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Increase in serum choline levels predicts for improved progression-free survival (PFS) in patients with advanced cancers receiving pembrolizumab

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ABSTRACT

Background Recent studies have demonstrated that T cells can induce vasodilation in a choline-acetyltransferase dependent manner, leading to an increase in T cell migration to infected tissues in response to viral infection, but its role in cancer is unclear. Choline acetyltransferase catalyzes the production of acetylcholine from choline and acetyl-CoA, however, acetylcholine is challenging to quantify due to its extremely short half-life while choline is stable. This study aims to correlate serum choline levels in patients with advanced solid tumors receiving pembrolizumab with treatment outcomes.

Methods Blood samples were collected at baseline and at week 7 (pre-cycle 3) in patients treated with pembrolizumab in the INvestigator-initiated Phase 2 Study of Pembrolizumab Immunological Response Evaluation phase II trial (NCT02644369). Samples were analyzed for choline and circulating tumor DNA (ctDNA). Multivariable Cox models were used to assess the association between choline and overall survival (OS) and progression-free survival (PFS) when including $\Delta ctDNA_{C3}$ (the change in ctDNA from baseline to cycle 3), cohort, PD-L1 expression and tumor mutation burden (TMB). An independent validation cohort from the LIBERATE study (NCT03702309) included patients on early phase trials treated with a PD-1 inhibitor.

Results A total of 106 pts were included in the analysis. With a median follow-up of 12.6 months, median PFS and OS were 1.9 and 13.7 months, respectively. An increase in serum choline level at week 7 compared with baseline ($\Delta choline_{C3}$) in 81 pts was significantly associated with a better PFS (aHR 0.48, 95% CI 0.28 to 0.83, p=0.009), and a trend toward a better OS (aHR 0.64, 95% CI 0.37 to 1.12, p=0.119). A combination of $\Delta ctDNA_{C3}$ and $\Delta choline_{C3}$ was prognostic for both OS and PFS. Multivariable analyses show $\Delta choline_{C3}$ was a prognostic factor for PFS independent of $\Delta ctDNA_{C3}$, cohort, PD-L1 and TMB. In the independent validation cohort (n=51), an increase in serum choline at cycle 2 was associated with a trend to improved PFS.

Conclusions This is the first exploratory report of serum choline levels in pan-cancer patients receiving pembrolizumab. The association between improved PFS and $\Delta choline_{C3}$ suggests a possible role for the cholinergic system in the regulation of antitumor immunity. Further pre-clinical and clinical studies are required to validate this finding.

Trial registration number NCT03702309.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The acetylcholine pathway has recently been shown to play an important role in the regulation of immune cell function in infection.

WHAT THIS STUDY ADDS

⇒ An increase in serum choline levels was significantly associated with an improved progression-free survival and a trend toward an improved OS in patients with advanced cancers receiving pembrolizumab.

⇒ This is the first exploratory report of serum choline levels in a pan-cancer cohort of patients receiving immune checkpoint inhibitors, and suggests that the acetylcholine pathway may play a role in immune regulation in cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ The current study suggests a potential role for serum choline as a novel biomarker of response to immune checkpoint blockade in cancer.

INTRODUCTION

Immune checkpoint inhibitors (ICI) targeting programmed cell death protein 1 (PD-1), its ligand (programmed death-ligand 1; PD-L1), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) have been approved by health regulatory authorities in many countries and are now considered standard

treatment for multiple solid and hematologic malignancies, in both curative and palliative settings. However, only a subset of patients benefit from ICI therapy. Objective response rates to anti-PD-1/L1 agents alone vary widely by tumor type, but overall average approximately 20% among tumor types with demonstrated efficacy.[1] Further, these agents are associated with a unique toxicity profile, which can result in significant morbidity and mortality.[2] There is a need to further elucidate the molecular mechanisms of response and resistance and to develop predictive biomarkers, of which few are validated at present.[3]

While the most promising biomarker studies to date come from randomized, prospective trials, these studies are often limited in the scope of genomic and immune correlatives because they use archival specimens, and seldom include on-treatment biological specimens to obtain a mechanistic understanding of the dynamic antitumor immune response. The phase II INvestigator-initiated Phase 2 Study of Pembrolizumab Immunological Response Evaluation (INSPIRE) trial (NCT02644369), involving patients with advanced solid tumors treated with pembrolizumab, sought to address this limitation by facilitating a dynamic, longitudinal assessment of the antitumor immune response using multiomic biomarkers to provide insights into the molecular correlates of ICI response and resistance.[4] For example, results from the INSPIRE trial showed that changes in circulating tumor DNA (ctDNA) during pembrolizumab treatment correlate with clinical outcome.[5] Integration of dynamics of both ctDNA and tumor burden on radiological imaging offers insights into immunogenomic mechanisms of pembrolizumab response or resistance.[6]

Metabolic signaling molecules and pathways, such as the choline pathway, have recently been implicated in the regulation of immune responses.[7] Choline is an essential nutrient predominantly used for the synthesis of essential lipid components of cell membranes such as phosphatidylcholine (PC). It is also a precursor for acetylcholine (ACh), a well-established neurotransmitter. In nerve terminals, ACh is synthesized from acetyl coenzyme A (acetyl CoA) generated during glycolysis and choline, in a reaction catalyzed by choline acetyltransferase (ChAT). Physiologically, ACh regulates numerous biological processes including movement, digestion, heart rate, respiration and other autonomic functions via the neuronal cholinergic system.[8–10] Recent findings suggest that ACh also plays important roles in the modulation of immune cell function in response to infection.[7 11 12] During infection, antigen-specific CD4 +T lymphocytes can facilitate transendothelial migration of T cells into affected tissues through T cell ChAT-dependent vasodilation.[13–15]

Cox *et al* recently described the potential role of T-cell derived ACh in a diverse array of pathological processes including cancer (figure 1).[7] Additional reports have described a potential link between the choline pathway and metabolic reprogramming in the tumor microenvironment (TME), possibly augmenting immune responsiveness.[16–20] As such its role as a potential biomarker of response and toxicity to ICI has emerged as an area of interest. ACh is challenging to quantify as it has an extremely short half-life in vivo due both to its innate instability and the ubiquitous presence of the enzyme ACh esterase (AChE), which cleaves ACh to choline.[21] Choline, however, is stable and represents a more viable option to interrogate. We report the first attempt to correlate changes in serum choline levels with treatment outcomes in patients with advanced solid tumors receiving pembrolizumab. Changes in serum choline levels were also explored in an independent validation cohort of patients treated with PD-1 ICIs in early phase trials.

