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Original Article

Lipid dysregulation after hematopoietic stem cell transplant

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Authorship Contributions

J.K. and S.M.D. were responsible for study concept, design and methodology; K.R.S., X.Z., L.L. and N.L. and E.W. performed the investigations; S.M.D and J.K. were responsible for visualization; S.M.D., S.J. K.M., and J.K. supervised the study; A.L. performed the statistical analysis and J.K., S.M.D., S.J., A.L., A.S., K.C.M., D.R., K.R.S., X.Z and E.W. wrote the original draft, reviewed and edited the final manuscript.

ABSTRACT

Transplant-associated thrombotic microangiopathy (TA-TMA) is a serious complication of allogeneic hematopoietic stem cell transplantation, primarily driven by endothelial injury and complement activation. Statins, combined with other drugs, are commonly used as prophylaxis against endothelial injury in some parts of the world but a mechanism of action is not clearly defined. We hypothesized that dysregulation of lipids, or their precursors, ceramides, might be an important mechanism of endothelial injury, and that statins might ameliorate that dysfunction. We measured plasma ceramide species at baseline and day 14 in pediatric and young adult allo-HSCT recipients. Ceramide species in general were increased in those who later developed endothelial injury, manifest as TA-TMA. These findings highlighted ceramides as markers of endothelial stress, prompting us to explore whether statin prophylaxis could favorably modulate lipid and ceramide pathways. A single-arm phase I trial of pravastatin prophylaxis was also performed in patients at elevated risk of endothelial injury due to high body mass index to assess lipid and ceramide modulation over time. Multiple ceramide species were elevated in patients who developed TA-TMA and showed strong correlations with ST2 but not with sC5b-9. While ceramides were associated with TA-TMA in univariate models, only ST2 remained significant in multivariable analysis. Addition of ceramide levels to ST2 only modestly improved prediction of later TA-TMA in ROC analysis. Pravastatin prophylaxis was associated with distinct shifts in lipoprotein and ceramide profiles, potentially reflecting modulation of endothelial function. Pravastatin may alter ceramide and lipoprotein pathways in a clinically meaningful way, contributing to their role in endothelial protection.

INTRODUCTION

Hematopoietic stem cell transplant (HSCT) is the sole curative modality for specific pediatric malignant and benign hematologic disorders. Despite advances in supportive care and transplant conditioning, life-threatening complications from endothelial damage secondary to HSCT procedures can lead to substantial morbidity and mortality. Endothelial damage plays a pivotal role in the induction of injury syndromes specific to HSCT including transplant-associated thrombotic microangiopathy (TA-TMA), veno-occlusive disease and graft-vs-host disease (GVHD). These complications cumulatively affect up to 70% of patients receiving allogeneic-HSCT (allo-HSCT)[1-3]. Inherent non-modifiable risk factors (e.g. race) also contribute to the development and progression of TA-TMA[4,5]. We have previously reported an increased risk of endothelial injury in children with high BMI who have received HSCT[5]. This observation, together with a proteomics study using an obese mouse model detected an upregulated in ceramide pathways after transplant (data not shown), drew our attention to a possible role for lipid metabolism, and ceramides in particular, in adverse outcomes after HSCT.

Ceramides are central to endothelial biology and inflammatory signaling and their modulation by statins has generated interest in their potential role as both therapeutic targets and predictive biomarkers of TA-TMA. Ceramides are a heterogeneous class of bioactive sphingolipids comprising a sphingoid base and a fatty acyl chain[6,7]. Ceramides play essential roles in maintaining endothelial barrier homeostasis and integrity[8]. Elevated ceramides increase oxidative stress, reduce nitric oxide (NO) production, induce mitochondrial dysfunction, modify endothelial activation and increase endothelial inflammation[9]. The different isoforms of ceramides have varying tissue distribution and function depending on their fatty acid chain lengths[10]. Ceramides are connected to numerous

inflammatory diseases, including systemic lupus erythematosus (SLE), diabetes mellitus, cardiovascular disease and even with

GVHD[11-14]. Interestingly, sphingosine-1-phosphate receptor (S1PR) modulators (e.g. Mocravimod) have been shown to diminish signaling that is needed for T cell migration to l3ymphoid organs and has been shown to reduce GVHD in murine models and in human studies[15,16]. This work highlights ceramides as a potential therapeutic target in the allo-HSCT setting[13,16].

Alongside ceramides, other lipid mediators may influence transplant outcomes. Despite increased recognition of the role of lipids in vascular inflammation, very few studies have examined lipidomic changes in HSCT recipients—particularly in the early post-transplant period when initial endothelial injury occurs—and associations with clinical outcomes. Limited prior investigations have focused on late dyslipidemia management and long-term cardiovascular complications after transplant[17,18]. High-density lipoprotein (HDL), for example, has been widely studied in the context of atherosclerosis and is typically associated with anti-inflammatory and vasculoprotective effects[19]. However, under certain pathological conditions, HDL-C may adopt proinflammatory characteristics[20,21]. To date, no studies have explored the functional role of HDL-C in HSCT or its relationship with transplant-related endothelial complications.

Prior reports have demonstrated that statins can reduce the incidence and severity of TA-TMA, likely through their protective effects on the endothelium[22]. Originally developed as lipid-lowering agents[23], statins are now recognized for their pleiotropic properties, including modulation of ceramide and metabolite profiles, improvement in endothelial function by increasing production of NO and reduction in inflammatory signaling[24-35]. These effects are particularly relevant in the setting of HSCT, where endothelial injury is a central driver of TA-TMA pathogenesis.

We have evaluated ceramides in the blood of children early after HSCT to determine whether similar alterations in ceramide pathways occur in human transplant recipients. We also investigated whether early post-transplant ceramide and lipid profiles differ between HSCT recipients who developed TA-TMA and those who did not, and the feasibility and impact of statin prophylaxis and children.

METHODS

Patient cohort

We studied ceramide species in a consecutive cohort of 135 pediatric and young adult patients undergoing first allogeneic HSCT (allo-HSCT) at Cincinnati Children's Hospital Medical Center between January 2013 and December 2021. Clinical data were obtained from an IRB-approved database, and blood samples were collected under informed consent and stored in an IRB-approved repository. Samples were prospectively collected at baseline (pre-conditioning) and on day +14 post-HSCT.

TA-TMA Diagnosis and Risk Stratification

Recipients were prospectively monitored for TA-TMA using weekly clinical assessments and laboratory screening (LDH, schistocytes, urine protein/creatinine ratio) from pre-conditioning through day +100. TA-TMA was diagnosed according to laboratory and clinical criteria published by Jodele et al[36].

Ceramide analysis using UPLC-MS/MS analysis

Plasma sphingolipids (20 µL) were extracted using a modified in-house protocol[37]. Quantification was performed by UHPLC coupled to triple-quadrupole mass spectrometry (Waters, Milford, MA) in Multiple Reaction Monitoring mode, using an Acquity UHPLC CSH C18 column (2.1 × 100 mm, 1.7 µm). Calibration curves were generated in charcoal-stripped human serum and processed identically to patient samples. Chromatography used a binary solvent system with gradient elution (acetonitrile/water and isopropanol/acetonitrile, each with 10 mM ammonium formate and 0.1% formic acid) at 0.4 mL/min, column temperature 55°C, total run time 20 min. Data were processed in MassLynx 4.1.

Safety and feasibility phase 1 study of pravastatin prophylaxis during HSCT

A prospective phase 1 trial (2023–2025; ClinicalTrials.gov NCT05524246, IND #162341) evaluated pravastatin prophylaxis during HSCT. Pravastatin (0.2–0.4 mg/kg/day; dose adjusted for calcineurin inhibitor use) was initiated at inpatient admission before conditioning and continued until day +35. Outpatients completed remaining doses at home, with compliance documented in study diaries.

Eligible patients were 2–25 years old, overweight/obese by CDC BMI criteria[38], scheduled for allogeneic HSCT, and able to take enteral medications. Exclusion criteria included pravastatin anaphylaxis, AST/ALT >3× ULN, GFR <50 mL/min/1.73 m², neuromuscular/metabolic disorders predisposing to rhabdomyolysis, or concurrent OATP1B1/1B3 substrate use. A consecutive cohort of HSCT patients not treated with pravastatin and matched as closely as possible by BMI were used as historical controls. Standard transplant supportive care procedures, including administration of total parenteral nutrition with intravenous lipid emulsions when clinically indicated (e.g., mucositis impairing oral intake), were followed in all patients regardless of study participation.

Primary endpoints were safety (NCI CTCAE v5.0) and feasibility (<70% adherence or withdrawal). Compliance was assessed via inpatient records and diaries. Secondary endpoints were day +100 TA-TMA incidence, acute GVHD, and overall survival. Exploratory endpoints included lipid/lipoprotein changes, with comparisons to 20 historical elevated-BMI controls. Because the majority of patients receive overnight total parenteral nutrition with intralipid, samples were often obtained shortly after lipid infusion. To minimize confounding, analyses emphasized within-person changes, with each patient serving as their own control.

Statistical Analysis

All available quantitative data were included in statistical analyses. For descriptive purposes, continuous variables are reported as median (IQR) given the presence of skewed distributions. Continuous variables were summarized as median (IQR) and categorical variables as frequency (%). Fisher exact and Wilcoxon tests compared categorical and continuous variables, respectively; Mann-Whitney U tested unpaired groups. For ceramide analyses, False Discovery Rate was controlled by the Benjamini-Hochberg method, with adjusted $p < 0.01$ considered significant. Other analyses used $p < 0.05$. In the pravastatin pilot trial, the prespecified secondary endpoint was incidence of TA-TMA by day +100. Separately, in the correlative cohort not treated with pravastatin, we calculated cumulative incidence curves for TA-TMA through 1 year to assess longer-term outcomes. Gray's competing risk method estimated 1-year TA-TMA cumulative incidence. Analyses were conducted in R v3.5.1.

