Gynecologic Oncology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Immunobiochemical pathways of neopterin formation and tryptophan breakdown via indoleamine 2,3-dioxygenase correlate with circulating tumor cells in ovarian cancer patients– A study of the OVCAD consortium

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HIGHLIGHTS

- Neopterin and tryptophan breakdown are associated with ovarian cancer CTCs.
- Increased plasma levels are related with an unfavorable outcome.
- Higher levels are observed in patients with CTCs at baseline.
- After treatment, higher levels are observed in CTC-positive patients with platinum-sensitive disease.

ARTICLE INFO

Article history:
Received 13 October 2017
Received in revised form 23 February 2018
Accepted 26 February 2018
Available online xxxx

Keywords:
Neopterin
Indoleamine 2,3-dioxygenase
Circulating tumor cells
Ovarian cancer
Immune system

ABSTRACT

Objective. Circulating tumor cells (CTCs) may represent a chronic stimulus for the immune system. In the present study we investigated the potential association of CTCs, the immune activation marker neopterin, and the ratio of kynurenine to tryptophan (Kyn/Trp) as a measure for tryptophan breakdown.

Methods. Neopterin, tryptophan and kynurenine levels were measured in plasma samples from patients with benign gynecological diseases ($n = 65$) and with primary advanced epithelial ovarian cancer (EOC) at diagnosis ($n = 216$) and six months after adjuvant platinum-based chemotherapy ($n = 45$) by an enzyme-linked immunosorbent assay and high performance liquid chromatography. The presence of CTCs had been assessed in a previous study by qPCR-based analysis of CTC-related transcripts in the blood. The respective plasma levels in EOC and benign samples were compared using a two-tailed Chi^2 or Fisher's exact test. The associations of the analytes and Kyn/Trp with clinicopathological parameters, platinum-sensitivity, and the presence of CTC-related transcripts were assessed using a two-sided *t*-test. Associations with patient outcome were evaluated using Cox regression analysis.

Results. In EOC, elevated Kyn/Trp and neopterin levels were associated with advanced disease, peritoneal carcinomatosis, ascites, sub-optimal debulking, poor response to therapy and worse outcome. Likewise, neopterin and Kyn/Trp were elevated in CTC-positive patients, both at diagnosis and at follow-up in platinum-sensitive disease.

Conclusions. We observed concomitant alterations of CTCs and immune system related biomarkers suggesting that immune responses along with increase of neopterin and Kyn/Trp concentrations are not necessarily only located at the site of the tumor, but may also go on in the circulation.

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https://doi.org/10.1016/j.ygyno.2018.02.020
0090-8258/

1. Introduction

Circulating tumor cells (CTCs) are crucial components in the formation of distant metastasis [1]. The presence of CTCs in the blood is an independent poor prognostic indicator of progression-free and overall survival in patients with various forms of cancer like breast, lung, pancreatic, and ovarian cancer. Beyond their mere enumeration, further phenotyping and genotyping of CTCs provided insights into their predictive role for assessing therapy sensitivity or resistance [2,3]. In addition to providing diagnostic, prognostic and predictive clinical information, CTCs may also increase our understanding of tumor heterogeneity.

The shedding of CTCs into the vasculature or lymphatics is associated with inflammatory processes, and most presumably the combined action of inflammatory mediators and tumor antigens might underlay the peripheral stimulation of immunocompetent cells. Peripheral immunological conditions are crucially involved in the fate of CTCs. Tumor escape is promoted by mechanisms that protect from elimination by cellular arms of the immune system, and their functional impairment e.g. by secretion of cytokines [4]. There are accumulating studies that focus on the crosstalk of peripheral immune surveillance systems and CTC survival. Recently, Mego et al. showed elevated CTC numbers in patients with inflammatory breast cancer to be associated with defects in adaptive immunity [5,6]. In order to further elucidate the crosstalk of CTCs and the immune system it is necessary to correlate the presence of CTCs with biomarkers that are functionally linked to the underlying immunological conditions and that have already been shown to monitor disease progression.

The cellular immune activation marker neopterin is well established as a most sensitive parameter to reflect immune activation in cancer patients [7]. Already decades ago, increased neopterin concentrations have been demonstrated in body fluids like blood, urine or ascitic fluid of a significant percentage of patients suffering from various types of cancer [8]. Moreover, increased neopterin was found as a significant and independent predictor of unfavorable prognosis for a variety of malignancies [8,9]. Urinary neopterin concentrations were assessed in ovarian cancer patients before and after primary therapy and were inversely correlated with progression-free and overall survival, further emphasizing the clinical significance of this biomarker [10–12].

Moreover, a significantly increased tryptophan breakdown along the kynurenine axis has been observed in patients suffering from various types of cancer, resulting in increased kynurenine to tryptophan ratios (Kyn/Trp) in plasma or serum [13,14]. This increased catabolism of tryptophan is largely attributed to the inflammation-induced expression and activity of the tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO-1), which is mainly induced in monocytes and dendritic cells [14,15]. In addition, stimulated IDO-1 expression by the tumor itself may contribute to the accelerated breakdown of tryptophan [16,17]. The T helper cell type 1 (Th1)-type cytokine interferon-γ (IFN-γ) is the major stimulus for both neopterin production and tryptophan breakdown during cell-mediated immune response, as was demonstrated in cultivated peripheral blood mononuclear cells from healthy donors [14].