MATERIALS AND METHODS

Patient selection and drug administration

INSPIRE is a multicohort basket trial of the anti-PD-1 antibody pembrolizumab in patients with advanced solid tumors (NCT02644369). A full description of enrolled patients and their characteristics has previously been reported.[5 6] To summarize, between April 2016 to May 2018, 106 patients were treated across five parallel cohorts: (A) squamous cell carcinoma of the head and neck (n=19), (B) triple negative breast cancer (TNBC) (n=22), (C) epithelial ovarian cancer type II (n=21), (D) malignant melanoma (n=12), and (E) mixed advanced solid tumors (n=32). Patients who had prior treatment with an anti-PD-1, anti-PD-L1, or anti-PD-L2 agent were excluded, however, prior therapy with anti-CTLA4 or T-cell co-stimulatory agents were allowed. Patients received pembrolizumab 200 mg intravenously every 3 weeks. Objective response was assessed every three cycles (9 weeks) using Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1. Adverse events were graded according to the NCI Common Terminology Criteria for Adverse Events V.4.03. The data collection cut-off date was March 14, 2021.

Tumor biopsies and blood collection

Fresh tumor biopsies were collected at baseline (day −10 to day 0 of study treatment) and at treatment cycles 2 or 3 (week 6 or 9).[4] Additional details regarding biopsy and processing have previously been described.[5] Peripheral blood samples were collected prior to- and on-treatment at week 7 (pre-cycle 3), where possible. Serum was separated and stored at −80°C until analysis.

Serum choline analysis

A total of 106 patients had blood samples available at baseline, and 81 patients also had on-treatment samples (figure 2). For each sample, 10 µL of 20 µg/mL D^9-choline and 0.5 mL acetone was added to 10 µL serum. The mixture was vortexed and centrifuged. The resulting supernatant was transferred and evaporated to dry under vacuum. The residual was reconstituted with 0.4 mL acetonitrile, 10 µL of which was injected onto the high-performance liquid chromatography (HPLC) system. The HPLC mass spectrometry system consists of a Shimadzu

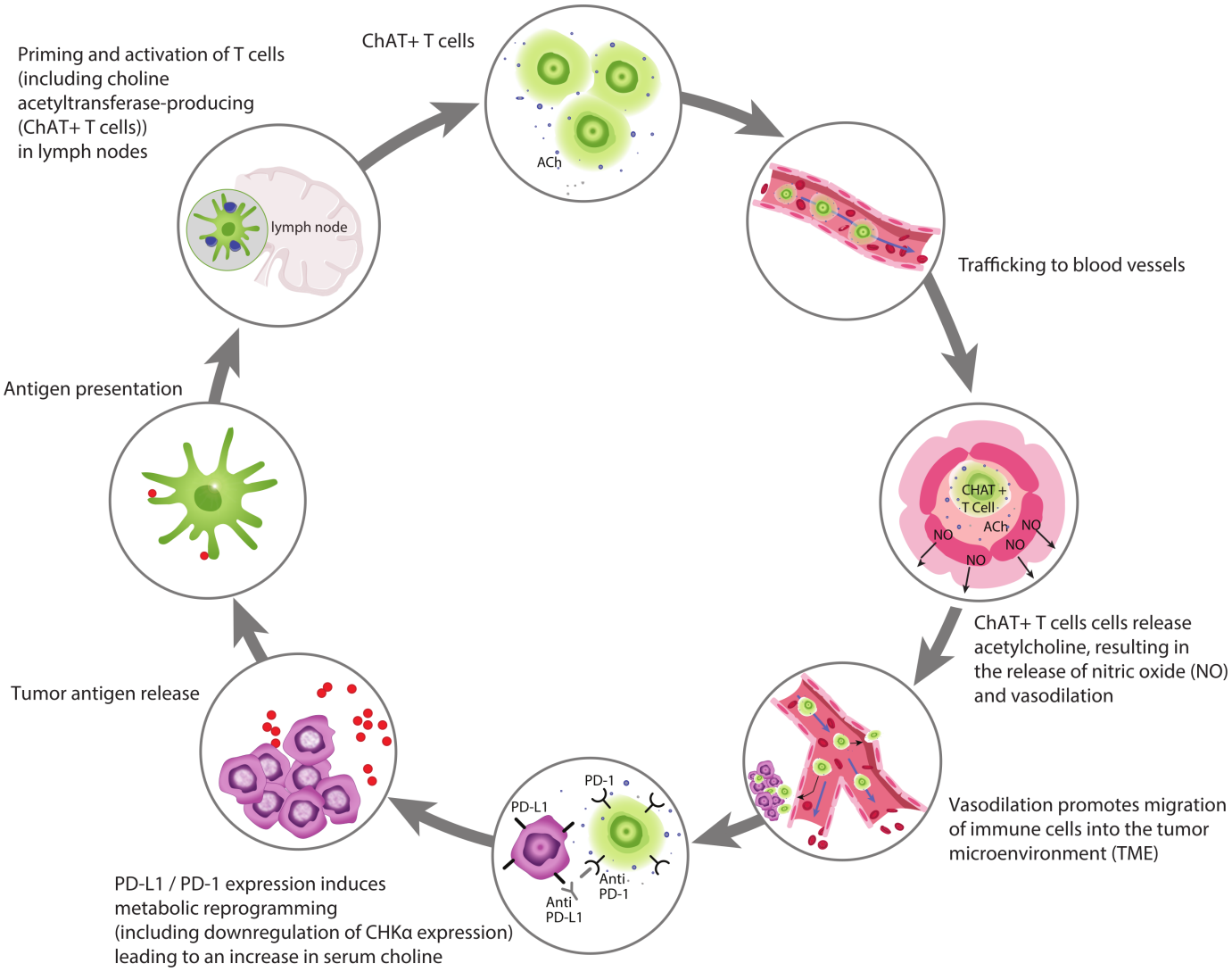

Figure 1 Tumor antigen release and presentation result in activation of T cells including ChAT-expressing T cells. ACh released from these cells acts on vascular endothelial cells, which release NO. NO induces vascular smooth muscle relaxation and vasodilation, facilitating the migration of immune cells into the TME and promoting the elimination of tumor cells. ACh, acetylcholine; ChAT, choline acetyltransferase; CHKα, choline kinase-α.

