A Role for Tryptophan-2,3-dioxygenase in CD8 T-cell Suppression and Evidence of Tryptophan Catabolism in Breast Cancer Patient Plasma

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Abstract

Tryptophan catabolism is an attractive target for reducing tumor progression and improving antitumor immunity in multiple cancers. Tumor infiltration by CD8 T cells correlates with improved prognosis in triple-negative breast cancer (TNBC) and a significant effort is underway to improve CD8 T-cell antitumor activity. In this study, primary human immune cells were isolated from the peripheral blood of patients and used to demonstrate that the tryptophan catabolite kynurenine induces CD8 T-cell death. Furthermore, it is demonstrated that anchorage-independent TNBC utilizes the tryptophan-catabolizing enzyme tryptophan 2,3-dioxogenase (TDO) to inhibit CD8 T-cell viability. Publicly available data revealed that high TDO2, the gene encoding TDO, correlates with poor breast cancer clinical outcomes, including overall survival and distant metastasis-free survival, while expression of the gene encoding the more commonly studied tryptophan-catabolizing enzyme, IDO1, did not. Metabolomic analysis, using quantitative mass spectrometry, of tryptophan and its catabolites, including kynurenine, in the plasma from presurgical breast cancer patients (n = 77) and 40 cancer-free donors (n = 40) indicated a strong correlation between substrate and catabolite in both groups. Interestingly, both tryptophan and kynurenine were lower in the plasma from patients with breast cancer compared with controls, particularly in women with estrogen receptor (ER)-negative and stage III and IV breast cancer.

Implications: This study underscores the importance of tryptophan catabolism, particularly in aggressive disease, and suggests that future pharmacologic efforts should focus on developing drugs that target both TDO and IDO1.

Introduction

Triple-negative breast cancer (TNBC) is an aggressive disease that lacks FDA-approved targeted therapies for the majority of patients (1). Importantly, TNBCs with high levels of immune infiltrate have more favorable prognoses (2, 3). Specifically, a high ratio of tumor-infiltrating cytotoxic CD8 T cells to immunosuppressive FoxP3+ T regulatory cells correlates with improved breast cancer-specific survival in chemotherapy-resistant TNBC (4). Indeed, TNBC has high expression of programmed cell death 1 ligand 1 (PD-L1) compared with other breast cancer subtypes, suggestive of evasion from immune attack (5–7). Active clinical trials are investigating the efficacy of targeting the PD-1/PD-L1 axis and other immune-directed interventions as treatments for TNBC; however, results in patients with TNBC treated with the anti-PD-1 agent pembrolizumab indicate promising but limited efficacy (8, 9).

We previously discovered that aberrant expression and activity of the hepatic tryptophan-catabolizing enzyme tryptophan-2,3-dioxygenase (TDO) in TNBC cell lines supports anchorage-independent cell growth and late-stage metastasis through an autocrine mechanism, whereby the tryptophan catabolite kynurenine activates the aryl-hydrocarbon receptor (AhR) in the breast cancer cells (10). However, it is clear that kynurenine also has suppressive paracrine effects on immune cells (11, 12). Most studies have focused on the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) in cancer, and current inhibitors of this pathway in clinical trials only target IDO activity. IDO transcript correlates with poor prognosis and increased microvessel density in breast cancer (13). However, TDO2 (the gene encoding TDO) is significantly higher in estrogen receptor (ER) negative (−) compared with ER+ breast tumors and correlates with overall survival in patients with breast cancer, suggesting a potential failure of current tryptophan-catabolizing agents in effectively targeting tumor cell enzymatic activity (10). Importantly, recent negative clinical trial results in melanoma (the ECHO-301 trial) and pancreatic cancer (ECHO-203) suggest that IDO inhibitors are not sufficient for the reversal of tumor immune evasion (14) and indicate that it may be necessary to target TDO as well.
Alterations in blood tryptophan and kynurenine levels have been examined in multiple human cancers (15–17). Lower serum tryptophan correlates with poor prognosis in melanoma, consistent with an increase in tryptophan catabolism (17). A small-scale study showed that plasma from postsurgical (lumpectomy or mastectomy) breast cancer patients has reduced tryptophan and elevated kynurenine compared with cancer-free controls, as measured by ultra-high pressure liquid chromatography (UHPLC), but importantly this study may not have been able to measure any potential effects of the primary tumor on systemic levels of these molecules (15). Another breast cancer study found relatively higher intratumoral kynurenine in ER− versus ER+ tumors (16).