Systemic activation of the T cell/monocyte axis is continuously triggered by secreted cytokine mediators like interferons. This chronically exaggerated cancer-associated inflammation is an energy and nutrient demanding process, which becomes limiting also for the immune cells, thus in long-term rendering the tumor defense less effective. The loss of immunocompetent cells in the tumor microenvironment represents the end stage of an antitumor immune defense because activated T cells have already undergone apoptosis or have become anergic. Accelerated tryptophan catabolism via IDO-1 plays a major role in the induction of regulatory T cells (Treg) thereby promoting immune escape [18]. It is yet unknown to which extent CTCs present in the circulation contribute to an increase of neopterin and Kyn/Trp during malignant tumor disease. Available data of CTC interactions with the different immune cell compartments in the periphery are still poor, and it is yet unknown to which extent CTCs present in the circulation may contribute to an immune activation cascade. The assessment of neopterin and tryptophan metabolism provides broader information on the underlying immune status than measurements of specific cytokines or immune cell subsets during malignant tumor disease. Hence, we investigated the potential associations of CTCs with immune activation in ovarian cancer, as reflected by neopterin formation and tryptophan breakdown.

2. Material and methods

2.1. Patients

Patients with primary epithelial ovarian cancer (EOC) were recruited from 2005 to 2008 within the multi-centered OVCAD study (www.ovcad.eu), a 6th Framework Program Project (LSHC-CT-2005-018698) of the European Union. All included patients were diagnosed at advanced stage (FIGO stage II-IV, according to the International Federation of Gynecology and Obstetrics), and received standard treatment consisting of debulking surgery and platinum-based adjuvant chemotherapy [19]. Six months after completion of the adjuvant treatment, the response to first-line treatment was evaluated according to the WHO criteria: progression of disease was defined as an at least two-fold increase in the nadir serum CA-125 concentration according to the GCIG criteria [20] and by radiological/clinical confirmation. Patients were classified as being platinum-sensitive if no recurrence or disease progression was observed within six months after completion of first-line chemotherapy. For the present study, patients with non-malignant diseases of the female genital tract were included as control group. Written informed consent was obtained from all patients. The study protocol was approved by the local ethics committees (EK207/2003, ML2524, HEK190504, EK366, and EK260).

2.2. Blood samples

Twenty mL of peripheral blood was taken by venipuncture at primary diagnosis of the disease (prior to treatment; i.e. baseline samples). From the EOC patients, an additional blood sample was drawn six months after completion of the adjuvant platinum-based chemotherapy (i.e. follow-up samples). The blood was collected into EDTA tubes. A two-layer density gradient centrifugation was performed to obtain a cell fraction enriched for mononuclear cells, which possibly would contain CTCs and a plasma layer as described previously [21]. The enriched cells were lysed using 350 μL RLT buffer (Qiagen). Both lysates and aliquots taken from the plasma layer were stored at −80 °C until further analysis.

2.3. CTC analysis

The analysis of CTC-related transcripts was performed as described in detail in [21]. In short, the total RNA was extracted from the density gradient enriched cells (RNeasy Mini Kit, Qiagen, Hilden, Germany), reverse transcribed into cDNA (M-MLV RT, Promega, Mannheim, Germany) and analyzed for the presence of 11 CTC-related transcripts using TaqMan-based quantitative PCR. Peptidyl-prolyl isomerase C (cyclophilin C or PPIC) mRNA was one of the selected transcripts.

2.4. Determination of neopterin concentrations

Plasma neopterin was measured by a commercially available ELISA (BRAHMS Diagnostica, Berlin, Germany) with a sensitivity of 2 nmol/L neopterin and inter-assay coefficients of variation ranging from 4.7 to 8.5% [22].

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2.5. Determination of tryptophan and kynurenine

HPLC on reversed phase was applied for tryptophan and kynurenine measurements as described [23]. Briefly, plasma samples were diluted with potassium phosphate buffer containing L-nitro-tyrosine as internal standard. Protein was removed by precipitation with trichloroacetic acid. After injection of 100 μL, separation was performed on a reverse phase C18 column (Lichrocart, 5 μm, 55 × 4 mm, Merck, Darmstadt, Germany) using sodium acetate buffer (15 mmol/L, pH 4.0) with 3% acetonitrile and a flow rate of 0.9 mL/min at a temperature of 25 °C on a Varian ProStar liquid chromatography system equipped with autosampler Model 400 (Varian, Palo Alto, CA, USA). L-kynurenine was monitored by its UV-absorption at 360 nm (UV-detector SPD-6A, Shimadzu, Japan). Tryptophan was detected by its natural fluorescence at 285 nm excitation and 365 nm emission wavelengths (ProStar fluorescence-detector Model 360, Varian). Peaks were identified by comparing their retention time to those of an albumin-based calibrator containing 10 μmol/L L-kynurenine and 100 μmol/L L-tryptophan, which was prepared in the same manner as the plasma samples. To estimate the IDO-1 activity, the kynurenine to tryptophan ratios (Kyn/Trp) was calculated.