LC-20AD system coupled with a SCIEX QTRAP 3200 mass spectrometer (AB SCIEX LP, Concord, Canada). Separation was achieved using a Alltima C18 reversed-phase column (100×4.6 mm, 3.0 µm particles) (Hichrom, Leicestershire, UK) with mobile phases of A: 5 mM ammonium acetate and B: 5 mM ammonium acetate in acetonitrile/methanol (1:1) at a flow rate of 0.7 mL/min. Choline and D^9-choline were monitored at mass/charge (m/z) 104.1 to 60.1, and 113.2 to 69.0, respectively. The lower limit of quantitation is 100 ng/mL (online supplemental figure 1).

ctDNA analysis

Assessment of ctDNA using the bespoke Signatera assay in INSPIRE patients has previously been reported.[5] The change in ctDNA ($\Delta ctDNA_{C3}$) was defined as the percentage change in absolute ctDNA levels in plasma at cycle 3 from baseline. ctDNA clearance was defined as undetectable ctDNA (less than 2 of 16 variants below detection threshold) at any time during pembrolizumab treatment. A total of 72 patients had paired choline and ctDNA samples available for analyses (figure 2).

Tumor mutation burden quantitation

DNA extracted from digested tumor biopsies were sequenced with Illumina HiSeq 2000/2500 at the Princess Margaret Genomic Center and the Princess Margaret - Ontario Institute of Cancer Research Translational Genomics Laboratory in Toronto, Canada. Additional details regarding sequencing and data processing have previously been described.[5]

Immunohistochemistry analysis

For baseline biopsies only, formalin-fixed paraffin-embedded blocks were used for PD-L1 immunohistochemistry (IHC) (clone 22C3) on 4–5 µm sections mounted on positively charged ProbeOn slides (QualTek, Goleta, CA). QualTek provided a Modified Proportion Score (MPS)

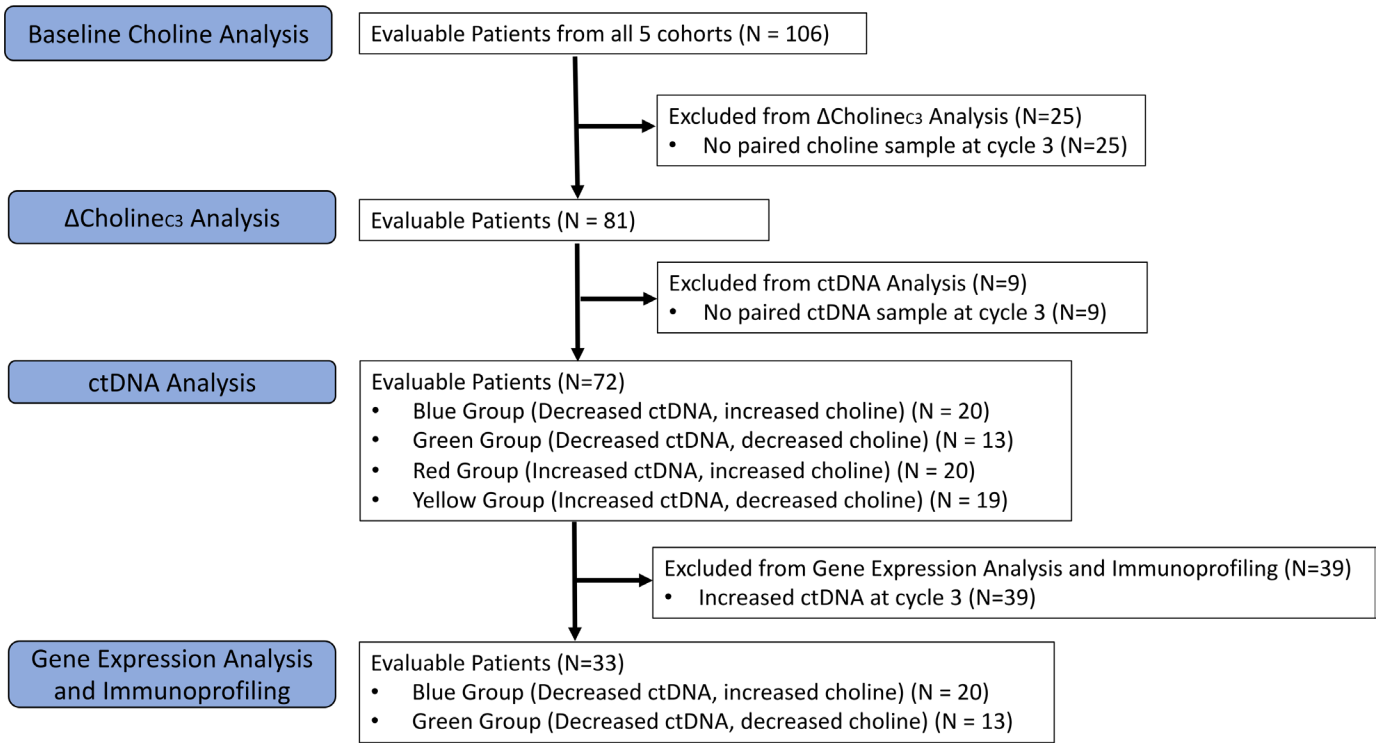

Figure 2 CONSORT diagram. CONSORT, Consolidated Standards of Reporting Trials; ctDNA, circulating tumor DNA.

indicating the proportion of PD-L1-expressing tumor cells and mononuclear inflammatory cells such as T, B, NK, macrophages and dendritic cells within tumor nests.[4]

Formalin-fixed, paraffin-embedded blocks of baseline and on-treatment tumor biopsies were used for multispectral fluorescent IHC (FL-IHC). The 4–5 µm sections were cut and mounted onto positively charged slides. Each slide was stained with a 6-marker panel composed of pan-cytokeratin (or melanoma cocktail and SOX10 for melanoma tissues), CD3, CD8, FOXP3, CD20, and CD68 using the OPAL 7-color IHC kit (catalog #: NEL811001KT; Akoya Biosciences, Marlborough, MA, USA) following the manufacturer's protocol (see online supplemental table 1 for antibody dilution information). Multiplex staining was done on an IP-FLX autostainer (BioCare) using sequential addition of monoclonal antibodies followed by fluorescent labeling with OPAL dyes. Stained slides were scanned using the Vectra 3 imaging system (Akoya Biosciences). Regions of interest containing evaluable tumor were selected by a study pathologist. Necrotic areas were also excluded from our analysis. Images were analyzed using inForm Software (Akoya Biosciences; V.2.2.0) to quantify immune cells, which are reported as cells/mm^2.