The issue of whether decreased tryptophan or increased kynurenine levels in breast cancer patient plasma reflect the activity of tumor tryptophan-catabolizing enzymes remains unresolved. Here, we demonstrate that kynurenine increased primary human CD8 T-cell death and that conditioned media from anchorage-independent TNBC cells suppressed primary human CD8 T-cell function in a TDO-dependent manner, suggesting a mechanism of enhanced immune evasion during metastasis. Furthermore, we quantified tryptophan and kynurenine, as well as a series of tryptophan catabolites, in plasma from 77 presurgical breast cancer patients using UHPLC-MS and detected significant differences in plasma from women with breast cancer versus no breast cancer, ER− versus negative tumors, and with increasing disease stage.

Materials and Methods

Cell culture

BT349 cells, purchased from ATCC in 2008 and authenticated prior to use by short tandem repeat DNA profiling (Promega), were grown in RPMI1640 medium with 10% FBS, penicillin/streptomycin, and insulin supplementation. Suspension culture was achieved using plates coated with 12 mg/mL poly-(2-hydroxyethyl methacrylate) (Sigma-Aldrich) reconstituted in 95% ethanol. Ethanol evaporated overnight, and plates were washed with PBS prior to use.

Primary human T-cell isolation and activation

Blood was collected by venipuncture into tubes with heparin (BD Biosciences Vacutainer Systems) from donors with written informed consent under a University of Colorado Anschutz Medical Campus Colorado Institutional Review Board (COMIRB)-approved protocol. Lymphocytes were isolated by adding 10 mL of Ficoll-Paque PLUS (GE Healthcare) per 20 mL of blood diluted in Hank's balanced salt solution, then centrifuging at 2,000 rpm for 25 minutes without deceleration. CD8 T-cells were resuspended in PBS containing 0.5% BSA and 2 mmol/L EDTA for selection using positive isolation kits (Dynabeads, EMD Millipore). CD8+ T-cells were activated in RPMI containing 10% FBS, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and HEPES. T-cells were activated in plates coated with 0.5 μg/mL of CD3 antibody (clone OKT3, eBioscience) and 1.0 μg/mL of soluble CD28 antibody (clone CD28.2, eBioscience) for 5 days. For intracellular staining, T-cells were stimulated for 4 hours after the activation using phorbol-12-myristate-13-acetate (PMA) at 20 ng/mL, ionomycin at 1 μg/mL, and GolgiStop (BD Biosciences).

Cell staining and flow cytometry

T cells were stained with antibodies for CD3 (clone HIT3a, BioLegend) and CD8 (clone RPA-T8, BioLegend) at a 1:100 dilution and an intracellular stain for IFNγ (clone 4S.B8, eBioscience) at a 1:50 dilution. Intracellular staining was achieved using an intracellular fixation and permeabilization buffer set (eBioscience). Dead cells were detected by positivity for a viability dye according to manufacturer’s instructions (Fixable Viability Dye BV421, eBioscience). Flow cytometry was conducted using an LSR II (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Inc.).

TDO2 and AhR inhibition

The TDO2 inhibitor 680C91 (Sigma-Aldrich) or the AhR antagonist CH-223191 (Tocris Bioscience) prepared in dimethyl sulfoxide (DMSO) were used.