RESULTS

Patient and transplant characteristics

Demographic and transplant related characteristics in the initial HSCT cohort in which ceramides were measured, including 135 consecutive allo-HSCT recipients, are shown in Table 1. A majority of

study subjects were white, with a higher proportion of males (54.1%). Most transplants were performed for non-malignant indications (71.9%). A majority of patients received an unrelated donor graft (69.6%), and bone marrow was the most common stem cell source (51.9%).

Plasma ceramides after hematopoietic stem cell transplant

Ceramide levels baseline (pre-conditioning) and at day 14 from HSCT are described in Figure 1. Ceramide species are categorized by the length of the fatty acyl chain, (e.g. C16Cer, C18Cer). Baseline concentrations of C16Cer (median 122.4 nmol/L, range 10-562.2 nmol/L) significantly increased by day 14 (median 326.1 nmol/L, range 142.5-928.9 nmol/L, $p<0.0001$) (Figure 1A). Significant elevation in concentrations of C18Cer, C20Cer, C24:1Cer and C26:1Cer at day 14 were also observed (Figure 1B-1E).

Plasma ceramide changes in TA-TMA

Given the established role of ceramides in promoting endothelial dysfunction[39], we next examined whether specific ceramide alterations were associated with the development of TA-TMA, a complication driven by endothelial injury. Figure 2 shows concentrations of ceramide species at baseline and day 14 from HSCT in patients later diagnosed with TA-TMA compared to patients without TA-TMA. We anticipated that day 14 ceramide levels would be elevated in those with TA-TMA but were surprised to find that baseline levels of ceramide species were also elevated in patients later diagnosed with TA-TMA compared to patients who never developed TA-TMA. While day 14 ceramide levels universally increased in all patients, patients with TA-TMA had more elevated concentrations of ceramides at day 14 compared to patients who never developed TA-TMA (Figure 2A-2E). Supplementary Tables 1 and 2 list baseline and day 14 concentrations of all measured ceramide species in patients with and without TA-TMA. Because of collinearity among ceramide species and

multiple comparisons being made, we set our level of statistical significance to $p<0.01$, and caution should be used in considering whether particular ceramide species contribute more or less to our findings. Ceramide species that were significantly elevated at baseline in patients with later TA-TMA included C16Cer, C18Cer, C20Cer, C22Cer and C24:1 Cer. At day 14, patients who developed TA-TMA had significantly higher levels of C16Cer, C16-OHCer, C18Cer, C20Cer and C24:1Cer compared to patients without TA-TMA (Supplementary Table 2).

Univariate and multivariate analysis of association of ceramides and TA-TMA

Univariate analyses and multivariate analyses were performed to evaluate the association between individual and multiple ceramide species and the development of TA-TMA (Table 2). Univariate analysis showed C16Cer (OR 3.44, 95% CI 1.62-8.34, p=0.003), C18Cer (OR 2.44, 95% CI 1.33-5.07, p=0.009), C20Cer (OR 2.41, 95% CI 1.30-5.13, p=0.012), C22Cer (OR 2.11, 95% CI 1.20-4.17, p=0.17), C24:1Cer (OR 2.61, 95% CI 1.38-5.81, p=0.009) and C26:1Cer (OR 2.2, 95% CI 1.28-4.10, p=0.007) were associated with increased risk of TA-TMA.

Multivariable logistic regression models were constructed to evaluate the association between ceramide species and TA-TMA. An initial multivariate model included C26:1Cer, C16Cer, and baseline ST2, a biomarker previously reported to be associates with TA-TMA[40] (Table 2). Multivariate analysis demonstrated that only baseline ST2 was associated with increased risk for developing TA-TMA (OR 2.21, 95% CI 1.07-5.25, p=0.047). Due to limited sample size and potential multicollinearity between ceramide species, additional models were constructed including ST2 and one ceramide species at a time to improve model interpretability (Table 2). This multivariate analysis demonstrated that C16Cer (OR 2.77, 95% CI 1.16-7.37, p=0.029) and C26:1Cer (OR 2.03, 95% CI 1.06-4.16, p=0.04) were associated with increased risk for developing TA-TMA. Overall, none of the ceramide

species were better than ST2 at predicting future risk of TA-TMA, in agreement with ROC analyses, shown in Supplementary Figure 1. We performed a similar univariate and multivariate analysis evaluating the association between ceramide levels and GVHD but did not observe any significant associations.

Pilot study of pravastatin prophylaxis during HSCT

We wanted to identify a prophylactic strategy that could be used routinely in children to mitigate endothelial injury mediated at least in part by elevation of ceramides. Ceramides are found in LDL particles alongside cholesterol, and lowering of cholesterol through statin treatment has been shown to decrease circulating ceramide levels in humans[41]. Pravastatin was chosen as previous success has been reported in reducing TA-TMA in adult HSCT recipients using pravastatin in combination with ursodiol[22].

Twenty-five patients with elevated BMI (median BMI above the 85th percentile for age) were enrolled in the study during HSCT, and 20 were evaluable for study endpoints. Patient disposition is shown in Supplementary Figure 2. Demographics of the pravastatin cohort and historical controls matched by BMI are described in Table 4. The pravastatin cohort was balanced by sex (45% female, 55% male) and predominantly White (85%). Most patients in the pravastatin cohort underwent transplant for malignancy (50%) or marrow failure (25%), with a median age at HSCT of 15.5 years. Most patients in the pravastatin group received fully matched grafts (65%) from unrelated donors (60%) and bone marrow or peripheral blood used equally as stem cell sources. Conditioning regimens were split evenly between myeloablative and reduced intensity approaches. GVHD prophylaxis was primarily CNI-based (75%). Intralipid was used for parenteral nutrition in most (85%) of patients. TA-TMA occurred in 55% of the pravastatin group. Acute GVHD by day 100 occurred in 30% of the pravastatin cohort with most patients developing only grade I acute GVHD (83.3%).

A median of 37 doses of pravastatin per patient were administered (IQR, 37-44). Mean medication adherence was 83.4% (IQR 80-100%) for the entire cohort. Five patients stopped treatment early due to low medication adherence (<70%) and were replaced in the study.

Adverse effects

Pravastatin was found to be safe and well tolerated. No attributable severe adverse events (SAEs) occurred. All SAEs possibly, probably, or definitely related to pravastatin treatment are described in Supplemental Table 3. Only one episode of grade 4 ALT elevation was possibly attributed to pravastatin. This patient was on other additional hepatotoxic medications including acetaminophen and posaconazole. Once these agents were discontinued, the patient had complete resolution of liver enzyme elevation making involvement of pravastatin less likely. One patient had possibly related grade 3 CPK elevation but also resolved without issue. No other attributable toxicities occurred. We observed grade 3 acute kidney injury (n=4); grade 3 AST/ALT elevation (n=18) and grade 4 AST/ALT elevation (n=2), all determined to be not attributed to pravastatin.

Ceramide changes in the pravastatin pilot study

We wanted to determine the effects of pravastatin on ceramide levels in this pilot study. Targeted mass spectrometry of ceramides was performed only in pravastatin-treated patients to assess longitudinal changes; control samples were not analyzed with this platform. We measured levels of specific ceramide species that were significantly altered in patients who developed TA-TMA in our preliminary analysis (Figure 3). Day 14 ceramide levels increased from baseline in patients treated with pravastatin, similar to our original observations in the cohort of 135 consecutive HSCT patients.

Day 14 concentrations of C16Cer, C18Cer, C22Cer, C24:1Cer and C26:0Cer were markedly increased from baseline in the pravastatin treated patients, indicating that in this limited pilot, pravastatin did not reduce ceramide elevation. C24Cer levels were not different at baseline compared to day 14.

Lipid profile changes in patients receiving pravastatin prophylaxis during HSCT

Ceramides are closely linked to lipoprotein metabolism and transport, and statins are well known to modify plasma lipoprotein levels, so we assessed changes in lipid profiles during pravastatin prophylaxis. First, we measured HDL levels in 20 HSCT recipients who received pravastatin and in 20 HSCT recipient controls who did not receive pravastatin. Levels were measured at baseline and days 7, 14 and 35 from HSCT (Figure 4). Baseline concentrations of HDL did not differ in pravastatin recipients (median 38 mg/dL, range 19-87 mg/dL) or controls (median 34 mg/dL, range 15-62 mg/dL, p=0.44). HDL levels declined at day 7 with a nadir at day 14 after HSCT in all pravastatin recipients. The overall trend in HDL levels in controls was similar, although there was an increase in HDL levels at day 14 compared with day 7 in 5 controls. However, HDL levels were not significantly different in the pravastatin (median 16.5 mg/dL, range 5-32 mg/dL) and control groups (median 16 mg/dL, range 5-65 mg/dL, p=0.97). By day 35, HDL levels were significantly higher in pravastatin treated patients (median 30.5, range 6-71 mg/dL) compared to controls (median 23 mg/dL, range 8-53 mg/dL, p=0.05).

To ensure that observed associations were not confounded by lipid pathway modulation from pravastatin prophylaxis, we evaluated the relationship between lipid levels and TA-TMA risk in patients not exposed to pravastatin (n = 86). The 1-year cumulative incidence (CI) of TA-TMA was

higher in patients with baseline HDL-C below the median compared to those above the median (50% vs. 29%, p = 0.20; Figure 5A), though this difference was not statistically significant. At day 14, the CI of TA-TMA was lower in patients with HDL-C below the median compared to those above the median (29% vs. 50%, p = 0.57; Figure 5B). Among all lipid parameters, baseline LDL-C levels below the median were significantly associated with increased TA-TMA risk (Figure 5C; p = 0.01). No significant differences in TA-TMA incidence were observed based on triglyceride levels at either time point (Figure 5E–F).