RNA-seq analysis

Detailed descriptions of RNA extraction, sequencing, and data processing have been published previously.[4 6] Gene-expression was quantified by RSEM (V.1.3.0) in units of transcripts per million from 100 bp paired-end RNAseq reads mapped to the GRCh38 human reference by STAR (V.2.4.2.a) and batch-corrected using ComBat.[22–24] Tumor infiltrating T cell abundances were inferred using CIBERSORT in absolute mode.[25]

Selected genes of interest included those involved with choline uptake (*CHT1* (*SLC5A7*), *CTL1* (*SLC44A1*), *CTL2* (*SLC44A2*)), choline metabolism (*CHKA*, *PCYT1A*, *PCYT1B*), ACh production, transport and signaling (*CHAT*, *ACHE*, *SLC18A3*, *CHRNA7*, *CHRNA9*, *CHRM3*), immune related genes (*IL21, IL21R, JAK1, JAK3, STAT1,* and *STAT3*) and genes involved in vasodilation (*NOS3, GUCY1A1, GUCY1A2*).

Flow cytometry

Peripheral blood mononuclear cells or tumor single cell suspensions were stained for immune markers of interest. Data were acquired using a 5-laser LSR Fortessa X-20 (BD, Mississauga, Ontario, Canada). Immunophenotyping data were analyzed using FlowJo V.10 (Treestar, Ashland, Oregon, USA). Additional details on the optimized flow cytometry panels have previously been reported.[4]

Independent validation cohort

In order to validate the findings related to choline analysis from the INSPIRE cohort, we evaluated blood samples collected from patients enrolled in an institutional liquid biopsy collection study, Liquid Biopsy Evaluation and Repository Development at Princess Margaret (LIBERATE) (NCT03702309). Briefly, between December 2017 to March 2020, 51 patients with 24 different tumor types were treated on early phase clinical trials involving an anti-PD-1 antibody alone or in combination with other investigational treatments. Objective response based on RECIST V.1.1 criteria and survival

outcomes were retrospectively collected. These blood samples were collected prior to treatment and on-treatment at week 3 or 4 (pre-cycle 2).

Statistical analysis

Summary statistics were reported for patient and disease characteristics, with median and range for categorical variables and frequency and percentage for continuous variables. Both baseline choline levels (defined as higher vs lower than the median value) and $\Delta choline_{C3}$ (defined as an increase or decrease from baseline at cycle 3) were evaluated as potential prognostic markers for overall survival (OS) and progression-free survival (PFS) outcomes. OS and PFS were estimated using the Kaplan-Meier method. Bivariable Cox proportional hazards models adjusting for cohort and multivariable Cox models including cohort, PD-L1 expression (as a percentage) and tumor mutation burden (TMB) (as a continuous variable on log scale), were fitted to assess the association between choline levels and outcomes. Fisher's exact test was used to assess the association between choline levels and treatment response, and between choline levels and grade ≥2 immune related adverse event (irAE) occurrences. For patients with both choline and ctDNA samples available (figure 2), multivariable Cox models were fitted to assess if $\Delta choline_{C3}$ is an independent prognostic factor for OS or PFS when including $\Delta ctDNA_{C3}$, cohort, PD-L1 expression and TMB.

Additional exploratory subgroup analyses including gene expression analysis, multiplex IHC and flow cytometry was performed in 33 patients that showed a decrease in ctDNA at cycle 3 compared with baseline (figure 2). Differences in baseline, on-treatment, and changes in levels of selected genes of interest and T cell scores between selected groups of patients were evaluated using Wilcoxon rank-sum test (change is defined as difference at cycle 2 or 3 compared with baseline among patients with measurements available at both time points). All tests were two sided and a $p \leq 0.05$ was considered statistically significant. All analyses were performed using R (R Core Team, 2020).[26]

RESULTS

A total of 106 patients were included in analyses of baseline choline (figure 2). The median follow-up time was 12.6 months (range: 0.6–58.1 months) for the INSPIRE cohort (online supplemental table 2). The median age was 59 years (range: 21–82 years), 66 patients were female (62%). The median PFS was 1.9 months and median OS was 13.7 months. All patients had an ECOG performance status 0 or 1.

A total of 81 patients were included for analyses of $\Delta choline_{C3}$ (figure 2). Demographic data for this cohort are provided in online supplemental table 3. There were no significant associations between choline levels (either baseline levels or $\Delta choline_{C3}$) and treatment response by RECIST V.1.1 in terms of objective response or clinical benefit (not shown). However, the combination of $\Delta choline_{C3}$ and cycle 3 RECIST response can predict OS (p=0.001, online supplemental figure 2). Among these 81 patients, 22 patients reported ≥grade 2 irAEs. There was no statistically significant correlation between $\Delta choline_{C3}$ and ≥grade 2 irAE (p=0.59).

Bivariable choline analyses

In bivariable analysis adjusted for cohort, baseline serum choline levels did not correlate with PFS (aHR 1.13, 95% CI 0.72 to 1.77, p=0.587) or OS (aHR 1.03, 95% CI 0.64 to 1.64, p=0.912) (figure 3A,B). However, a positive $\Delta choline_{C3}$ was significantly associated with an improved PFS (aHR 0.48, 95% CI 0.28 to 0.83, p=0.009) compared with a negative $\Delta choline_{C3}$, and a trend toward a better OS (aHR 0.64, 95% CI 0.37 to 1.12, p=0.119) (figure 3C,D).

Multivariable analyses

In multivariable analysis, a positive $\Delta choline_{C3}$ was significantly associated with an improved PFS (HR 0.45, 95% CI 0.25 to 0.78, p=0.0051) after including cohort, PD-L1 status and TMB as covariates.

Correlation with ctDNA analyses

A total of 72 patients had paired choline and ctDNA samples available for analyses (figure 2). Demographic data for this cohort are provided in online supplemental table 4. ctDNA levels and choline levels did not correlate at baseline or cycle 3. Four subgroups were created based on changes in ctDNA and choline levels: (1) increase in ctDNA, increase in choline (red group, n=20), (2) decrease in ctDNA, increase in choline (blue group, n=20), (3) increase in ctDNA, decrease in choline (yellow group, n=19), and (4) decrease in ctDNA, decrease in choline (green group, n=13). A combination of $\Delta ctDNA_{C3}$ and $\Delta choline_{C3}$ was shown to be prognostic for both OS and PFS (figure 4).

Multivariable Cox regression analyses show $\Delta choline_{C3}$ was an independent prognostic factor for PFS (p<0.001) when including cohort, PD-L1, TMB and $\Delta ctDNA_{C3}$ (table 1), but not statistically significant for OS (p=0.11) (not shown).