2.6. Statistics

Statistical analysis was performed by IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. The level of significance was set at $p < 0.05$. Neopterin, tryptophan, and kynurenine concentrations, as well as the Kyn/Trp ratio measured in the plasma samples from patients with EOC and benign diseases were compared to previously published reference values, which had been set at the 95th percentile of the kynurenine concentrations and of Kyn/Trp and at the 5th percentile of the tryptophan concentrations, respectively, measured in 100 healthy donors [24]. The differences in proportions of levels above that reference value between the EOC and the benign group were assessed with the two-tailed Chi^2 or Fisher's exact test where appropriate. The two-sided *t*-test was performed to assess the relationship between the lg(2)-transformed neopterin, tryptophan, and kynurenine concentrations, as well as the lg(2)-transformed Kyn/Trp ratio and the clinicopathological characteristics (including PPIC/CTC positivity) of the patients. Likewise, the one-way ANOVA analysis was performed to compare the lg(2)-transformed concentration of each analyte and FIGO stages. A paired *t*-test was used to compare the lg(2)-transformed concentrations in paired plasma samples taken at baseline and follow-up. The correlation between the lg(2) transformed concentrations of the respective analytes and the lg(10) transformed concentrations of the ovarian tumor marker CA-125 was assessed using the Pearson's correlation coefficient. Clinical endpoints were progression-free survival (PFS) and overall survival (OS). Both endpoints were defined as the time period between blood draw (baseline: prior to surgery, follow-up: six months after completion of adjuvant chemotherapy) and first recurrence or death due to any cause, respectively. Kaplan-Meier survival analyses and log-rank testing were used to compare survival outcomes of the "high" and "low" neopterin, kynurenine, tryptophan, and Kyn/Trp patient subgroups (stratified by the median concentration of each analyte in the EOC patient cohort). Cox proportional hazards regression was used to determine univariate and multiple hazards ratios for PFS and OS. Both in Kaplan-Meier and Cox regression analyses, patients who died shortly after primary surgery or during primary adjuvant chemotherapy were excluded. Additionally, patients whose date of recurrence had not been documented were excluded for PFS. Covariates included in the multiple Cox regression analysis were patient age, FIGO stage, residual tumor mass after surgery, peritoneal carcinomatosis, ascites, and neopterin, kynurenine, tryptophan, and Kyn/Trp ("high" vs. "low") as categorical variables. The model was built using a forward stepwise method by entering all variables at a *p*-value of <0.05 and removing them at a *p*-value of >0.10.

3. Results

3.1. Patient characteristics

After several analyses using plasma samples from the OVCAD study cohort ($n = 275$) had already been undertaken, plasma samples were available from a total of 233 patients for the present study. The majority of the samples were taken at diagnosis (216 patients). Follow-up plasma samples taken six months after completion of the primary adjuvant treatment were available in just a few cases (45 patients), and for 28 of these patients a baseline blood sample taken at diagnosis was available as well (paired samples). The 233 patients (216 with baseline or paired samples, plus 17 with a follow-up sample only) were primarily diagnosed with FIGO stage IIIC (72.1%) and IV (17.2%) disease. The most prevalent histological tumor type was serous epithelial cancer (87.6%). Six months after completion of adjuvant treatment, 77.7% of the patients were classified as platinum-sensitive. The median follow-up time was 71 months. Plasma samples from 65 patients with non-malignant diseases (61.5% ovarian cystadenoma) of the female reproductive tract were included as benign controls. The EOC patients were significantly older than the control group (median 58.0 years, IQR 50.0–67.0 years vs. median 48.2 years, IQR 44.3–63.9 years, $p = 0.002$). An overview of the clinicopathological characteristics of the benign control group and the OVCAD study cohort is given in Table 1.

3.2. Neopterin, tryptophan and kynurenine in EOC and benign plasma

At primary diagnosis, the baseline plasma concentration of neopterin, but not of kynurenine was significantly higher in the EOC samples than in the benign control group. Tryptophan concentrations were significantly decreased, and likewise, the Kyn/Trp ratio was significantly higher in the cancer patients (Fig. 1). Comparing the levels of the respective analytes to the previously published reference values established in 100 healthy individuals [24] showed that neopterin and kynurenine levels above the reference value of 9.10 nmol/L and 2.48 μmol/L, respectively, as well as an elevated Kyn/Trp ratio higher than 35.1 μmol/mmol occurred more often in the malignant group than in the benign control group (neopterin: 47.7% vs. 29.2%, $p = 0.008$; kynurenine: 18.1% vs. 4.6%, $p = 0.008$; Kyn/Trp: 84.7% vs. 23.1%, $p < 0.001$). Accordingly, tryptophan levels below the reference value of 53.1 μmol/L occurred more often in cancer patients (94.4% vs. 70.8%, $p < 0.001$).

In the EOC group, higher levels of neopterin at baseline and Kyn/Trp were significantly associated with older age, more advanced disease stage, with the presence of ascitic fluid and peritoneal carcinomatosis, and with residual disease after surgery (Table 2, Suppl. Table 2). Baseline neopterin and Kyn/Trp levels were only weakly correlated with CA-125 concentrations (neopterin: Pearson's $R = 0.194$, $p = 0.005$; Kyn/Trp: $R = 0.254$, $p < 0.001$).

3.3. Association of neopterin, tryptophan and kynurenine with response to treatment

At follow-up, significantly higher neopterin levels and Kyn/Trp ratios were observed in the patients who had been diagnosed at an advanced stage and with peritoneal carcinomatosis, and who had not been optimally debulked (Table 2, Suppl. Table 3). We observed a moderate and strong correlation between neopterin levels and Kyn/Trp ratios and CA-125, respectively (neopterin: Pearson's $R = 0.417$, $p = 0.004$; Kyn/Trp: $R = 0.713$, $p < 0.001$). Additionally, we observed similar plasma levels in patients with benign diseases and platinum-sensitive patients, but not in patients who suffered from recurrence within six months after treatment. Regarding neopterin, tryptophan, and kynurenine concentrations no significant difference was found between baseline and follow-up plasma in any of the groups (Fig. 1). However,

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Table 1
Clinicopathological characteristics of the benign control group and the OVCAD study cohort. The blood samples were drawn at diagnosis (baseline) from patients with benign conditions (n = 65) and from patients with primary advanced EOC (OVCAD study cohort, n = 216). From 28 EOC patients an additional blood sample was taken six months after completion of the adjuvant therapy (paired samples), and from 17 just the follow-up sample was available, resulting in a total number of 45 follow-up samples.