Additional lipoprotein and triglyceride levels are presented in Supplementary Figure 3. Median baseline cholesterol levels were non-significantly lower in between pravastatin treated patients (115.5 mg/dL, range 39-355 mg/dL) compared with controls (144.5 mg/dL, range 95-193 mg/dL, p=0.07). Day 7 cholesterol levels increased significantly from baseline in pravastatin treated patients (median 154.5 mg/dL, range 103-209 mg/dL, p=0.02), while levels in controls were similar to baseline (152 mg/dL, range 109-273 mg/dL, p=0.19). Patients treated with pravastatin had significantly elevated total cholesterol levels at day 14 (216 mg/dL, range 77-405 mg/dL) compared to controls (150 mg/dL, range 71-227 mg/dL, p=0.03). Cholesterol levels at day 35 were not different between cases (200 mg/dL, range 200-460 mg/dL) and controls (162.5 mg/dL, range 104-238 mg/dL, p=0.21). Baseline triglycerides were significantly decreased in pravastatin patients (median 78 mg/dL, range 39-122 mg/dL) compared to controls (median 126.5 mg/dL, range 55-564 mg/dL, p=0.006). Day 7 triglyceride concentrations significantly increased overall in the pravastatin group compared to baseline (median 167 mg/dL, range 65-479 mg/dL, p<0.0001) and increased in controls on days 14 (median 175.5 mg/dL, range 87-979 mg/dL) and 35 (median 280 mg/dL, range 74-831 mg/dL). Triglyceride levels in pravastatin treated patients decreased at day 14 compared with day 7 (median 66 mg/dL, range 20-134 mg/dL) and remained lower by day

35 (median 81 mg/dL, range 17-171 mg/dL). A large majority of children in this study received parenteral nutrition including an infusion of lipids known to alter triglyceride levels so caution must be used in considering these data, as we have no ability to distinguish the effects of this infusion from changes due to the HSCT process or use of statins.

Baseline low density lipoprotein (LDL) levels were higher in pravastatin treated patients (median 144 mg/dL, range 70-228 mg/dL) compared to controls for unclear reasons (median 77 mg/dL, range 51-118 mg/dL, $p<0.0001$). Day 7 LDL levels decreased significantly in the pravastatin group (median 93 mg/dL, range 29-151 mg/dL, $p<0.0001$) from baseline but remained similar in controls (median 83 mg/dL, range 44-196 mg/dL, $p=0.31$). LDL levels at day 14 (median 82 mg/dL, range 22-176 mg/dL) and day 35 from HSCT in controls remained consistently lower than pravastatin treated patients at the same timepoints. On day 14, pravastatin treated patients had significantly elevated LDL level (median 129.5 mg/dL, range 129.5-222 mg/dL) compared to controls (82 mg/dL, range 22-176 mg/dL, $p=0.002$). Similarly, on day 35, pravastatin treated patients had significantly increased LDL levels (median 166.5 mg/dL, range 84-242 mg/dL), compared to controls (median 84.5 mg/dL, range 42-156 mg/dL, $p<0.0001$).

DISCUSSION

In this current study we investigated ceramides as potential mediators of TA-TMA. Our study demonstrated that specific ceramide species were markedly increased at baseline and at day 14 from HSCT in patients who developed TA-TMA compared to patients without TA-TMA. Long chain ceramides (C16Cer and C18Cer) and very long chain ceramides (C20-C24Cer) are the most altered in patients with TA-TMA, in agreement with other studies that have shown ceramides to be elevated in other disease states involving immune dysregulation and inflammation[10,11,42,43]. Ceramide

accumulation is believed to contribute to endothelial dysfunction and inflammation in numerous disease states but has not previously been studied in HSCT[12,44]. Increased C16Cer and C18Cer have been associated with increased cardiovascular risk by inducing endothelial dysfunction through oxidative stress and hindering nitric oxide production leading to vascular inflammation and endothelial cell activation[45,46]. Accumulation of very long chain ceramides (C22Cer and C24:1Cer) has been associated with endothelial dysfunction through the disruption of vascular homeostasis[43]. These reports support our finding of increased ceramides on day 14 in patients with TA-TMA. Intriguingly, we found that ceramides were elevated prior to HSCT in patients who later got TA-TMA, indicating ongoing endothelial abnormality before the start of transplant. Correlative analyses revealed robust positive associations between these ceramides and ST2, a known marker of endothelial activation, but not with sC5b-9, implicating ceramides in endothelial rather than complement-mediated injury[39]. We considered whether measurement of ceramide level prior to transplant might be a useful biomarker of risk of TA-TMA, but univariate, multivariate and ROC analyses did not indicate increased benefit of ceramide levels over the more easily measured existing TA-TMA risk biomarker, ST2.

Statin-based endothelial prophylaxis is widely used in Europe; we considered whether using statins as a prophylactic strategy that would modify ceramides[22]. In our pilot study, pravastatin was well-tolerated with no severe adverse events attributable to treatment. Medication adherence was as would be expected in a pediatric trial of an oral therapy, with five patients discontinuing pravastatin due to low adherence. We saw an increase in ceramides in pravastatin recipients from baseline to day 14, similar to those seen in patients not receiving statin, suggesting that ceramide modification may not be an important part of statin-based endothelial prophylaxis.

In parallel, pravastatin treatment was associated with fluctuations in HDL-C, LDL-C, total cholesterol, and triglycerides. Interestingly, we found that lower baseline LDL levels were significantly associated with a higher cumulative incidence of TA-TMA**,** a finding that runs counter to conventional understanding but is supported by emerging literature[47,48]. Oxidized LDL (oxLDL) is known to directly mediate endothelial activation, promote leukocyte adhesion, and impair nitric oxide signaling through eNOS uncoupling[49]. However, native LDL also plays critical physiological roles, including the delivery of antioxidant-rich cargo and membrane components that may buffer against oxidative and inflammatory insults**.** Thus, insufficient baseline LDL may reflect a state of endothelial vulnerability that predisposes to TA-TMA following transplant-related stress. This observation underscores the need for further investigation into whether extremely low LDL levels, especially in the context of immune activation and endothelial stress, may be maladaptive.

Our study has both strengths and limitations. In our correlative biology studies, this is the first to report a link between ceramides with endothelial injury after HSCT. In parallel, the phase I pilot trial also demonstrated that pravastatin prophylaxis is feasible and safe in a pediatric population and produces measurable shifts in ceramides and lipid profiles. Although using baseline values as internal controls mitigates some confounding from TPN, residual effects of intravenous lipids on circulating lipid species cannot be fully excluded. Further limitations include, the correlative study was observational, and the phase I pilot study was small and non-randomized. This underscores the need for larger, randomized phase II studies incorporating longitudinal lipoprotein and ceramide profiling to validate these findings. Such trials will be essential to validate these findings, establish efficacy, and further dissect the mechanisms by which lipid remodeling and endothelial integrity intersect in TA-TMA pathogenesis.

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FIGURES AND TABLES

Table 1. Patient demographics and transplant characteristics

	Patient cohort n=135
Sex, n (%)	
Female	62 (45.9)
Male	73 (54.1)
Age at HSCT[#] in years, median (range)	6.8 (0.3-28.8)
Race, n (%)	
Caucasian	123 (91.1)
African American	7 (5.2)
Asian	2 (1.5)
Mixed	3 (2.2)
Ethnicity, n (%)	
Hispanic or Latino	130 (96.3)
Not Hispanic or Latino	5 (3.7)
Diagnosis, n (%)	
Malignancy	38 (28.1)
Marrow Failure	39 (28.9)
Immune Deficiency	30 (22.2)
Benign Hematology	21 (15.6)
Genetic/Metabolic	7 (5.2)
Stem Cell Source, n (%)	
Bone Marrow	70 (51.9)
PBSC*	54 (40)
Cord	11 (8.1)
Donor Type, n (%)	
Related	41 (30.4)
Unrelated	94 (69.6)
Degree of Match, n (%)	
Fully matched	101 (74.8)
Mismatched	34 (25.2)
Conditioning Regimen, n (%)	
Myeloablative	84 (62.2)
Reduced Intensity	50 (37)
None	1 (0.8)
GVHD[^] Prophylaxis, n (%)	
Calcineurin-based	91 (67.4)
T-cell depletion	44 (32.6)
TA-TMA[&], n (%)	
TA-TMA	52 (38.5)
No TA-TMA	83 (61.5)

TA-TMA risk category, n (%)	*n=52*
Low risk	4 (7.7)
Moderate risk	20 (38.5)
High risk	28 (53.8)
Days from HSCT to TA-TMA diagnosis, median (range)	31 (-12 to 378)

[#]HSCT: hematopoietic stem cell transplant; *PBSC: peripheral blood stem cell; [^]GVHD: graft-vs-host-disease; [&]TA-TMA: transplant-associated thrombotic microangiopathy.