Breast cancer patient plasma

Blood was acquired as described above from presurgical breast cancer patients with written informed consent under a COMIRB- and HRPO-approved protocol (COMIRB 15-2225 and HRPO A-18613). The characteristics of the cohort are described in Supplemental Table S1.

UHPLC-MS–targeted metabolic analysis of tryptophan catabolism

Tryptophan ([15N2]) and kynurenine (ring-D6, 3,3-D2) were purchased from Cambridge Isotope Laboratories, Incorporated. To measure linearity over 5 orders of magnitude, lysis buffer (LB), a 5:3:2 ratio of MeOH/acetonitrile:H2O, and 0.1% formic acid aliquots were each spiked with varying ranges of heavy-labeled tryptophan and kynurenine (50 μmol/L, 5 μmol/L, 500 nmol/L, 50 nmol/L, 5 nmol/L, 500 pmol/L) and analyzed by ultra-high pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS). Plasma (20 μL) was extracted at 1:5 and 1:10 in LB containing 5 μmol/L heavy-labeled tryptophan and kynurenine as described previously, then diluted 1:5, 1:10 and 1:20 with H2O and run on UHPLC-MS (18, 19). Sample extracts were analyzed via UHPLC-MS (Vanquish, Q Exactive – Thermo Fisher Scientific) using C18 reverse-phase chromatography and positive electrospray ionization (ESI; refs. 18, 20). A Kinetex C18 column, 2.1x150 mm, 1.7 μm particle size (Phenomenex) was used and equipped with a C18 guard column (Phenomenex). The method is a variant of previously published methods (21). Samples were resolved at 45°C with a gradient elution over 4 minutes, flowing at 450 μL/minute. Mobile phase A is 0.1% formic acid in water, mobile phase B is 0.1% formic acid in acetonitrile. 5% B and 95% A is held from 0.00 to 0.50 minutes. From 0.50 to 1.10 minutes, B increases to 95% B and 5% A. This condition is held from 1.10 to 2.75 minutes. From 2.75 to 3.00 minutes A decreases to the initial condition of 5% B and 95% A and is held from 3.00 to 4.00 minutes. The Q Exactive mass spectrometer was operated in positive ion mode using electrospray ionization, scanning in Full MS mode (1 jscm) from 100 to 1,500 m/z at 70,000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas. Calibration was performed prior to analysis using the Pierce Positive Ion Calibration Solution (Thermo Fisher Scientific). Metabolite assignments and heavy isotopeologue detection for absolute quantitation against internal standards were determined against in-house standard libraries and KEGG database searches through Maven (22). Technical reproducibility was assessed by...
monitoring internal heavy-labeled standard mixes as reported (18). Calculation of absolute quantification for measured metabolites was performed using the following formula: [light] = (abundance light) / (abundance heavy) * [heavy] (dilution factor) where dilution factor is 10 for an extraction of 10 μL plasma in 90 μL lysis buffer.

Statistical analyses
Prism GraphPad Version 7.02 was used for all statistical analyses. All tests are two-sided with statistical significance set at P < 0.05. Statistical tests and sample sizes are described in the figure legends. *, P < 0.05; **, P < 0.01; †††, P < 0.001; ††††, P < 0.0001.

Results
Primary human CD8 T-cell response to kynurenine and cancer cell conditioned media
Infiltration into breast tumors by cytotoxic CD8 T cells correlates with improved prognosis in ER+ breast cancer (4, 23–25). To test the effect of kynurenine on primary human peripheral blood cytotoxic T cells, CD8+ cells isolated from peripheral blood of healthy human donors (Fig. 1A) were treated with increasing doses of kynurenine over a 5-day T-cell receptor (TCR) activation protocol. At 50 and 100 μmol/L kynurenine, CD8 T-cell death increased as measured by a fixable viability dye (Fig. 1B). While CFSE analysis of live CD8 T cells indicated no change in proliferation (Fig. 1C), demonstrating a direct impact of kynurenine specifically on human CD8 T-cell viability. To test whether kynurenine acts through AhR to affect CD8 T-cell viability, CD8 T cells were treated with increasing doses of kynurenine with or without the AhR antagonist CH-223191 (26). Treatment with CH-223191 significantly reduced the cytotoxic effect of 50 μmol/L kynurenine (Fig. 1D and E), indicating that kynurenine acts in part on CD8 T cells through AhR.