	Control group n (%)	**OVCAD study cohort n (%)**		
Time of blood draw	Baseline	Baseline	Follow-up	Baseline and follow-up
Total	65 (100)	216 (100)	45 (100)	28 (100)
Patient age				
< 55	43 (66.2)	83 (38.4)	14 (31.1)	8 (28.6)
≥ 55	22 (33.8)	133 (61.5)	31 (68.9)	20 (71.4)
Histology				
Ovarian cystadenoma	40 (61.5)			
Functional ovarian cyst	5 (7.7)			
Endometriosis cyst	5 (7.7)			
Non-gynae inflammatory changes	4 (6.2)			
Salpingitis	3 (4.6)			
Leiomyoma uteri	3 (4.6)			
Teratoma ovarii	2 (3.1)			
Exploratory laparascopy w/o pathology	2 (3.1)			
Other benign condition	2 (3.1)			
Epithelial serous ovarian carcinoma		189 (87.5)	41 (91.1)	26 (92.9)
Mixed epithelial ovarian carcinoma		10 (4.6)	2 (4.4)	2 (7.1)
Endometrioid ovarian carcinoma		8 (3.7)	1 (2.2)	0
Undifferentiated ovarian carcinoma		7 (3.2)	1 (2.2)	0
Clear cell ovarian carcinoma		2 (0.9)	0	0
Figo stage				
IIa–IIIb		22 (10.2)	2 (4.4)	0
IIIc		159 (73.6)	36 (80.0)	26 (92.9)
IV		35 (16.2)	7 (15.6)	2 (7.1)
Histologic grade				
1		10 (4.6)	2 (4.4)	2 (7.1)
2		53 (24.5)	8 (17.8)	7 (25.0)
3		152 (70.4)	35 (77.8)	19 (67.9)
Unknown		1 (0.5)	0	0
Peritoneal carcinomatosis				
No		74 (34.3)	16 (35.6)	12 (42.9)
Yes		141 (65.3)	29 (64.4)	16 (57.1)
Unknown		1 (0.5)	0	0
Ascites				
No		54 (25.0)	6 (13.3)	6 (21.4)
Yes		161 (74.5)	39 (86.7)	22 (78.6)
Unknown		1 (0.5)	0	0
Residual disease after surgery				
No		152 (70.4)	36 (80.0)	22 (78.6)
Yes		64 (29.6)	9 (20.0)	6 (21.4)
Platinum-sensitivity				
No		48 (22.2)	10 (22.2)	6 (21.4)
Yes		167 (77.3)	35 (77.8)	22 (78.6)
Unknown		1 (0.5)	0	0

the spreading is high in particular in the EOC group at baseline, and paired samples were not available for all patients.

To assess whether indeed quantitative changes of neopterin formation or tryptophan metabolism may reflect response to treatment, the neopterin levels and Kyn/Trp ratios in paired plasma samples taken at diagnosis and six months after completion of therapy ($n = 28$) were compared. In samples taken from platinum-sensitive patients ($n = 22$) significantly lower Kyn/Trp ratios were observed after treatment than at baseline (mean difference − 0.30, $p = 0.037$), whereas no difference was seen in the neopterin concentrations, and in patients who had already suffered from recurrent disease (see Suppl. Table S4).

3.4. Association of neopterin, tryptophan and kynurenine with outcome

For each analyte assessed at baseline, the patients were assigned to a "high" and "low" group according to whether the concentration of the respective analyte was below or above the median concentration in all EOC patients. Both the "high" neopterin and the "high" Kyn/Trp group had a significantly shorter median OS and PFS than the respective "low" groups. The "low" tryptophan group had a shorter OS, but not PFS; in contrast, the "high" kynurenine group had shorter PFS, but similar OS (Fig. 2).

Moreover, "high" Kyn/Trp was found to have an independent prognostic impact on survival in the multi-variate Cox regression (PFS: HR 1.588, 95% CI 1.127–2.239, $p = 0.008$; OS: HR 1.386, 95% CI 1.055–1.821, $p = 0.019$) after adjusting for patient age, FIGO stage, residual tumor mass after surgery, peritoneal carcinomatosis, and ascites, which are known prognostic parameters in ovarian cancer [25]. In contrast, "high" neopterin was not a significant prognostic factor in the multivariate Cox-regression analysis (Table 3).

Furthermore, the potential prognostic impact of neopterin, tryptophan and kynurenine at follow-up was assessed in a Cox regression analyses by including the respective concentrations as continuous variables: Higher kynurenine concentrations and a higher Kyn/Trp had a negative impact on both PFS and OS. Higher neopterin was related with poor OS but not PFS (Supplementary Table S1). Due to the rather small number of patients, a multivariate Cox regression analysis was not performed.

3.5. CTCs and neopterin, tryptophan and kynurenine concentrations

In our recent study, a blood sample was classified as being "CTC-positive", if at least one of the chosen transcripts (including *PPIC*) was over-expressed beyond the defined threshold; over-expression of the PPIC