Table 2. Univariate analysis of ceramides and TA-TMA

Univariate analysis		
Variable	**Odds ratio (95% CI)**	***p*-value**
C16Cer	3.44 (1.62-8.34)	**0.003**
C16-OHCer	1.67 (0.99-3.07)	0.074
C16-DHCer	1.09 (0.65-1.84)	0.726
C18Cer	2.44 (1.33-5.07)	**0.009**
C18 DHCer	1.30 (0.79-2.21)	0.298
C20Cer	2.41 (1.30-5.13)	**0.012**
C22Cer	2.11 (1.20-4.17)	**0.017**
C24:1Cer	2.61 (1.38-5.81)	**0.009**
C24Cer	1.56 (0.93-2.79)	0.105
C24 DHCer	1.01 (0.60-1.67)	0.974
C24-OHCer	1.10 (0.66-1.87)	0.717
C26:1Cer	2.2 (1.28-4.10)	**0.007**
C26:0Cer	1.63 (0.95-3.11)	0.097
ST2	2.52 (1.32-5.76)	**0.013**
Multivariate analysis of multiple ceramides and baseline ST2 in development of TA-TMA		
Variable	**Odds ratio (95% CI)**	***p*-value**
C16Cer	2.03 (0.69-6.51)	0.211
C26:1Cer	1.48 (0.65-3.48)	0.351
ST2	2.21 (1.07-5.25)	0.047
Multivariate analysis using single ceramide species and baseline ST2 in development of TA-TMA		
Variable	**Odds ratio (95% CI)**	***p*-value**
C16Cer	2.77 (1.16-7.37)	0.029
ST2	2.23 (1.07-5.38)	0.048
Variable	**Odds ratio (95% CI)**	***p*-value**
C18Cer	1.80 (0.84-4.11)	0.142
ST2	2.27 (1.14-5.35)	0.038
Variable	**Odds ratio (95% CI)**	***p*-value**
C20Cer	1.69 (0.79-3.89)	0.191
ST2	2.34 (1.19-5.44)	0.028
Variable	**Odds ratio (95% CI)**	***p*-value**
C24:1Cer	2.02 (0.93-4.92)	0.093
ST2	2.29 (1.18-5.26)	0.029
Variable	**Odds ratio (95% CI)**	***p*-value**

Table 3. Demographics and transplant characteristics of pravastatin treated patients and controls

	Pravastatin (n=20)	Controls (n=20)
Sex, n (%)		
Female	9 (45)	9 (45)
Male	11 (55)	11 (55)
Race, n (%)		
White	17 (85)	17 (85)
African American	1 (5)	2 (10)
Asian	1 (5)	1 (5)
Mixed	1 (5)	0
Ethnicity, n (%)		
Hispanic or Latino	0	0
Not Hispanic or Latino	20 (100)	20 (100)
Diagnosis, n (%)		
Malignancy	10 (50)	7 (35)
Marrow failure	5 (25)	7 (35)
Hemoglobinopathy	2 (10)	1 (5)
Genetic/metabolic	1 (5)	0
Inborn error of Immunity	2 (10)	5 (25)
Median age at HSCT[!], years (IQR)[#]	15.5 (11.2-17.6)	13.7 (8.3-19.8)
Pre-transplant BMI[@], kg/m^2, median (IQR)	29.7 (16.9-46.2)	24.5 (21.7-29.6)
Pre-transplant BMI percentile, kg/m^2, median (IQR)	97.8 (91.5-98.6)	97.2 (90.6-97.7)
Pre-transplant BMI Z-score, kg/m^2, median (IQR)	2.09 (1.4-2.3)	1.9 (1.3-2)
Donor type, n (%)		
Related	8 (40)	6 (30)
Unrelated	12 (60)	14 (70)
Degree of match, n (%)		
Fully matched	13 (65)	14 (70)
Mismatched	7 (35)	6 (30)
Stem cell source, n (%)		
Bone marrow	10 (50)	11 (55)
Peripheral blood	10 (50)	9 (45)
Conditioning regimen, n (%)		
Myeloablative	9 (45)	9 (45)
Reduced intensity	11 (55)	11 (55)
GVHD[$] Prophylaxis, n (%)		
CNI[^]-based	15 (75)	12 (60)
Ex vivo T-cell depletion	5 (25)	8 (40)
Intralipid use during HSCT, n (%)	17 (85)	18 (90)
TA-TMA[&], n (%)	11 (55)	7 (35)
Day 100 acute GVHD, n (%)		
All	6 (30)	3(15)
Grade 1	5 (83.3)	2 (66.7)
Grade 2	1 (16.7)	1 (33.3)

[!]HSCT: hematopoietic stem cell transplant; [@]BMI: body mass index; [#]IQR: interquartile range; [$]GVHD: graft-vs-host disease; [^]CNI: calcineurin inhibitor; TPN: total parenteral nutrition; [&]TA-TMA: transplant-associated thrombotic microangiopathy

FIGURE LEGENDS

Figure 1. Representative ceramide changes in all patients at baseline and day 14 after HSCT

Concentrations of C16Cer, C18Cer, C20Cer, C24:1Cer and C26:1Cer in plasma from all patients at baseline (purple) and day 14 (blue). Data are shown as median with interquartile range.

Figure 2. Representative ceramide changes in patients with TA-TMA.

Concentrations of C16Cer, C18Cer, C20Cer, C24:1Cer and C26:1Cer in plasma at baseline and day 14 from HSCT in patients with no TA-TMA (blue) and TA-TMA (red). Data are shown as median with interquartile range. TA-TMA: transplant-associated thrombotic microangiopathy

Figure 3. Ceramide concentration changes in pilot pravastatin study

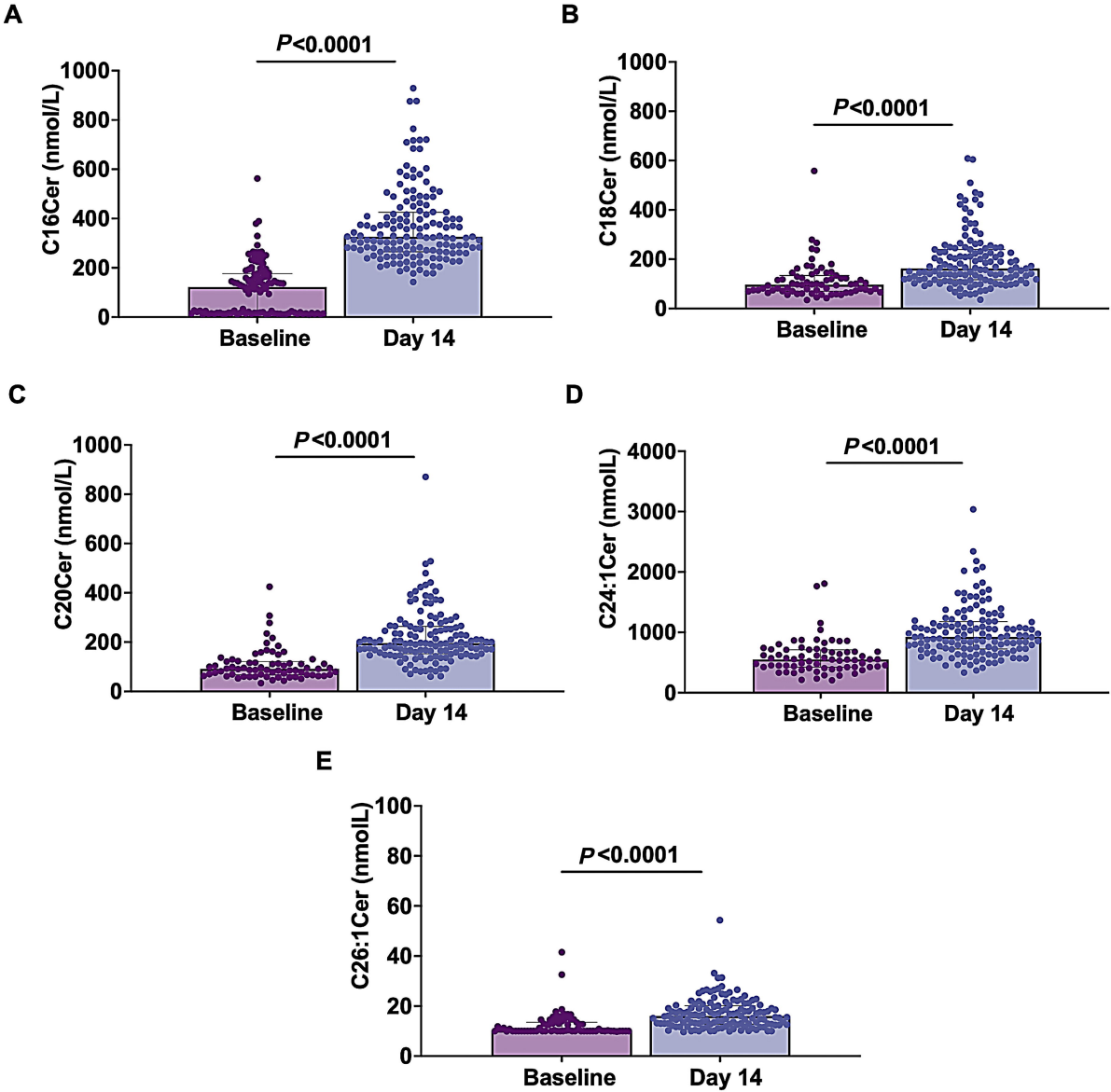
Ceramide species quantified by mass spectrometry in pravastatin-treated patients at baseline and day 14. No control ceramide data were available. Notably, long-chain ceramides C24 and C26 decreased under pravastatin while other species increased. Concentrations of C16Cer, C18Cer, C20Cer, C22Cer, C24Cer, C24:1Cer and C26Cer in plasma at baseline (orange) and day 14 (black) from HSCT in all pravastatin treated patients are shown. Data are shown as median with interquartile range.

