasma total carnitine: 32.5 to 73.6 μM; plasma carnitine ratio: 0.12 to 0.58; urinary total carnitine: 20.4 to 46.6 μmol/mmol/creatinine; urinary carnitine ratio: 0.7 to 3.4. full color online

mitochondrial respiration from chemotherapy-induced injury can be toxic to the mitochondria and induce the release of reactive oxygen species leading to inflammation and the development of transplant-related complications such as VOD and GVHD.[33,42]

Carnitine supplementation is currently not routine in HSCT; however, therapeutic use in HSCT should be considered as the anti-inflammatory effects of carnitine supplementation on restoring mitochondrial metabolism and reducing oxidative stress and inflammation through cytokine reduction has been established.[35,38,43,44] Carnitine at pharmacological doses has been shown to imitate the biological actions of glucocorticoids thus exerting an immunologic effect through activation of glucocorticoid receptors in reducing the release of inflammatory cytokines in cell studies.[44] The use of carnitine supplementation as anti-cytokine therapy to reduce inflammation could be a valuable addition to transplant protocols. Whether carnitine supplementation could reduce the inflammatory pathophysiology of GVHD and VOD without the negative sequelae associated with the use of anti-inflammatory agents post-HSCT should be investigated.

The carnitine ratio is sensitive to changes in mitochondrial metabolism.[8] Our unexpected findings of an association with mortality and abnormal PCR levels on day 28, either above or below the reference range, may be indicative of a strong disturbance in mitochondrial dysfunction arising from the side-effects of the conditioning therapy, as these patients had not been deemed at high risk of death before HSCT. The findings that an abnormal PCR level before or after day 28 was not indicative of mortality in all patients suggests that mitochondrial disturbances occurring on day 28 after the cumulative effects of chemotoxicity and metabolic stressors post-HSCT acted as a prognostic indicator of progression towards irreversible disease leading to death. In the patients with elevated PCR values, 1 had VOD, and 2 had viral infections at that time with 1 dying within the month. An increased PCR has been reported to reflect mitochondrial dysfunction causing inhibition of fatty acid oxidation and disruption in the oxidative processes and subsequent substrate utilization required for several metabolic pathways.[7,9,11,42,44] Carnitine ratios have a relevant role in both energy metabolism and the stress-induced response due to their role in regulating acetylation pathways.[20] Disturbances in carnitine homeostasis arising from metabolic stress may be revealed by either low total carnitine or an elevated acyl/free carnitine ratio.[9] We postulate that the high PCR during the day 28 period of VOD occurrence and infective processes was indicative of substantial metabolic stress leading to irreversible complications and death.

Alternatively, in those with a low PCR on day 28, 1 had developed a fungal infection and graft failure and the other had gastrointestinal losses in addition to having a low PTC at that time. These factors may have resulted in less availability of acylcarnitine for detoxification of organic acids and removal of toxic metabolites from the mitochondria leading to further cellular toxicity.[31] A larger study focusing on the association of altered carnitine ratios with changes in cellular homeostasis in HSCT is warranted to investigate the potential role of day 28 PCR as an indicator of significant metabolic stress and negative clinical outcomes.

Limitations of this study include the small number of patients and the heterogeneity of the type of transplants, thus decreasing the sample sizes of those that were at risk of developing GVHD and VOD. In addition, the low power of the study was also compounded for the UTC analysis where the percentage of urine samples returned was 66% of those requested. Also being a small observational descriptive study, we cannot assume causal relationships; however, it is possible that these associations may be suggestive of carnitine profiles as useful markers in pediatric HSCT, or for therapeutic carnitine supplementation as a potential addition to conditioning therapy.

Overall, our findings indicate that carnitine profiles tended to be more reflective of negative outcomes such as VOD, GVHD, and mortality rather than of changes in the dietary intake of carnitine. The lack of any association between low carnitine intake from PN and decreased carnitine profiles supports the role of carnitine more as a prognostic indicator of metabolic stress rather than of low carnitine intake.

An expanded understanding of carnitine profiles in HSCT would allow us to better delineate the importance of these potential new roles for carnitine in HSCT: carnitine profile changes as a prognostic tool and carnitine supplementation as cytokine therapy for improvement of metabolic and inflammatory outcomes in HSCT. Whether the use of therapeutic carnitine supplementation added to HSCT protocols as adjunctive prophylactic intervention could mitigate mitochondrial disruptions and inflammatory risks of the conditioning therapy through the strengthening of the mitochondria and reduction in cytokine release requires further investigation in larger studies.

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