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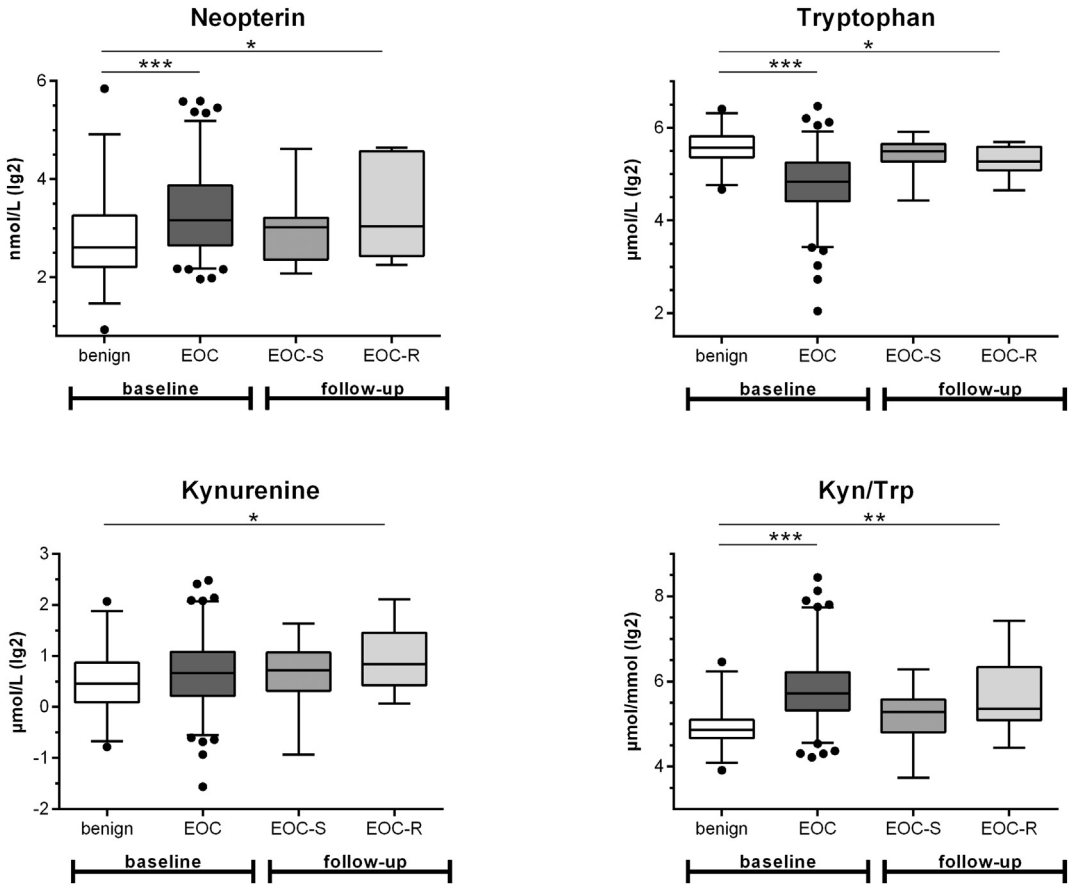

Fig. 1. Box plots showing the plasma concentrations of neopterin, tryptophan and kynurenine in patients with benign ovarian diseases ($n = 65$) and with EOC at baseline ($n = 216$) and follow-up. The follow-up samples are split into those obtained from platinum-sensitive (EOC-S, $n = 35$) and platinum-resistant (EOC-R, $n = 10$) patients. Likewise, the ratio of kynurenine to tryptophan (Kyn/Trp) as a measure of tryptophan metabolism is shown. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers indicate the 2.5th and 97.5th percentiles, outliers are represented by dots. *p*-Values were calculated using the two-sided *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

gene was observed in about two-thirds of the CTC-positive cases and correlated with worse prognosis [21]. At baseline, patients classified as being "PPIC/CTC-positive" presented with higher plasma neopterin concentrations and Kyn/Trp ratios, mostly due to significantly higher kynurenine levels (Fig. 3). At follow-up, we observed significantly higher neopterin concentrations in those patients who were PPIC/CTC-positive at that time-point; both kynurenine and tryptophan, as well as the Kyn/Trp ratio did not differ significantly between PPIC/CTC-positive and CTC-negative patients at follow-up (data not shown). In contrast, when we performed a subgroup analysis of platinum-sensitive patients ($n = 35$), we observed significant differences in neopterin, kynurenine and the Kyn/Trp ratio at follow-up (Fig. 3). The number of platinum-resistant or refractory patients ($n = 10$) was too small to assess the relationship of PPIC/CTCs and neopterin, kynurenine, and tryptophan in a reasonable way.

4. Discussion

In the present study we investigated the potential associations of CTCs with immune activation - as reflected by neopterin formation and tryptophan breakdown - in ovarian cancer. For this purpose, we fell back on the OVCAD study cohort as a well characterized collection of samples from patients with primary advanced EOC. We assessed the plasma levels of neopterin and the ratio of kynurenine to tryptophan (Kyn/Trp), and linked the results to findings from our previous study on the molecular detection of CTC-related transcripts (such as *PPIC*) in the same patients [21]. Furthermore, we investigated the associations between neopterin formation and tryptophan breakdown and clinicopathological characteristics, platinum-sensitivity, and patient survival.

In line with findings from other studies [9–14,26], we showed that both higher concentrations of neopterin and kynurenine, the first stable tryptophan catabolite, were related with advanced stage of the disease at diagnosis, the presence of ascitic fluid, tumor spread within the peritoneal cavity, and with a poor prognosis. Furthermore, we observed that response to treatment went hand in hand with a decrease of the tryptophan breakdown, albeit in a small number of patients with serial samples available.

In addition to the OVCAD study group we assessed neopterin, tryptophan and kynurenine in plasma samples from female patients with benign diseases, mainly of the reproductive tract. Though the percentage of patients presenting with low tryptophan levels was relatively high (approximately 70%), only 23.1% of the patients showed an elevated Kyn/Trp ratio as compared to healthy donors [24]. We conclude that the Kyn/Trp ratio may be a better biomarker for immune activation than the absolute tryptophan concentration, because IDO-1 activity rather than tryptophan alone is important for immune regulation. However, it is true that some moderate immune activation occurs also in patients with benign diseases.