We previously reported that TNBC cells surviving under anchorage-independent culture conditions increased expression and activity of TDO, resulting in increased intracellular and secreted kynurenine (10). In the present study, we asked whether such anchorage-independent cells have enhanced immune evasion. BT549 TNBC cells were cultured in attached (ATT) or suspended (SUS) conditions for 24 or 48 hours. Primary human CD8 T cells were cultured in these conditioned media (CM) with CD3 and CD28 antibodies for 5 days. CD8 T cells cultured in SUS CM were less viable than those cultured in ATT CM (Fig. 2A). Because kynurenine decreased IFNγ mRNA in murine cytotoxic T cells (27), we tested whether CM from attached versus suspended TNBC cells would affect human primary CD8 T-cell production of IFNγ. Indeed, the SUS CM significantly reduced IFNγ production compared with ATT CM (Fig. 2B and C). To determine whether the effects of SUS CM were due to increased TDO activity, TNBC cells were plated in ATT or SUS conditions with or without TDO-specific inhibitor 680C91 for 48 hours. While SUS CM again increased CD8 T-cell death, this effect was significantly abrogated when SUS cells were treated with TDO inhibitor 680C91 (Fig. 2D), suggesting that TDO activity is at least partially responsible for the CM effect. However, treating the CD8 T cells with the AhR antagonist CH-223191 did not rescue the effect of SUS CM, suggesting additional effects of TNBC TDO activity on the CD8 T cells (Fig. 2E).

TDO2 RNA expression correlates with breast cancer outcomes, and tryptophan catabolism is altered in breast cancer
We previously reported that TDO2 is significantly higher in breast cancer versus normal breast tissue and in ER+ versus ER- disease, and that above-median TDO2 correlated with poor overall survival in the Curtis and colleagues dataset (a dataset containing gene expression analysis from 2,000 breast tumors, extracted through the data-mining platform Oncomine; refs. 10, 28). In this study, we used the KM Plotter tool to investigate breast cancer outcomes associated with both TDO2 and IDO1 expression (29). High TDO2 in primary breast cancer correlated with worse overall survival and reduced distant metastasis-free survival (Fig. 3A), while IDO1 did not (Fig. 3B). Consistent with our findings that breast cancer cells have elevated tryptophan catabolism during anchorage independence, we also found, using the Curtis and colleagues dataset (28), that the gene encoding the amino acid transporter LAT1 (SLC5A) (which uptakes tryptophan and other large neutral amino acids from the microenvironment into the cell) was significantly elevated in breast cancer versus normal breast (Supplementary Fig. S1A), in ER+ versus ER- disease (Supplementary Fig. S1B), and in higher grade breast cancer (Supplementary Fig. S1C). We also found using the Curtis and colleagues dataset and KM Plotter tool that above-median LAT1 was associated with worse overall (Supplementary Fig. S1D and S1E) as well as distant metastasis-free (Supplementary Fig. S1F) survival as compared with below-median.