Exploratory subgroup analyses

Additional exploratory subgroup analyses were performed in those patients demonstrating a decrease in ctDNA and an increase in serum choline (figure 4 (blue group)) and patients with a decrease in ctDNA and a decrease in choline (figure 4 (green group)) (figure 2) (online supplemental table 5). Baseline characteristics between the two groups were compared, including gender, age, cohort, prior radiation therapy, prior lines of systemic therapy, previous immunotherapy treatment and MPS for PD-L1, however, no statistically significant differences were seen between the two groups (data not shown). Similarly, changes from baseline to cycle 3 in absolute neutrophil count (ΔANC), lymphocytes (Δlymphocytes) and neutrophil to lymphocyte ratio (ΔNLR) were not statistically significant different between these two groups

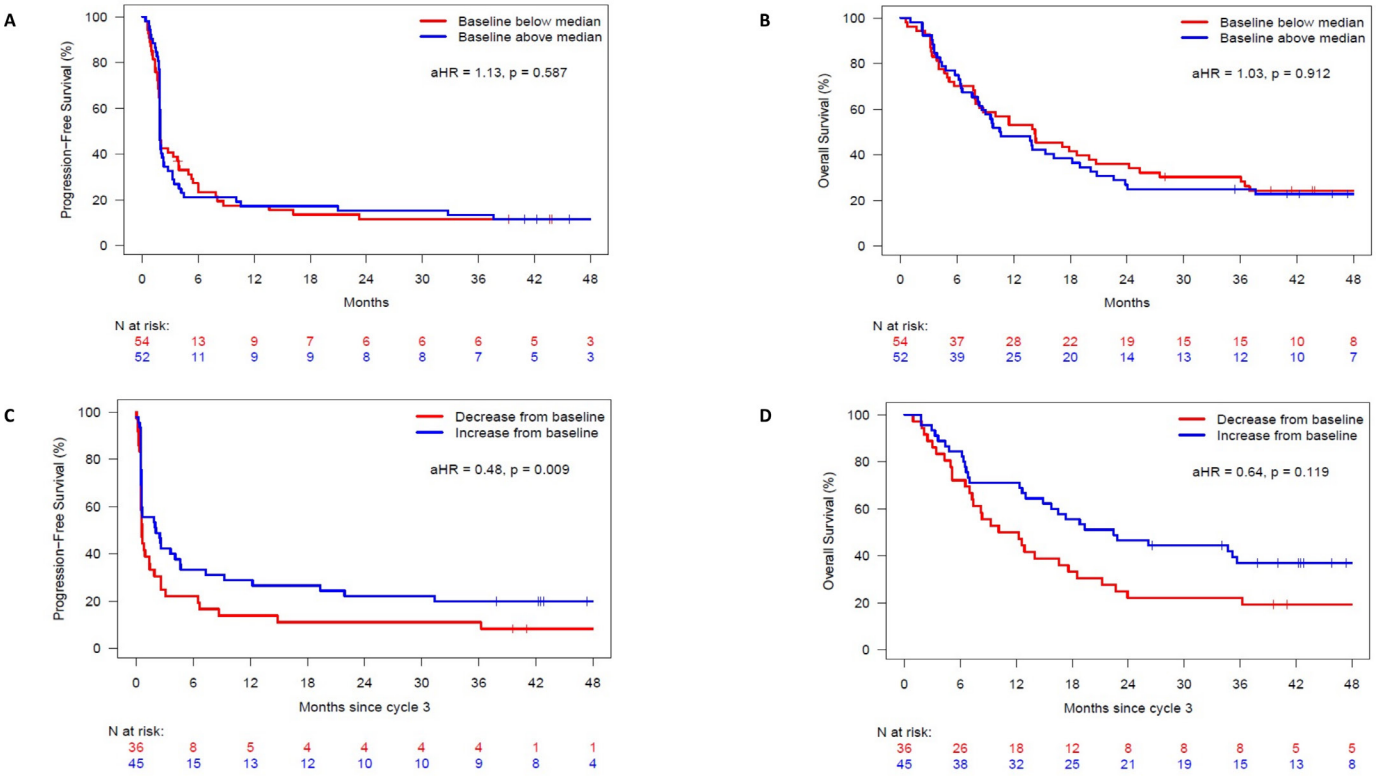

Figure 3 (A–D) Kaplan-Meier curves of PFS (A) and OS (B) in the entire cohort (n=106) stratified at the median choline value. Kaplan-Meier curves of PFS (C) and OS (D) among patients with both baseline and cycle 3 choline values (n=81) stratified according to increase vs decrease since baseline. OS, overall survival; PFS, progression-free survival.

(online supplemental figure 3A). These results suggest that changes in choline observed in the current study are independent from the fluctuations in white blood cell counts during treatment.

Gene expression analyses

Genes known to be associated with choline and ACh-related pathways were identified and grouped according to metabolic function, RECIST V.1.1 response rates, and blue (patients with a decrease in ctDNA and an increase in serum choline) and green groups (patients with a decrease in ctDNA and a decrease in choline), however, no significant differences were detected in gene expression patterns between baseline and on-treatment tumor samples in the 14 patients with available tissue for analysis (online supplemental figures 4,5). One of the selected genes of interest involved in ACh production, transport and signaling, Cholinergic Receptor Muscarinic 3 (*CHRM3*), was found to be significantly upregulated in