The main finding of our study is that the presence of *PPIC*-positive CTCs was related with elevated neopterin levels and accelerated tryptophan breakdown. High plasma neopterin levels were shown to indicate the activation status of the immune system in the periphery, and render it most likely that the increase of kynurenine and Kyn/Trp was due to an enhanced IDO-1 activity as well [13,14]. The observed association of neopterin and Kyn/Trp and CTC presence supports the view that there is a chronic immune stimulation going on in the patients [14], which may be driven by the CTCs, e.g. by presenting tumor antigens or secretion of cytokines, and which could activate IDO-1. However, an

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Table 2
Neopterin and Kyn/Trp at baseline and follow-up. The median concentration of neopterin and the median ratio of Kyn/Trp as a measure of tryptophan metabolism are shown, as well as their correlation with patient and tumor characteristics. The difference between patient subgroups was assessed using the two-sided t-test. IQR: inter-quartile range; n.a.: not assessed.

	Baseline					Follow-up				
	N	Neopterin [nmol/L] median (IQR), *p*-value		Kyn/Trp [μmol/mmol] median (IQR), *p*-value		N	Neopterin [nmol/L] median (IQR), *p*-value		Kyn/Trp [μmol/mmol] median (IQR), *p*-value	
Patient age										
<55	83	8.2 (5.4–12.1)	*0.004*	46.2 (35.9–70.5)	*0.037*	14	8.2 (5.2–21.2)	*0.646*	36.4 (27.2–45.9)	*0.354*
≥55	133	10.0 (7.3–16.0)		54.7 (44.4–77.4)		31	7.8 (5.1–10.9)		39.9 (31.1–48.2)	
FIGO stage										
IIa–IIIb	22	8.6 (5.6–14.6)	*0.005*	44.3 (35.9–65.5)	*<0.001*	4	5.4 (4.5–8.0)	*0.076*	26.4 (15.4–39.6)	*0.004*
IIIc	159	8.8 (5.7–13.5)		51.2 (38.7–70.7)		34	8.0 (5.1–10.0)		39.4 (31.9–45.9)	
IV	35	13.2 (8.2–21.5)		68.5 (52.2–103.0)		7	13.5 (5.6–24.2)		72.7 (25.7–83.6)	
Histologic grade										
1–2	63	8.6 (5.6–11.9)	*0.149*	49.9 (34.7–74.8)	*0.822*	10	7.5 (5.7–16.7)	*0.756*	42.9 (38.3–49.8)	*0.479*
3	152	9.5 (6.5–16.1)		53.7 (41.3–74.5)		35	8.1 (5.1–10.9)		35.7 (28.1–47.9)	
Peritoneal carcinomatosis										
No	74	8.5 (5.3–11.9)	*0.004*	46.1 (34.6–61.0)	*<0.001*	16	5.7 (4.8–8.8)	*0.039*	36.3 (23.8–43.0)	*0.025*
Yes	141	9.6 (7.0–17.1)		56.4 (43.5–87.9)		29	8.3 (5.8–18.3)		40.1 (32.8–53.1)	
Ascites										
No	54	8.5 (5.3–10.9)	*0.026*	44.4 (36.3–57.6)	*<0.001*	6	7.3 (5.2–12.2)	*0.736*	43.8 (38.7–53.6)	*0.438*
Yes	161	9.1 (6.6–15.6)		55.4 (41.9–82.0)		39	8.1 (5.1–13.5)		36.5 (28.0–47.9)	
Residual disease after surgery										
No	152	8.6 (6.0–14.3)	*0.038*	50.1 (37.7–70.0)	*0.004*		n.a.		n.a.	
Yes	64	10.5 (7.9–17.3)		58.1 (46.5–93.2)						
Platinum-sensitive disease										
Yes		n.a.		n.a.		35	8.1 (5.1–9.3)	*0.300*	36.5 (28.0–44.7)	*0.028*
No						10	8.3 (5.4–23.7)		57.1 (36.4–80.8)	

p-values are based on a two-sided t-test and on one-way ANOVA.

influence of the second tryptophan-degrading enzyme tryptophan 2,3-dioxygenase (TDO) cannot be excluded. TDO is a substrate-activated enzyme, which is usually restricted to the liver where it is involved in the regulation of systemic tryptophan concentrations, but some tumors have been shown to acquire expression of this enzyme [27]. Tumoral expression of IDO-1 and/or TDO may further promote immune escape mechanism such as the differentiation of Tregs.

Both, neopterin formation and IDO-1-mediated tryptophan breakdown represent important immune effector pathways. Deprivation of the essential growth factor tryptophan leads to diminished synthesis of new proteins and thereby attenuates cell growth, which is beneficial in terms of inhibiting tumor growth but on the long term leads also to a decreased proliferation of T cells. In addition to this direct effect, IDO-1 catabolite kynurenine and downstream products such as 3-hydroxyanthranilic acid or quinolinic acid interfere with T cell activation and survival and may lead to apoptosis [28] and T cell unresponsiveness [29]. Likewise, it has been shown that IDO-1 activity correlates with the induction of a Treg phenotype and this may play a major role in the initiation and maintenance of an immunosuppressive environment [30]. These effects may occur both locally at the tumor site [31] and in circulation [14]. Both inflammatory mediators released into circulation (e.g. by tumor infiltrating lymphocytes) and the presence of antigenic CTCs may represent a chronic stimulus for the induction of the T cell/monocyte axis and thus for IDO-1 mediated tryptophan breakdown and neopterin formation [8,14,31].