To further understand how tryptophan catabolism is altered in breast cancer subtypes, we measured plasma concentrations of tryptophan and its main catabolites, including kynurenine using UHPLC-MS (Supplementary Table S1). In the plasma from cancer-free “normal” women, the concentration of tryptophan ranged from 69.50 to 294.49 μmol/L, while in women with breast cancer, the range was 38.04–260.77 μmol/L. Meanwhile, the plasma concentration of kynurenine in normal women ranged from 3.46 to 13.61 μmol/L, and from 2.19 to 19.83 μmol/L in women with breast cancer. Strikingly, there was a significant positive correlation between tryptophan and kynurenine both in plasma from normal women (Fig. 4A and B) and women with breast cancer (Fig. 4B). A comparison of the plasma concentrations of tryptophan and kynurenine in women with breast cancer versus normal controls demonstrated that women with breast cancer had significantly reduced tryptophan (mean = 141.39 μmol/L) compared with normal (mean = 173.32 μmol/L; Fig. 4C). Interestingly and surprisingly, the concentration of kynurenine was also significantly lower in the plasma of women with breast cancer (mean = 7.12 μmol/L) compared with normal controls (mean = 9.02 μmol/L; Fig. 4D).

Given previous reports that TDO2 is significantly higher in ER- versus ER+ disease, the plasma tryptophan and kynurenine concentrations in women with ER+ and ER- breast cancer were compared next (10). There was no significant difference in either plasma tryptophan or kynurenine in women with ER+ and ER- disease (Fig. 4C and D), though lower levels of tryptophan and anthranilate were observed in plasma from women with ER+, HER2+ disease (Supplementary Fig. S2). However, patients with ER+ breast cancer had significantly reduced plasma tryptophan compared with normal controls, while patients with ER- breast cancer did not (Fig. 4C). Plasma kynurenine from both ER+ and ER- breast cancer subsets was significantly lower than that from normal controls (Fig. 4D). Of note, these decreases were more marked in women with nonpregnancy-associated breast cancer.
Figure 1.
Increased kynurenine leads to primary human CD8 T-cell death, which is reversed by an AhR antagonist. A, Representative example of gating strategy for flow cytometric analysis: CD8 T cells were gated on the basis of double positivity for CD8 (PE-Cy7) and CD3 (PerCP-Cy5.5). Cell death was determined by positive staining for the fixable viability stain BV421. Proliferation was measured by CFSE dilution. B, Cells were activated with CD3 and CD28 antibodies for 5 days in the indicated concentration of kynurenine and CD8 T-cell death was determined. Each dot represents cells from one human donor. N = 9 donors, mean with SD, one-way ANOVA. C, Cells were treated with kynurenine as described in B and assayed for CD8 T-cell proliferation. N = 9 donors, one-way ANOVA. D, Representative images (10× magnification) of CD8 T cells on activation day 5 that were treated with indicated concentrations of kynurenine and either 10 μmol/L of the AhR antagonist CH-223191 (AhRa) or DMSO. E, CD8 T cells were activated, treated with 10 μmol/L AhRa or vehicle control (DMSO), and assayed for cell death. N = 6, mean with SD, paired t tests.
(PABC) in comparison with patients with PABC (where PABC is defined as a diagnosis with 5 years of pregnancy), with the highest relative levels of tryptophan (and its catabolites hydroxytryptophan, kynurenine, and kynurenic acid) detected in normal women who did not give birth within the past 5 years. Interestingly, women without a cancer diagnosis (normal), who had not given birth within the past 5 years, had intermediate levels of tryptophan and its catabolites in between the levels observed in normal women who had given birth within the past five years and PABC/non-PABC patients (Supplementary Fig. S3).

To examine whether either plasma tryptophan or kynurenine correlated with breast cancer aggressiveness, breast cancer stage at diagnosis was used. Both tryptophan (Fig. 4E) and kynurenine (Fig. 4F) were significantly lower in stage III/IV breast cancer patient plasma than in plasma from normal controls, while there was no difference in either tryptophan or kynurenine when comparing stage 0/I patients versus normal (Fig. 4E and F).

Interestingly, there was also a decrease in the kynurenine catabolite kynurenic acid in patients with stage III/IV breast cancer compared with patients with stage I/II disease (Supplementary Fig. S4).