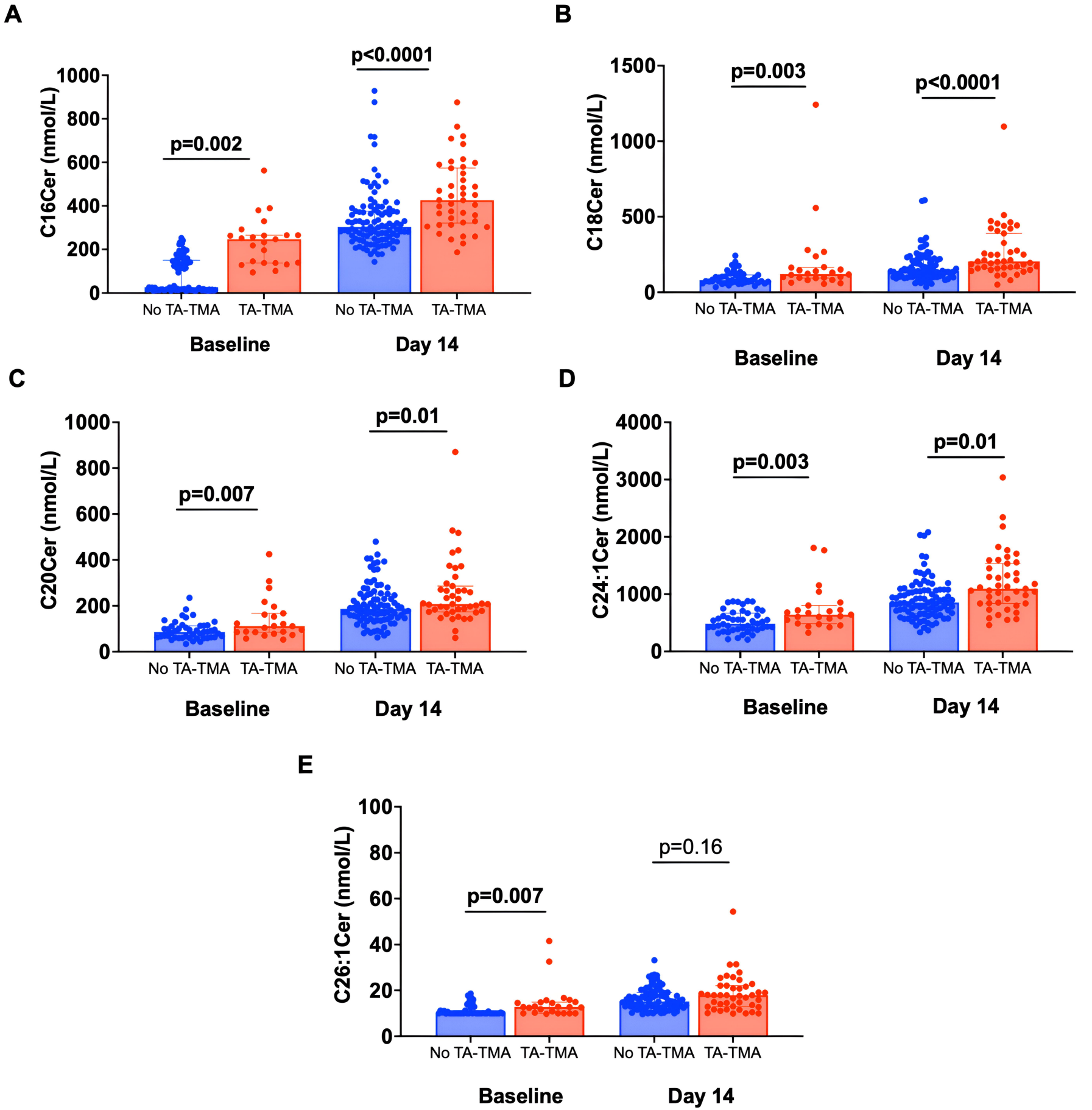
Figure 4. High-density lipoprotein cholesterol dynamics in pravastatin treated patients and controls.

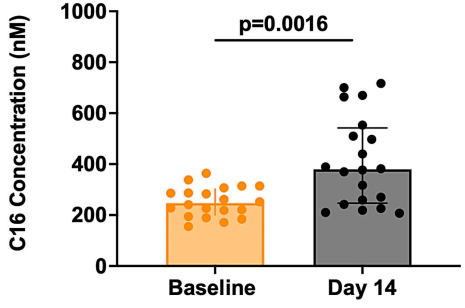
HDL-C concentration changes are shown for cases (purple) and controls (green) at baseline (pre-conditioning), days 7, 14 and 35 after HSCT. Each colored line represents an individual patient. The black-dashed line represents the median HDL-C concentration at each timepoint. HDL-C changes over time are represented in (A) cases and (B) controls. Each colored line represents a single patient and their respective changes in HDL-C over time. (C) Comparisons of the concentrations of HDL-C at baseline, days 7, 14 and 35 are shown. HDL-C: high-density lipoprotein cholesterol

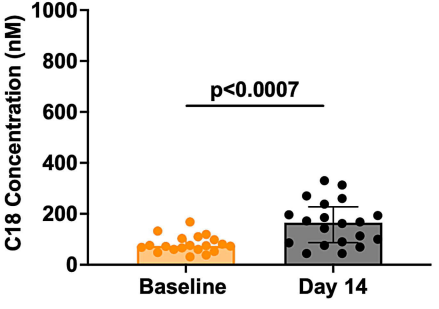
Figure 5. Lower baseline LDL-C is associated with increased 1-year cumulative incidence of TA-TMA after HSCT.

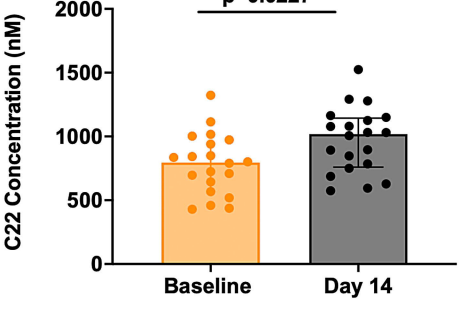
Cumulative incidence of transplant-associated thrombotic microangiopathy (TA-TMA) within 1 year following hematopoietic stem cell transplantation (HSCT) is shown stratified by lipid levels. (A, C, E) Curves represent patients with baseline levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides either above the median (dashed line) or below the median (solid line). (B, D, F) Curves show TA-TMA incidence based on day 14 post-HSCT levels of the same lipid parameters. A significant association was observed between low baseline LDL-C and increased TA-TMA incidence (C, $p = 0.01$), while other lipid markers did not show statistically significant associations. Differences in cumulative incidence were evaluated using Gray's test.

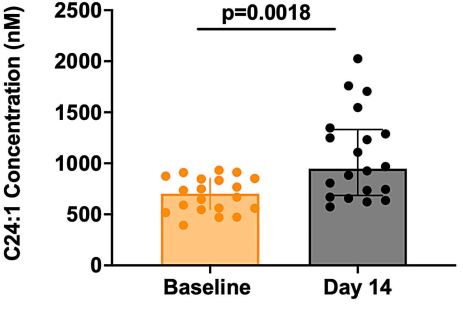

A
P<0.0001
C16Cer (nmol/L)
1000
800
600
400
200
0
Baseline
Day 14
B
P<0.0001
C18Cer (nmol/L)
1000
800
600
400
200
0
Baseline
Day 14
C
P<0.0001
C20Cer (nmol/L)
1000
800
600
400
200
0
Baseline
Day 14
D
P<0.0001
C24:1Cer (nmolL)
4000
3000
2000
1000
0
Baseline
Day 14
E
P<0.0001
C26:1Cer (nmolL)
100
80
60
40
20
0
Baseline
Day 14

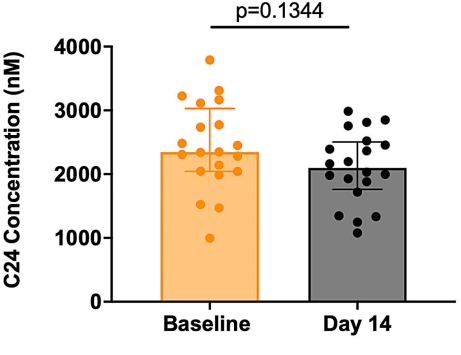

A
C16Cer (nmol/L)
1000
800
600
400
200
0
p=0.002
p<0.0001
No TA-TMA
TA-TMA
No TA-TMA
TA-TMA
Baseline
Day 14
B
C18Cer (nmol/L)
1500
1000
500
0
p=0.003
p<0.0001
No TA-TMA
TA-TMA
No TA-TMA
TA-TMA
Baseline
Day 14
C
C20Cer (nmol/L)
1000
800
600
400
200
0
p=0.007
p=0.01
No TA-TMA
TA-TMA
No TA-TMA
TA-TMA
Baseline
Day 14
D
C24:1Cer (nmol/L)
4000
3000
2000
1000
0
p=0.003
p=0.01
No TA-TMA
TA-TMA
No TA-TMA
TA-TMA
Baseline
Day 14
E
C26:1Cer (nmol/L)
100
80
60
40
20
0
p=0.007
p=0.16
No TA-TMA
TA-TMA
No TA-TMA
TA-TMA
Baseline
Day 14