CTCs are shed by the tumor and spread via the lymphatic or the blood system throughout the body. They are considered as the origin of distant metastasis as they were found in the peripheral blood of patients suffering from various metastatic diseases, including ovarian cancer [32]. The clinical relevance of CTCs in ovarian cancer has long been underrated as the direct peritoneal spread in the abdominal cavity was considered as the main route of metastasis. However, there has been increasing evidence that the hematogenous spread of ovarian cancer may play a more relevant role than has been assumed for years. The first scientists who have provided such evidence were South Korean researchers who highlighted the importance of tumor cell dissemination via the peripheral blood using a parabiosis mouse model. They showed that omental metastases are caused by active travelling of the ovarian cancer cells in the blood stream and not by passive exposure of exfoliate cancer cells [33]. In addition, a recent study showing the unique tropism of injected ovarian cancer cells for the murine xenograft ovaries [34] indicated that hematogenous spread in ovarian cancer may be more common than previously assumed. Several studies investigating the clinical potential of CTCs in ovarian cancer management confirmed that CTC counts correlated with survival and had prognostic value [25], which however also depends on the phenotypic characterization and the isolation protocol [21,35].

The potential association of IDO-1 and the progression of ovarian cancer has just recently been discussed by several studies: Nonaka showed in mouse xenograft models that the injection of an IDO-1 overexpressing ovarian cancer cell line led to a markedly increased formation of intraperitoneal tumors as compared to the control cell line [36], and in a murine lung cancer model, it has been shown that IDO-1 supported neovascularization led to an increased metastatic burden [37]. Thus, CTCs can indeed represent a functional link between peripheral immune activation and tumor activity.

In our study, we observed that at baseline the plasma concentrations of neopterin and kynurenine were significantly higher in those patients who were classified as being “CTC-positive” due to over-expression of the PPIC gene in their blood cell isolates. Six months after treatment, which consisted of surgery and adjuvant platinum-based chemotherapy, the correlations remained significant in platinum-sensitive patients. This finding could hint towards a still ongoing immune activation due to the continuously present PPIC/CTCs, while peripheral inflammation might be driven towards resolution in the CTC-negative patients. However, it cannot be excluded that there is an additional relation between the observed immune activation and suppression with therapy-related processes, e.g. while cytotoxic therapies are generally considered to be immunosuppressive, the antigenic material that

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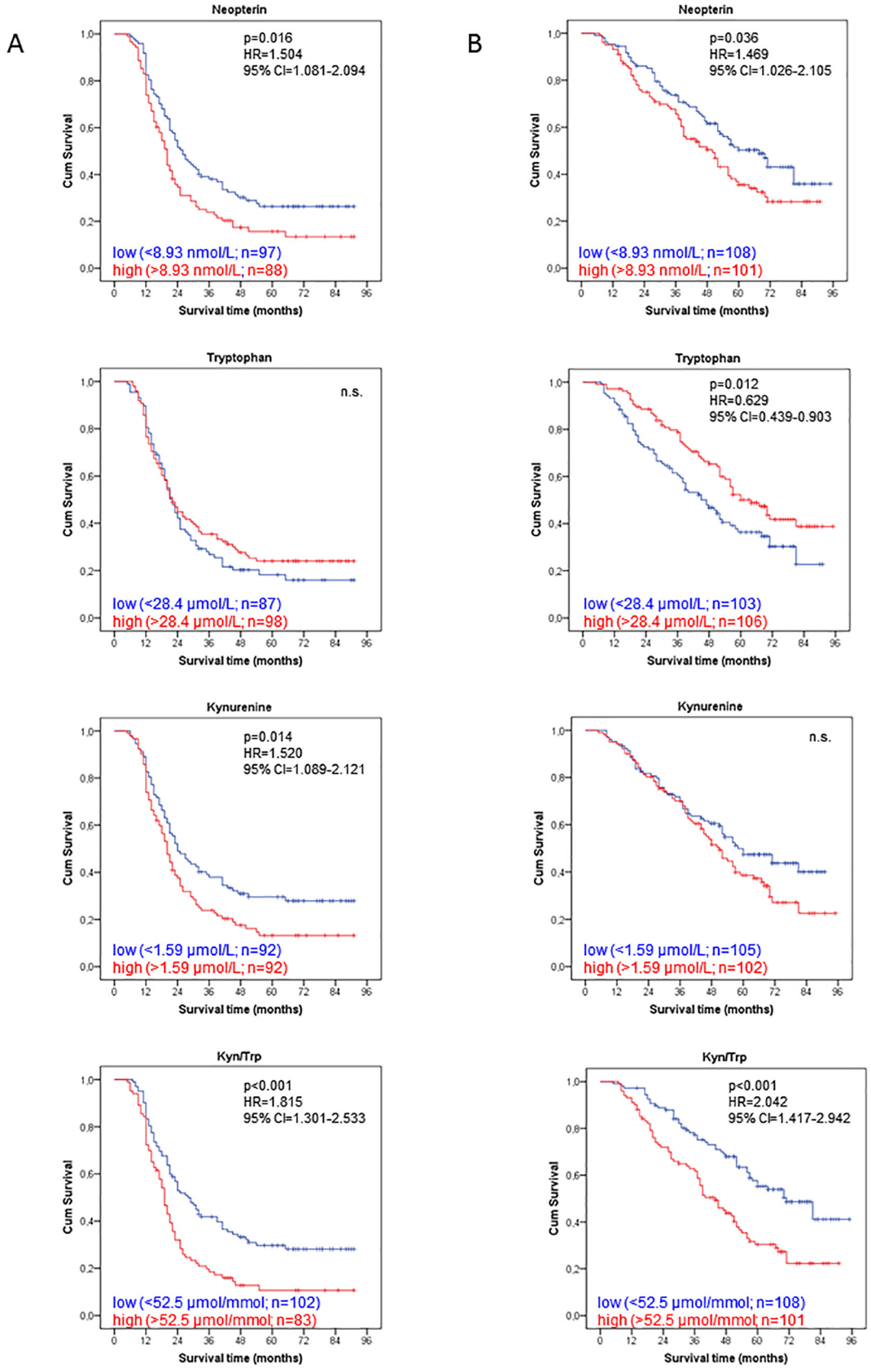

Fig. 2. Survival analysis of EOC patients stratified by the level of neopterin, tryptophan, kynurenine, and by the Kyn/Trp ratio in "high" (above the median) and "low" (below the median). Kaplan-Meier curves showing the differences in progression-free (A) and overall survival (B). Hazard risks, 95% CI, and *p*-values were calculated using a Cox regression.