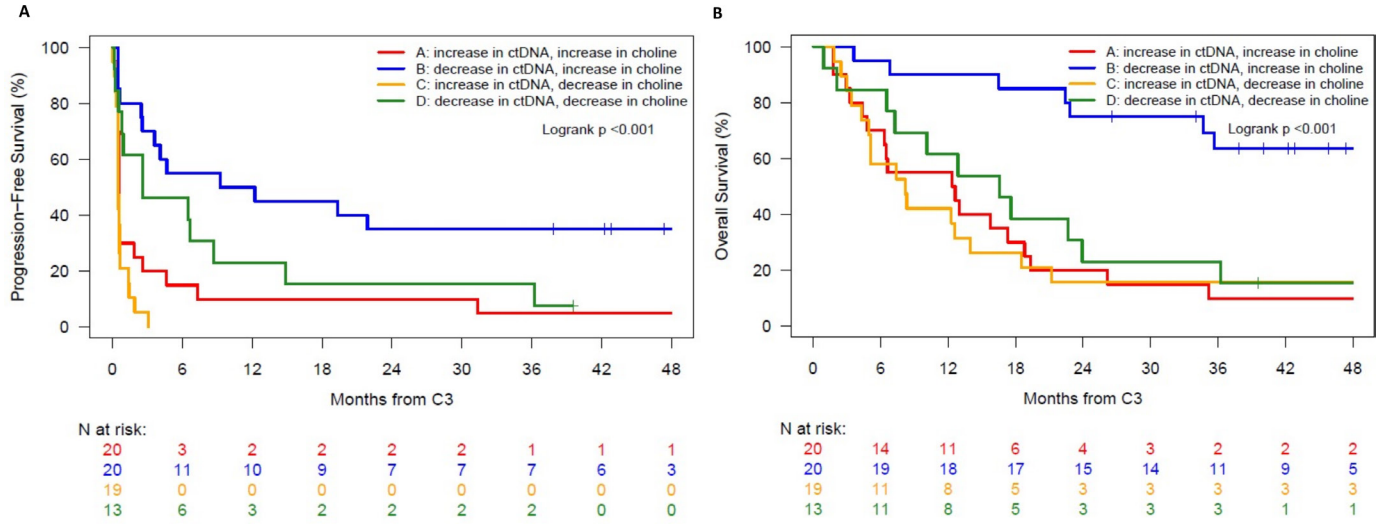

Figure 4 Kaplan-Meier curves of PFS (A) and OS (B) stratified by $\Delta ctDNA_{C3}$ (increase vs decrease) and $\Delta choline_{C3}$ (increase vs decrease). ctDNA, circulating tumor DNA; OS, overall survival; PFS, progression-free survival.

Table 1 Multivariable Cox regression analysis of association between $\Delta choline_{C3}$ and progression-free survival, adjusting for cohort, PD-L1, TMB and $\Delta ctDNA_{C3}$

Covariate	HR (95% CI)	P value	Global p value
$\Delta choline_{C3}$			**0.001**
Decrease from baseline	Reference		
Increase from baseline	0.35 (0.20 to 0.64)		
Cohort			**0.001**
A	Reference		
B	4.64 (1.74 to 12.37)	**0.0022**	
C	2.99 (1.13 to 7.86)	**0.027**	
D	0.74 (0.22 to 2.47)	0.63	
E	0.9 (0.41 to 1.95)	0.78	
PD-L1 (per percentage point)	0.99 (0.98 to 1)		0.17
log TMB	0.81 (0.59 to 1.12)		0.21
$\Delta ctDNA_{C3}$			**0.001**
Increase from baseline	Reference		
Decrease from baseline	0.33 (0.18 to 0.59)		

ctDNA, Circulating tumor DNA; PD-L1, Program Death-Ligand 1; TMB, tumor mutation burden.

patients with decreased choline and decreased ctDNA (green group) after anti-PD-1 treatment. In this group, the increase in *CHRM3* from baseline to week 7 was statistically significant (p=0.01) (online supplemental figure 5).

Immunoprofiling

T cell inference comparisons were also performed. There were no statistically significant differences in the quantities of T cell populations from RNAseq data in tumor biopsies between patients from blue (patients with a decrease in ctDNA and an increase in serum choline) and green (patients with a decrease in ctDNA and a decrease in choline) sample groups (online supplemental figure 6). Flow cytometry analyses revealed no significant differences in CD3 or CD8 cells between the blue and green groups.

We performed FL-IHC on baseline (n=23) and on-treatment (n=9) biopsies from blue and green group patients in order to further evaluate the immune infiltration in the TME (figure 5). Scoring of immune cell subsets in tumor and stromal compartments showed the presence of CD8 +T cells (CD3 +CD8+), CD8- T cells (CD3 +CD8-), which could be CD4+, double-negative or γδ-T cells, potential FOXP3 +regulatoryT cells (Tregs; CD3 +CD8-FOXP3+), CD20 +B cells, and CD68 +macrophages. At baseline, biopsies from blue group patients had significantly more CD8- T cells and B cells in the stroma in comparison to the tumor (p≤0.05; paired t-test). We did not observe any other significant differences in the density of immune subsets between stroma and tumor compartments, and between blue and green groups at baseline or on-treatment. We calculated the ratio of CD3 +CD8+T cells to CD3 +CD8-FOXP3+T cells (potential Tregs) and saw that the blue group had a significantly higher ratio in the tumor compartment in comparison to the green group at baseline (p=0.0481) (figure 5A). The difference in the ratio of CD3 +CD8+T cells to CD3 +CD8-FOXP3+T cells was not significant in the stroma (p=0.0963), and we also did not observe any differences between groups in their on-treatment biopsies (figure 5B).

Independent validation cohort

From the LIBERATE cohort, a total of 102 samples from 51 patients were included in serum choline analyses. The median follow-up time was 11.7 months (range: 2.5–37.7 months). The median PFS was 1.9 months and median OS was 11.7 months. The objective response rate was 14%. Patient demographics and treatment characteristics are summarized in online supplemental table 6. Of note, only four patients were treated with an anti-PD-1 agent as monotherapy while the remainder were treated with immuno-oncology drug combinations.

Baseline serum choline levels did not correlate with PFS (HR 0.87, 95% CI 0.49 to 1.55, p=0.66) or OS (HR 0.81, 95% CI 0.42 to 1.58, p=0.54) (online supplemental figure 7). There were no significant associations between choline levels (either baseline levels or $\Delta choline_{C2}$ (defined as an increase or decrease from baseline at cycle 2)) and treatment response by RECIST 1.1 in terms of objective response or clinical benefit (not shown). However, a positive $\Delta choline_{C2}$ was associated with a trend to an improved PFS (HR 0.73, 95% CI 0.41 to 1.32, p=0.3) compared with negative $\Delta choline_{C2}$ (figure 6). A non-statistically significant trend toward a better OS (HR 0.78, 95% CI 0.40 to 1.51, p=0.44) was also observed (figure 6). As in INSPIRE, no differences in ΔANC, Δlymphocytes and ΔNLR between increase and decrease in $\Delta Choline_{C2}$ were seen (online supplemental figure 3B).