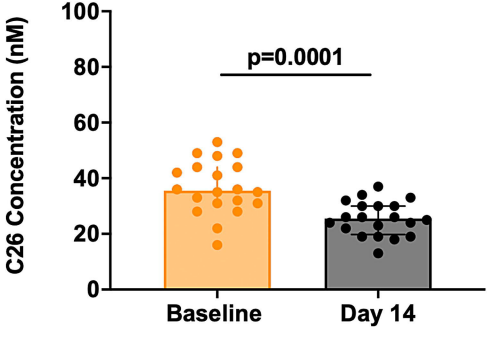

C16 Concentration (nM)
1000
800
600
400
200
0
p=0.0016
Baseline
Day 14

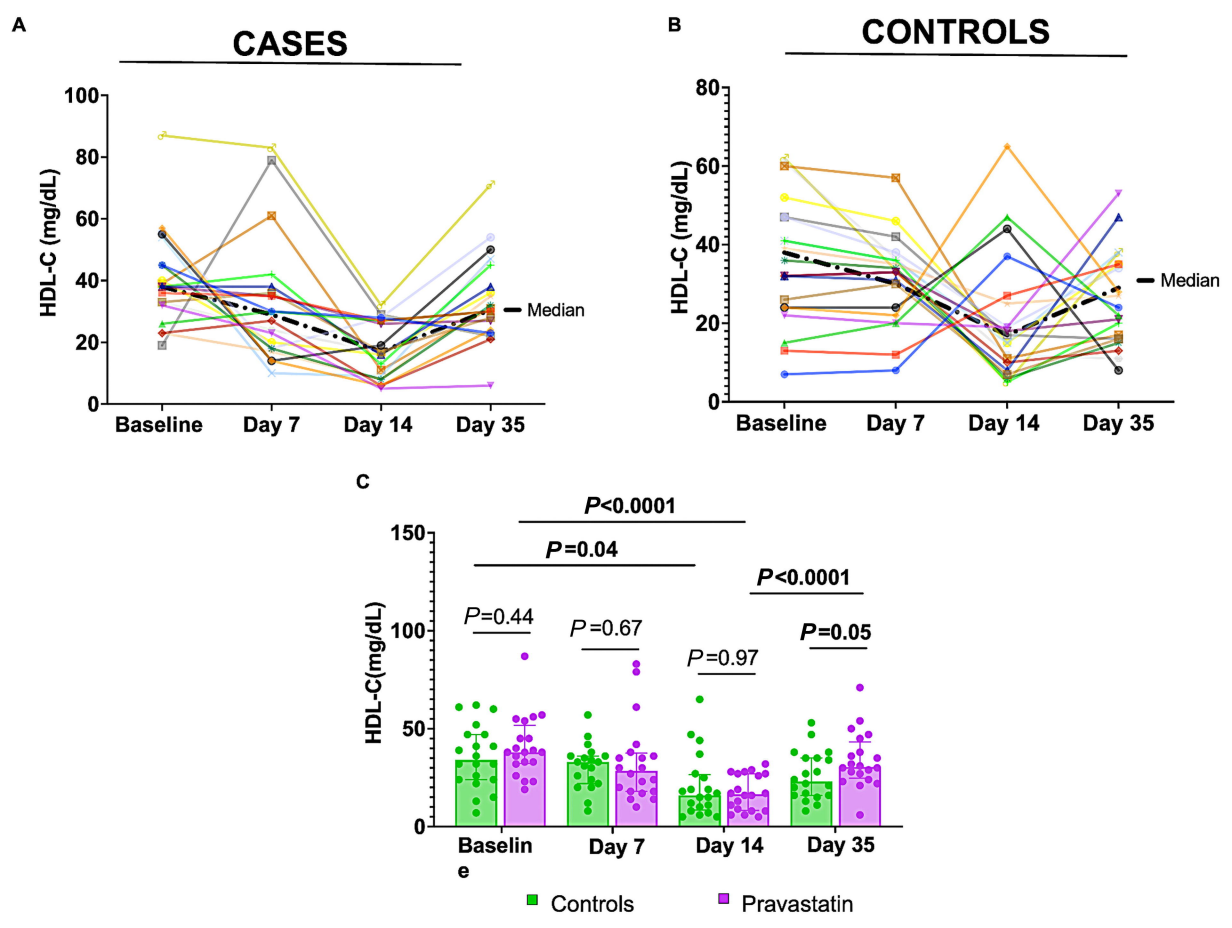

C18 Concentration (nM)
1000
800
600
400
200
0
p<0.0007
Baseline
Day 14

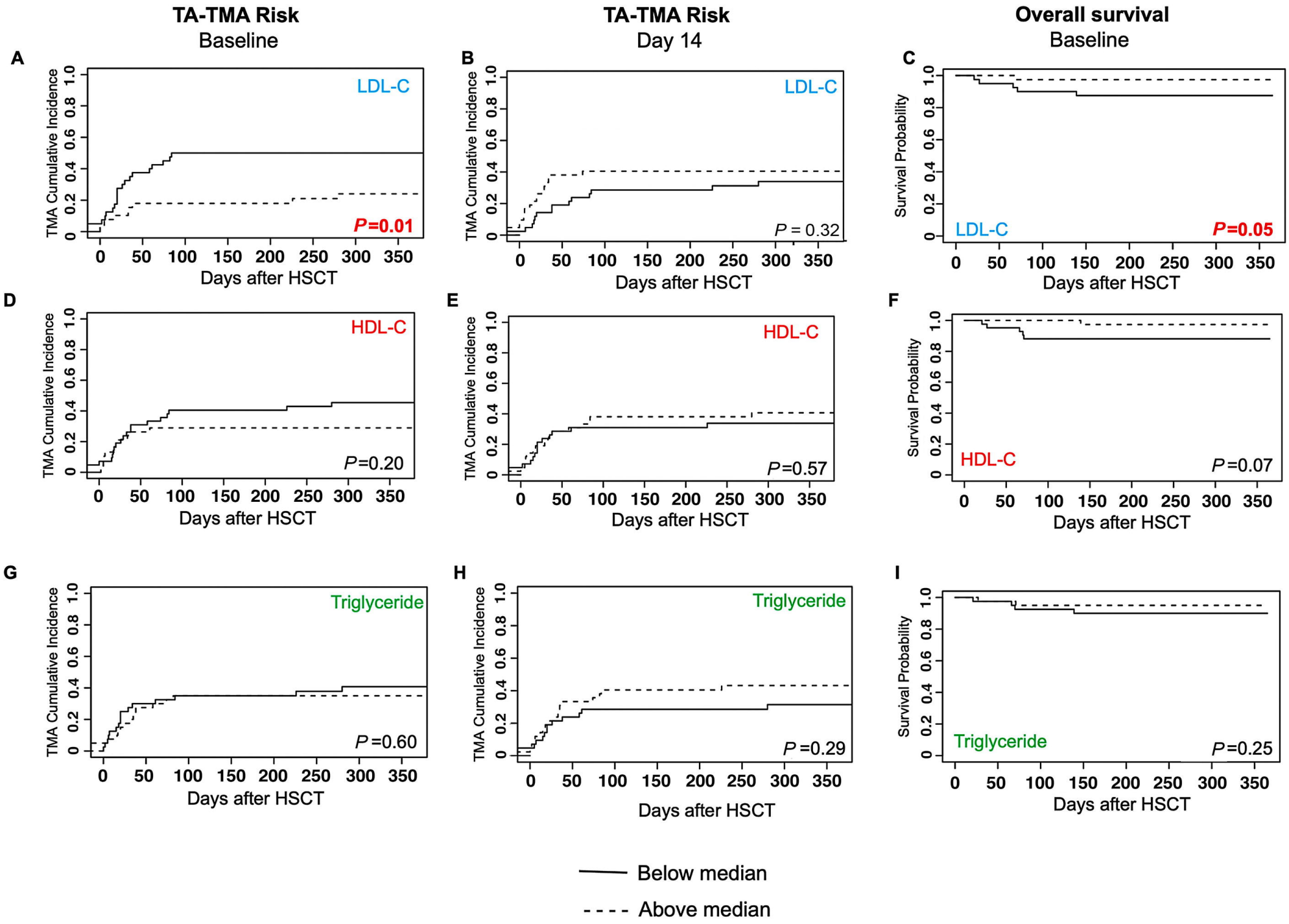

C22 Concentration (nM)
2000
1500
1000
500
0
p=0.0227
Baseline
Day 14


C24:1 Concentration (nM)
2500
2000
1500
1000
500
0
p=0.0018
Baseline
Day 14


C24 Concentration (nM)
4000
3000
2000
1000
0
p=0.1344
Baseline
Day 14


C26 Concentration (nM)
100
80
60
40
20
0
p=0.0001
Baseline
Day 14


A
CASES
HDL-C (mg/dL)
100
80
60
40
20
0
Baseline
Day 7
Day 14
Day 35
Median
B
CONTROLS
HDL-C (mg/dL)
80
60
40
20
0
Baseline
Day 7
Day 14
Day 35
Median
C
P<0.0001
P=0.04
P<0.0001
P=0.05
P=0.44
P=0.67
P=0.97
HDL-C(mg/dL)
150
100
50
0
Baseline
Day 7
Day 14
Day 35
Controls
Pravastatin


TA-TMA Risk
Baseline
TA-TMA Risk
Day 14
Overall survival
Baseline
A
B
C
D
E
F
G
H
I
LDL-C
HDL-C
Triglyceride
TMA Cumulative Incidence
Survival Probability
Days after HSCT
P=0.01
P = 0.32
P=0.05
P=0.20
P=0.57
P=0.07
P=0.60
P=0.29
P=0.25
Below median
Above median