To investigate whether either plasma tryptophan or kynurenine were reduced in the plasma of patients with breast cancer versus normal controls, led us to investigate whether there were differences in nicotinamide, the final metabolite in the kynurenine pathway of tryptophan catabolism, between breast cancer patient and normal plasma. To do this, we calculated the ratio of tryptophan/nicotinamide in our dataset. We found that the tryptophan/nicotinamide ratio was significantly lower in the plasma from patients with breast cancer compared with normal controls, suggesting a potential overall increase in tryptophan catabolism not limited to the conversion of tryptophan to kynurenine (Fig. 4G). While the average relative abundance of nicotinamide was slightly elevated in breast cancer patient plasma compared

Figure 2.

The effect of conditioned media from suspended TNBC cells on primary human CD8 T cells is similar to that of purified kynurenine, and is reversed by TDO2 inhibitor 680C91. A, Conditioned media was collected from BT549 TNBC cells grown in attached (ATT) or suspended (SUS) culture conditions for either 24 or 48 hours as indicated. CD8 T cells were isolated from the blood of normal donors and activated for 5 days with CD3 and CD28 antibodies in the conditioned media. Each line represents the response of CD8 T cells from one donor. Cell death was measured as described in Fig. 1A and B. N = 9, paired t tests. B, Representative flow cytometric analysis of CD8 T cells for IFNγ production is shown. C, IFNγ production by CD8 T cells cultured in conditioned media for 48 hours. N = 7, paired t tests. D, CD8 T cells were activated in conditioned media and either 0.1 μmol/L 680C91 (to inhibit TDO activity) or vehicle control (DMSO). Each line represents the response of CD8 T cells from one human volunteer donor. N = 5, paired t tests. E, CD8 T cells were activated in conditioned media and treated with either 10 μmol/L CH-223191 (to inhibit AhR activation) or vehicle control (DMSO). N = 5, paired t tests.
with controls, this difference was not statistically significant (Fig. 4H). However, the ratio, which represents each individual's substrate (tryptophan) versus metabolite, is likely more meaningful given the person-to-person variability.

To examine whether tryptophan catabolism correlated with outcomes in this breast cancer dataset, we divided the patient data into tryptophan-low and tryptophan-high groups based on the median concentration of plasma tryptophan, and compared the progression-free survival of these groups, where progression was defined as local, regional, or distant recurrence, or patient death. While there was no significant difference in progression-free survival between these groups in patients with breast cancer with either ER$^+$ (Fig. 5A) or ER$^-$/C0 (Fig. 5B) disease, there was a trend that did not reach statistical significance toward patients with lower than median plasma tryptophan having poorer outcomes (Fig. 5B), consistent with a negative impact of reduced tryptophan in ER$^-$ disease.

**Discussion**

While tryptophan catabolism is known to mediate tumor immune evasion, the impact of IDO or TDO activity on CD8 T cells was thought to occur through indirect suppression via regulatory T-cell activation. We demonstrate here that the direct impact of kynurenine on primary human CD8 T cells, and that this effect is abrogated by the specific TDO inhibitor 680C91, indicating that anchorage-independent TNBC cells are highly immunosuppressive in part through TDO-mediated activity. A high ratio of cytotoxic CD8 T cells to Treg cells correlates with improved clinical outcomes in TNBC (30, 31). Thus, the power of TDO-positive metastatic TNBC cells to suppress CD8 T cells both directly and indirectly via the induction of Tregs (12) could critically contribute to disease progression. Interestingly, the concentration of kynurenine in the conditioned media was lower than the concentration of purified kynurenine that reduced CD8 T-cell viability, a phenomenon that could be explained by the milieu of other factors that are secreted by suspended TNBC cells that might have immunomodulatory effects (10).