DISCUSSION

While the introduction of ICIs has been an important addition to the treatment armamentarium in various advanced malignancies, novel strategies to augment the immune response and enhance T cell trafficking into the TME are needed. Recently the study of blood metabolite composition, or metabolomics, has become a powerful tool in measuring both global and dynamic changes in metabolic responses in several medical diseases including cancer.[27–31] There is now increasing interest in cancer metabolism, particularly choline metabolism, which is emerging as a metabolic hallmark of cancer.[32 33] To our knowledge, this is the first exploratory report of serum choline levels in pan-cancer patients

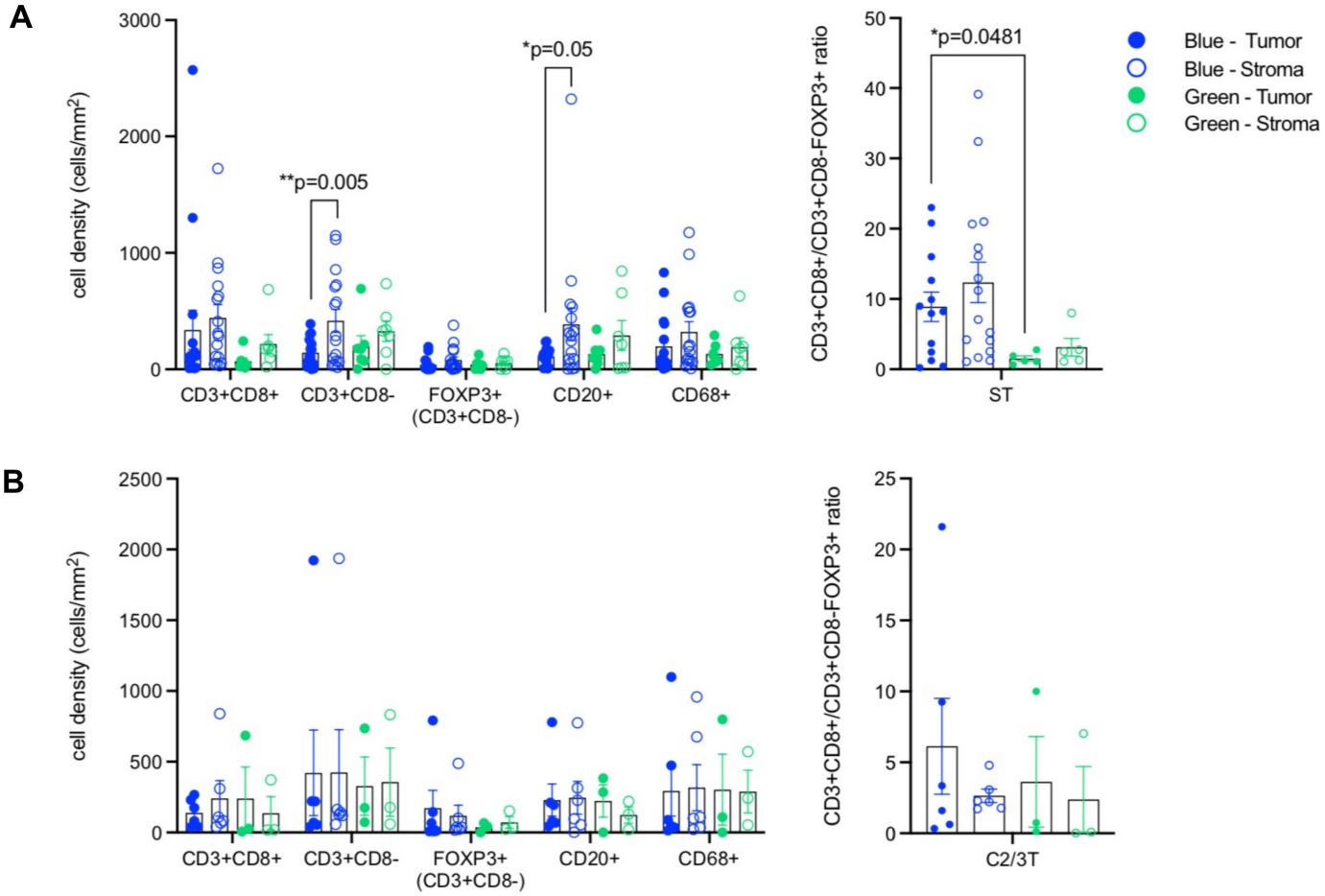

Figure 5 Multispectral fluorescent immunohistochemistry (FL-IHC) analysis of patient biopsies. (A) Baseline (n=23) and (B) on-treatment (n=9) biopsies from blue (patients with a decrease in ctDNA and an increase in serum choline) and green (patients with a decrease in ctDNA and a decrease in choline) sample groups were stained with a panel composed of pan-cytokeratin (or melanoma cocktail and SOX10 for melanoma tissues), CD3, CD8, FOXP3, CD20, and CD68. Tumor and stroma were identified by a study pathologist. Single-cell segmentation was performed and the indicated immune subsets were quantified in tumor and stroma using InForm Software. The ratio of CD3 +CD8+T cells to CD3 +CD8-FOXP3+T cells (Tregs) was also determined in tumor and stroma. ST, screening tissue; C2/3T, cycle 2/3 tissue. *P≤0.05, **p≤0.01, paired Student's t-test. ctDNA, circulating tumor DNA.

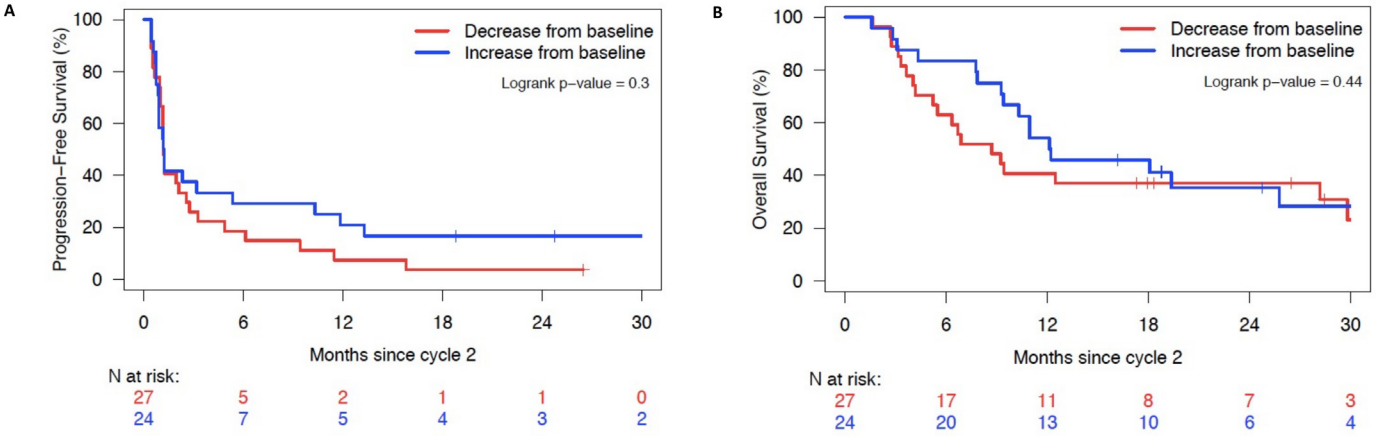

Figure 6 Kaplan-Meier curves of PFS (A) and OS (B) in patients with both baseline and cycle 2 choline values treated with at least an anti-PD-1 agent within early phase trials stratified according to increase versus decrease since baseline (n=51). OS, overall survival; PFS, progression-free survival.