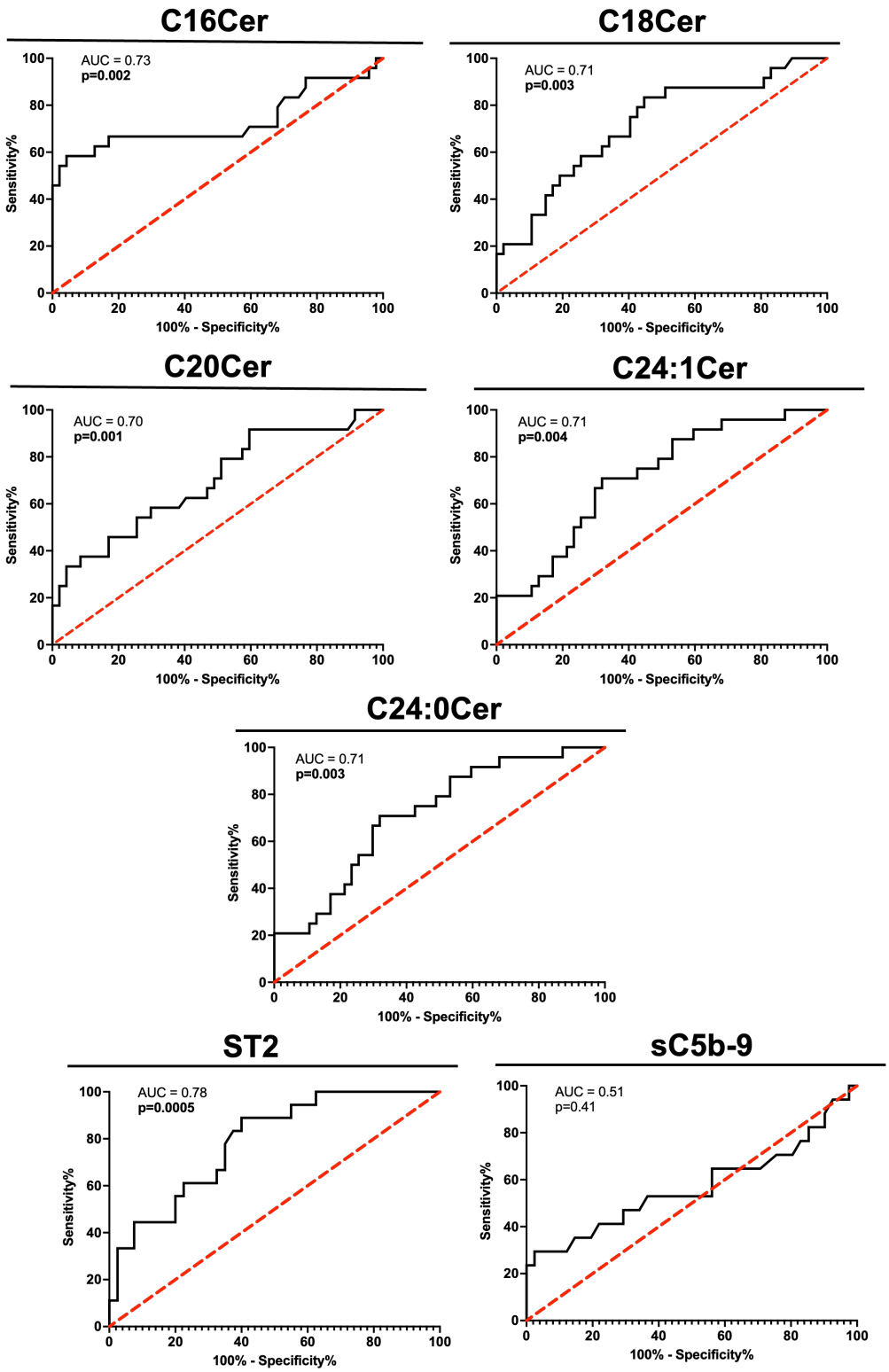
Supplementary Table 1. Baseline concentrations of all ceramide species analyzed in cohort

	TA-TMA	No TA-TMA	*p*-value
C16Cer (nmol/L)	248.56 (94.33-2289)	152 (93.95-252.65)	**0.001**
C16-OHCer (nmol/L)	12.65 (9.94-176)	13.01 (9.94-19.69)	0.15
C16 DHCer (nmol/L)	25.67 (11.12-647.5)	24.47 (9.82-79.89)	0.76
C18Cer (nmol)	120.25 (56.23-1241.5)	80.81 (34.66-242.09)	**0.003**
C18 DHCer (nmol/L)	14.36 (9.52-298.5)	13.92 (9.52-46.87)	0.30
C20Cer (nmol/L)	111.46 (52.74-1598)	86.1 (33.7-235.05)	**0.009**
C22Cer (nmol/L)	607.56 (237.01-5466.5)	416.89 (190.67-1329.53)	**0.008**
C24:1Cer (nmol/L)	657.22 (326.8-4359)	485.87 (206.33-878.46)	**0.003**
C24Cer (nmol/L)	1527.94 (646.04-9745.5)	1312.24 (503.46-2825.71)	0.13
C24 DHCer (nmol/L)	82.19 (14.43-1535)	85.5 (15.96-630.39)	0.91
C24-OHCer (nmol/L)	34.49 (11.72-201.5)	29.9 (7.81-88.81)	0.44
C26:1Cer (nmol/L)	13.03 (9.77-41.5)	12.58 (9.92-18.65)	0.02
C26:0Cer (nmol/L)	18.6 (10.77-73.5)	14.32 (9.74-22.88)	0.01
Sphingosine (nmol/L)	26.72 (9.69-52.44)	21.04 (14.36-86.17)	0.12

Supplementary Table 2. Day 14 concentrations of all ceramide species analyzed in cohort

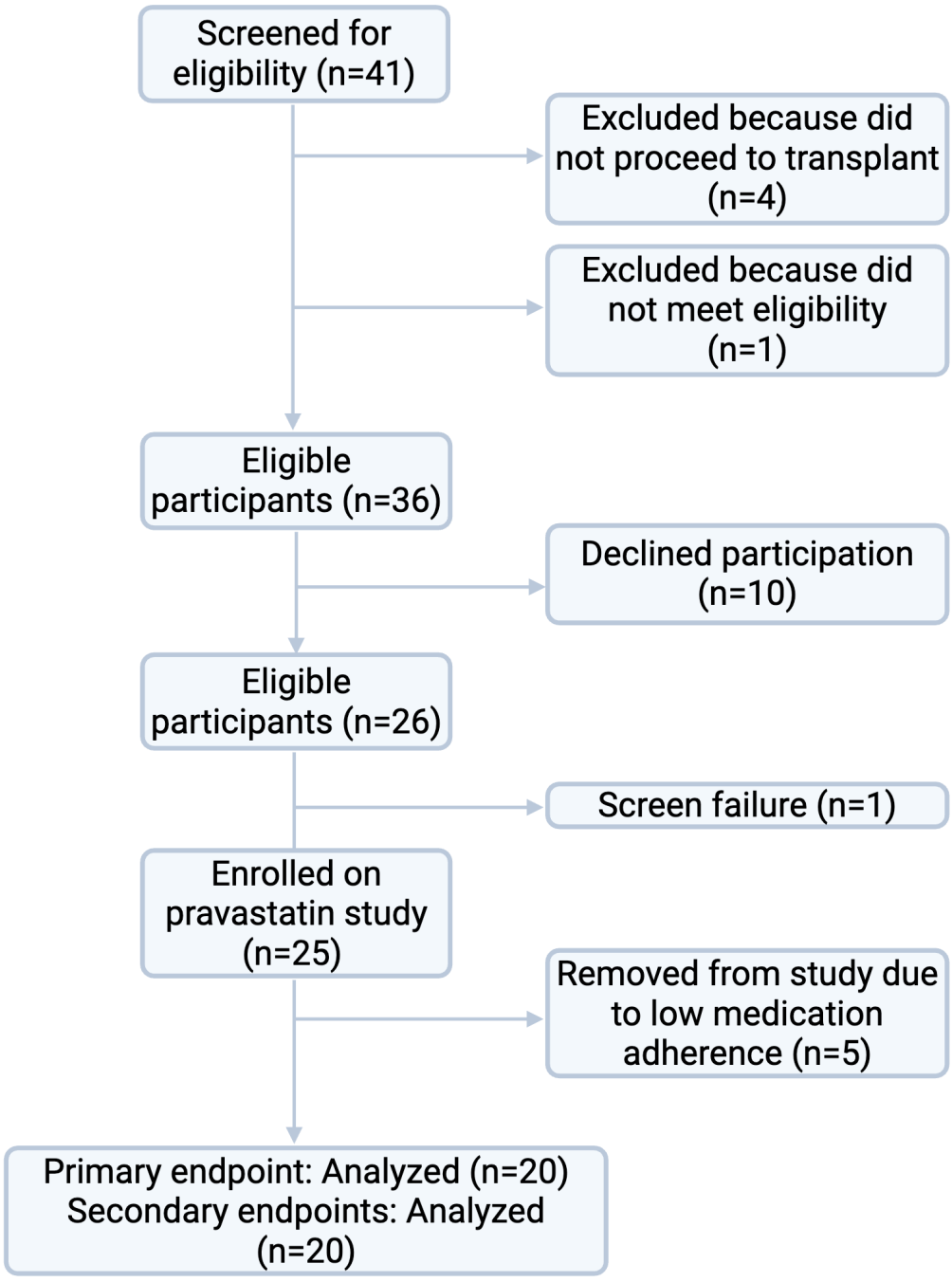
	TA-TMA	No TA-TMA	*p*-value
C16Cer (nmol/L)	469.4 (226.98-1387.35)	318.05 (142.51-928.93)	**0.0007**
C16-OHCer (nmol/L)	28.18 (14.09-124.66)	16.98 (9.76-66.67)	**<0.0001**
C16 DHCer (nmol/L)	44.11 (19.09-477.66)	29.19 (14.46-72.85)	0.06
C18Cer (nmol)	211.49 (136.34-1097.08)	140.85 (35.72-360.74)	**0.0008**
C18 DHCer (nmol/L)	29.78 (11.28-233.48)	16.3 (9.87-53.04)	0.03
C20Cer (nmol/L)	270.43 (173.72-870.43)	191.91 (62.71-479.87)	**0.002**
C22Cer (nmol/L)	854.71 (506.03-2517.78)	722.2 (300.24-1645.37)	0.06
C24:1Cer (nmol/L)	1178.22 (551.35-2341.62)	887.57 (333.13-2031.35)	**0.009**
C24Cer (nmol/L)	1678.37 (1058.81-4420.17)	1612.78 (610.78-3334.26)	0.43
C24 DHCer (nmol/L)	64.62 (33.46-790.48)	73.06 (21.03-216.27)	0.57
C24-OHCer (nmol/L)	61.31 (33.66-138.54)	60.41 (12.17-186.18)	0.53
C26:1Cer (nmol/L)	19.91 (14.8-31.24)	17.25 (10.21-33.16)	0.15
C26:0Cer (nmol/L)	21.55 (12.25-50.92)	19.63 (9.59-42.07)	0.59
Sphingosine (nmol/L)	29.06 (18.37-60.45)	20.54 (14.36-87.51)	0.62

Supplementary Figure 1. ROC analyses of C16Cer, C18Cer, C20Cer, C24:1Cer and C26Cer levels at baseline in plasma from patients with TA-TMA.