Although inhibition of TDO activity in suspended TNBC cells abrogated the effects of conditioned media on the viability and function of CD8 cells, inhibition of the receptor for kynurenine, AhR, in the CD8 cells was not sufficient to mitigate this effect. One possible explanation for the differential effects of TDO as compared with AhR inhibition is that the effect of TDO activity on CD8 T cells is due to the combined effects of both tryptophan depletion and kynurenine secretion. Kynurenines combined with tryptophan depletion downregulate the TCR zeta-chain and decreases cytokine production in murine CD8 T cells (27), and IDO-mediated tryptophan depletion inhibits mTOR and PKC-$\delta$ activation to induce murine T-cell anergy and autophagy (32). AhR antagonism would interfere with kynurenine-mediated AhR activation, but would not abrogate tryptophan depletion.

To our knowledge, this study includes the largest metabolomic analysis of tryptophan and kynurenine in plasma from presurgery breast cancer patients, allowing assessment of the impact of an
Tryptophan and kynurenine are reduced in breast cancer plasma. UHPLC/MS was used to measure tryptophan and kynurenine concentrations and nicotinamide abundance in the plasma from normal women and women with breast cancer. The concentrations of tryptophan and kynurenine correlate significantly and positively in both normal (A, n = 40) and breast cancer patient (B, n = 77) plasma by Pearson and Spearman tests. Comparison of tryptophan (C) and kynurenine (D) concentrations in plasma from normal women, all breast cancer patients in the cohort, and ER⁺ versus ER⁻ breast cancer patients. Mean with SD, *, *P < 0.05; **, P < 0.01, one-way ANOVA and Tukey multiple comparisons test. Comparison of tryptophan (E) and kynurenine (F) concentrations plotted by breast cancer stage is shown. Mean with SD, one-way ANOVA and Tukey multiple comparisons test. G, Comparison of the ratio of tryptophan (TRP) to nicotinamide between plasma from normal women and women with breast cancer. Mean with SD, ****, P < 0.0001, unpaired t test. H, Comparison of the relative abundance of nicotinamide in the plasma from normal and breast cancer patient plasma. Mean with SD, P = 0.11, unpaired t test.
existing breast tumor on systemic tryptophan and kynurenine levels. Given that others have reported higher kynurenine and lower tryptophan levels in breast cancer patients compared with healthy donors (15), our finding of a strong maintenance of balanced levels of substrate to catabolite in plasma from both breast cancer patients and cancer-free donors was unexpected. This may be attributable to the strong ability of the kidneys to handle increased kynurenine and it is documented that increased TDO/IDO activity does not necessarily result in increased plasma kynurenine (33, 34). Interestingly, we found that compared with healthy donors, tryptophan was only significantly lower in the plasma of patients with ER− tumors. This finding suggests a particularly important role of elevated tumor tryptophan catabolism in ER− disease. An expanded follow-up metabolic study that includes more patients with ER− and stage III/IV disease is imperative to determine whether plasma tryptophan and kynurenine reflect tumor enzymatic activity and correlate with outcome. Although currently limited by lack of quality antibodies for IHC, it is likely that measurement of TDO/IDO protein levels or activity in primary tumor and/or circulating tumor cells would be more informative than plasma levels of tryptophan and kynurenine. In addition, levels of the enzymes in tumors should be correlated with tumor-infiltrating lymphocyte composition in future studies. It is possible that further flux through the kynurenine pathway in tumors could account for the comparatively low kynurenine concentration in plasma from women with breast cancer. In a previous study, we found that expression of KYN1, the gene encoding kynureninase (an enzyme downstream of TDO) is elevated in suspended TNBC cell lines and in the context of increased inflammatory signaling (10), which would be consistent with tryptophan catabolism further down the pathway. Indeed, our finding that the tryptophan/nicotinamide ratio was significantly reduced in breast cancer patient plasma compared with controls is consistent with the possibility of an overall increase in tryptophan catabolic activity not limited to the initial step in the pathway. Metabolomic tracing of tryptophan could help elucidate the ultimate fate of this amino acid in tumors.

Development of better antibodies to measure TDO and IDO or gauge activity will be a significant hurdle to overcome in the quest for biomarkers indicative of patients who may benefit from tryptophan catabolism–targeted therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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