receiving pembrolizumab. In this study, we observed that an increase in choline levels was significantly associated with an improved PFS and a trend toward an improved OS, and the combination of $\Delta ctDNA_{C3}$ and $\Delta choline_{C3}$ was prognostic for both OS and PFS. Further, $\Delta choline_{C3}$ was an independent prognostic factor for PFS when including $\Delta ctDNA_{C3}$, cohort, PD-L1 and TMB in a multivariable analysis. Lastly, IHC analyses demonstrated significantly more CD8- T cells and B cells at baseline in the stroma when compared with tumor in patients with a decrease in ctDNA and an increase in serum choline. Additionally the ratio of CD3 +CD8+T cells to CD3 +CD8-FOXP3+T cells (potential Tregs) was significantly higher in baseline tumors of ctDNA-decreasing ($\Delta ctDNA_{C3}<0$) patients with increase serum choline compared with patients with serum choline decrease. These findings suggest a potential role for choline function and metabolism in altering vascular characteristics, possibly modifying the immune response.

Choline is best known for its role in the nervous system, where it is a precursor for the synthesis of the neurotransmitter ACh. More recently, it has been established that the non-neuronal cholinergic system and ACh play important roles in regulating the immune response, particularly vasodilation and modulation of immune cell function.[7 11] Altered vascular characteristics can have profound effects on the TME causing distorted expression of adhesion molecules and disruption of the chemokine and cytokine milieu, which may adversely impact T cell homing and migration.[34] Increased understanding of this intricate and complex circuitry may allow for a heightened T cell migratory capacity to sites of tissue injury. The role of choline function and metabolism, and its effect on the tumor vasculature and immune response, has emerged as an area of interest.

The current study provides further support that cholinergic activity may have prognostic implications, although the exact mechanisms remain unclear. Previous work by Wang *et al* analyzed metabolomic characteristics, including changes in choline levels, in patients with acute myeloid leukemia.[35] In these patients lower levels of choline were observed when compared with healthy controls, and the authors suggest this is the result of consumption of choline and its by-products during leukemic cell proliferation. This metabolic trend has also been observed in patients with hepatocellular carcinoma compared with cancer-free subjects.[36 37] The current study offers further support to this hypothesis, with improved survival observed in those patients with a decrease in ctDNA as well as an increase in serum choline. The increase in serum choline may be due to reduced choline metabolism by proliferating cancer cells, possibly reflecting efficacy of anti-PD-1 treatment and a reduction in tumor burden.

Exploratory subgroup analyses for patients demonstrating a decrease in ctDNA and an increase in serum choline (blue group), and patients with a decrease in ctDNA and a decrease in choline (green group), were performed in an effort to further elucidate the mechanisms of this response. IHC analyses reported significantly more CD8- T cells and B cells at baseline in patients in the blue group, specifically CD8- T cells (CD3+CD8-), which may represent CD4+ T cells. These findings were consistent with previous reports of CD4+ T cells being triggered to produce ACh in response to infection.[12 13] Further, preclinical studies have shown mice lacking CD4 +T cells succumbed to infection due to an inability to produce immune cell-derived ACh.[13] The increase in levels of CD8- T cells (potential CD4+ T cells) observed in the patients in the blue group suggests a similar cholinergic mechanism for regulating cellular migration into tissue in the cancer setting. In addition, the ratio of CD3+CD8+ T cells to CD3+CD8-FOXP3+ T cells (potential Tregs) was significantly higher in the blue group at baseline in comparison to the green group. Higher CD8+/Treg (or FOXP3) ratios have been associated with improved clinical outcomes, in non-immunotherapy settings, in several cancer types.[38–42]

Finally, enzymes associated with choline metabolism, such as choline kinase-α (CHKα), have been shown to be overexpressed in cancer and have been proposed as potential prognostic markers and therapeutic targets.[32] TCD-717, a CHKα inhibitor, has recently been evaluated in a first-in-human phase I clinical trial (NCT01215864). CHKα converts choline to phosphocholine and subsequently to PC which is the predominant phospholipid in mammalian cells.[43] The oncogenic role of CHKα has previously been described and its overexpression has been associated with tumor progression and metastasis.[16 20 44–46] Most recently, PD-L1 expression was shown to be inversely related to CHKα expression in human TNBC cells, with downregulation of CHKα expression resulting in increased PD-L1 expression.[20] Further, anti-PD-1 agents have previously been shown to reactivate cytotoxic T-cells and may contribute to the induction of PD-L1 expression in cancer cells.[47] There was no statistically significant downregulation of CHKα detected in the current study. However, paired baseline and on-treatment tumor samples were only available in 14 patients for the gene expression analysis, therefore, it is possible that our results are limited by the small number of available tissues.

Attempts to validate these results in an independent cohort of patients were made. In this cohort, we observed a trend toward a better PFS in patients with an increase in choline levels from baseline to cycle 2, however, it was not statistically significant. Patients in the validation cohort participated in early phase clinical trials and were treated with several different ICIs. With a small number of patients included in the validation cohort, power was limited to detect a statistically significant association with outcome. Both the INSPIRE cohort and the LIBERATE cohort are limited by their heterogeneity in terms of histology and prior therapies, and in the case of the LIBERATE cohort, there exist variations in treatment (anti-PD-1 monotherapy and combinations). In both cohorts, there was no significant correlation between changes observed in

choline levels with changes in lymphocyte or neutrophil counts, or in NLR, thus refuting the possibility that these immune cell populations were confounders as potential sources of choline and ACh. To validate our findings, additional correlative analysis of larger clinical datasets of patients treated with ICI would be informative. In addition, in vivo evaluations such as the study of choline-deficient mice, or the addition of choline to syngeneic models treated with ICI, may shed further mechanistic insights into the role of the choline pathway in immune modulation.

In summary, the current study provides additional evidence to support a relationship between the cholinergic system in the regulation of antitumor immunity, and its role as a biomarker in patients with cancer receiving immunotherapy agents warrants further investigation. Increased understanding of the intricate communication network that directs T cells into the TME and elimination of potential inhibitory factors may facilitate T cell migration into tumor tissue and augment the antitumor response. Novel combination strategies exploiting the effects of the choline metabolic pathway on both T cells and the tumor microvasculature may reduce pre-existing barriers in the TME that cannot be overcome with single agent ICI alone, and ultimately improve treatment outcomes in patients with cancer.

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