Supplementary Figure 1. ROC analyses of baseline C16Cer, C18Cer, C20Cer, C22Cer and C24:1Cer levels in plasma from patients with TA-TMA. Corresponding AUCs are shown in Table 4. Abbreviation: AUC, area under the curve; Cer, ceramide; ROC, receiver operating characteristic; ST2, suppression of tumorigenicity 2; sC5b-9, terminal complement complex

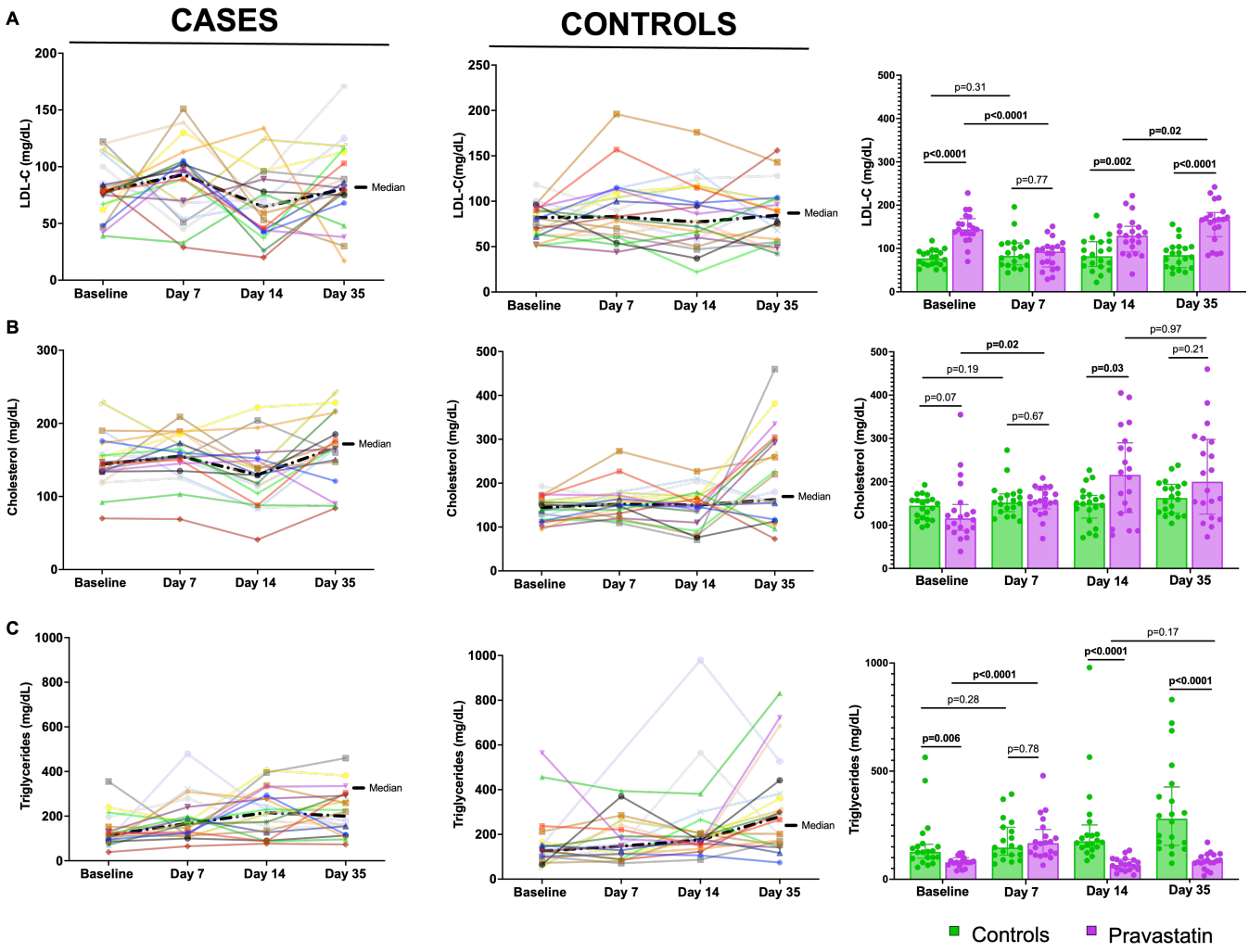
Supplementary Figure 2. CONSORT diagram



Supplementary Table 3. Serious adverse events.

SAE Term	# of Patients	Resolved?	Relationship to Pravastatin	Subject IDs	Number of Participants with Grade 1	Number of Participants with Grade 2	Number of Participants with Grade 3	Number of Participants with Grade 4	Number of Participants with Grade 5
Respiratory Failure	2	Yes	Unrelated	PRAV - 01, 06	N/A	N/A	N/A	2	N/A
Respiratory Failure	1	No (ongoing at end of study participation)	Unrelated	PRAV - 23	N/A	N/A	N/A	1	N/A
Somnolence	1	Yes	Unrelated	PRAV - 01	N/A	N/A	N/A	1	N/A
Cerebrospinal Fluid Leak	1	No (ongoing at end of study participation)	Unrelated	PRAV - 01	N/A	N/A	1	N/A	N/A
Hypertension	2	Yes	Unrelated	PRAV - 02, 22	N/A	N/A	1	1	N/A
Seizure	1	Yes	Unlikely	PRAV - 04	N/A	N/A	1	N/A	N/A
Ventricular Arrhythmia	1	Yes	Unrelated	PRAV - 07	N/A	N/A	1	N/A	N/A
Encephalopathy	1	Yes	Unrelated	PRAV - 07	N/A	N/A	1	N/A	N/A
Delirium	1	Yes	Unrelated	PRAV - 07	N/A	N/A	1	N/A	N/A
Leukocytosis	1	Yes	Unrelated	PRAV - 08	1	N/A	N/A	N/A	N/A
Maculo-papular Rash	1	Yes	Unrelated	PRAV - 08	N/A	1	N/A	N/A	N/A
Hypotension	1	Yes	Unrelated	PRAV - 12	N/A	N/A	1	N/A	N/A
Septic Shock	2	Yes	Unrelated	PRAV - 14, 18	N/A	N/A	N/A	2	N/A
Hypoxia	1	Yes	Unrelated	PRAV - 14	N/A	N/A	N/A	1	N/A
Diarrhea	1	Yes	Unrelated	PRAV - 16	N/A	N/A	1	N/A	N/A
Neck Pain	1	Yes	Unrelated	PRAV - 16	N/A	N/A	1	N/A	N/A
Breast Mass	1	Recovered with sequelae	Unrelated	PRAV - 16	1	N/A	N/A	N/A	N/A
Upper Respiratory Infection	1	Yes	Unrelated	PRAV - 17	N/A	N/A	1	N/A	N/A
Cytokine Release Syndrome	1	Yes	Unrelated	PRAV - 18	N/A	N/A	N/A	1	N/A
Anaphylaxis	1	Yes	Unrelated	PRAV - 18	N/A	N/A	N/A	1	N/A
Worsening Hypoxia	1	Recovered with sequelae (ongoing hypoxia AE after resolution of SAE (transfer from ICU back to BMT) - hypoxia eventually resolved	Unrelated	PRAV - 19	N/A	N/A	N/A	1	N/A
Acute Kidney Injury	1	Yes	Unrelated	PRAV - 22	N/A	N/A	1	N/A	N/A
Acute Kidney Injury	1	No (ongoing at end of study participation)	Unrelated	PRAV - 23	N/A	N/A	N/A	1	N/A
Worsening Acute Kidney Injury	1	Yes	Unrelated	PRAV - 22	N/A	N/A	1	N/A	N/A
Sinusoidal Obstructive Syndrome	1	Yes	Unrelated	PRAV - 23	N/A	N/A	N/A	1	N/A
Gastrointestional Graft Versus Host Disease	1	Yes	Unrelated	PRAV - 25	N/A	N/A	1	N/A	N/A
Facial Pain	1	Yes	Unrelated	PRAV - 26	N/A	1	N/A	N/A	N/A
Pruritus	1	Yes	Unrelated	PRAV - 26	N/A	N/A	1	N/A	N/A
Flushing	1	Yes	Unrelated	PRAV - 26	N/A	1	N/A	N/A	N/A
Suicidial Ideation	1	Yes	Unrelated	PRAV - 26	N/A	N/A	N/A	1	N/A

Supplementary Figure 3. Additional lipid dynamics in pravastatin treated patients and controls.



Supplementary Figure 3. LDL-C, cholesterol and triglyceride changes are shown for cases (purple) and controls (green) at baseline (pre-conditioning), days 7, 14 and 35 after HSCT. Each colored line on the graphs represents changes in LDL-C, total cholesterol and triglycerides at each timepoint. The black-dashed line represents the median HDL-C concentration at each timepoint. (A) LDL-C, (B) cholesterol and (C) triglyceride changes over time are represented. Each colored symbol represents a single patient and their respective changes in HDL-C over time. Bar graphs for each lipid exhibiting comparisons of the concentrations of LCL-C, cholesterol and triglycerides at baseline, days 7, 14 and 35 are shown.
LDL-C: low-density lipoprotein cholesterol