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Table 3
Cox's proportional hazard regression analysis for OS and PFS. All parameters (assessed at baseline) were included as categorical variables, and the hazard ratio for reduced PFS and OS in relation to the reference category is given for each parameter. * not included in the final multiple regression model; n.s. not significant.

	Progression-free survival			Overall survival		
	N	Univariate HR (95% CI) *p*-value	Multiple HR (95% CI) *p*-value	N	Univariate HR (95% CI) *p*-value	Multiple HR (95% CI) *p*-value
Patient age						
< 55 years	74	1	*n.s.*	82	1	1
≥ 55 years	111	1.586 (1.125–2.237) *0.009*		127	1.986 (1.338–2.948) *<0.001*	1.702 (1.144–2.533) *0.009*
FIGO stage						
IIa–IIIb	20	1	1	21	1	1
IIIc	139	1.983 (1.090–3.607)	1.684 (0.921–3.079)	154	2.721 (1.188–6.231)	2.193 (0.949–5.068)
IV	26	4.027 (1.994–8.131) *<0.001*	3.634 (1.779–7.425) *0.001*	34	6.620 (2.725–16.082) *<0.001*	4.414 (1.788–10.897) *0.001*
Residual disease after surgery						
No	140	1	*n.s.*	148	1	*n.s.*
Yes	45	1.965 (1.350–2.860) *<0.001*		61	1.976 (1.357–2.876) *<0.001*	
Peritoneal carcinomatosis						
No	68	1	1	72	1	1
Yes	116	1.703 (1.137–2.551) *0.010*	2.393 (1.649–3.473) *<0.001*	136	2.445 (1.597–3.745) *<0.001*	1.887 (1.216–2.929) *0.005*
Ascites						
No	47	1	*	53	1	*
Yes	137	1.663 (1.130–2.449) *0.010*		155	1.556 (0.994–2.436) *n.s.*	
Neopterin [nmol/L]						
Low (<median)	97	1	*n.s.*	108	1	*n.s.*
High (≥ median)	88	1.292 (1.049–1.592) *0.016*		101	1.319 (1.054–1.652) *0.016*	
Tryptophan [μmol/L]						
High (≥ median)	97	1	*	103	1	*
Low (<median)	88	0.995 (0.983–1.008) *n.s.*		105	0.712 (0.539–0.940) *0.017*	
Kynurenine [μmol/L]						
Low (<median)	92	1	*	105	1	*
High (≥ median)	92	1.449 (1.138–1.846) *0.003*		102	1.305 (0.999–1.704) *n.s.*	
Kyn/Trp [μmol/mmol]						
Low (<median)	102	1	1	108	1	1
High (≥ median)	83	1.406 (1.139–1.735) *0.001*	1.588 (1.127–2.239) *0.008*	101	1.618 (1.280–2.044) *<0.001*	1.386 (1.055–1.821) *0.019*

p-values are based on Cox regression analysis.

derives from dying tumor cells may trigger the evasion from immunosuppressive circuitries [38].

Importantly, we did not observe a correlation between the presence of PPIC/CTCs, neopterin and Kyn/Trp in those patients who were diagnosed with progressive disease during treatment or recurrence within six months after adjuvant treatment (data not shown). Moreover, both the neopterin concentrations and Kyn/Trp ratios were elevated irrespective of the CTC-status of these blood samples, suggesting that additional immune activating processes are ongoing in this patient subgroup as discussed above. However, due to the low number of patients in this sub-group ($n = 10$), these findings require further confirmation in larger cohorts.

Considerable effort has been made to dissect the immunogenic potential of ovarian tumors by gene expression profiling, revealing tumor subtypes ranging from immunologically active to inactive [35]. The classification of the immunogenic potential was based on the count of tumor infiltrating lymphocytes, thus local events. In order to improve classification, a wealth of research has been performed in order to characterize primary and secondary tumors and the immune cells in the tumor microenvironment on a molecular basis [33–35,38,39]. Yet, the interplay between local events at the tumor site and the immune activation in the periphery is not fully understood.

PPIC is involved in a variety of biological processes, including redox-related processes and inflammation, and is correlated with cancer pathogenesis without detailed knowledge about the specific mechanisms [40]. In our earlier study, we identified *PPIC* as a reliable indicator of CTC presence and of poor prognosis; thus, *PPIC* may serve as a biomarker for poor response to treatment and early detection of relapse [21]. Our present study shows that the presence of *PPIC*/CTCs, elevated neopterin levels and an increased tryptophan catabolism – both are in line with increased IDO-1 activity – coincide in EOC patients. Thus CTCs can represent a functional link to the immunological conditions underlying the tumor disease.

In conclusion, the observed concomitant alterations of CTCs and immune system related biomarkers suggest that immune responses along with increase of neopterin and Kyn/Trp concentrations are not necessarily only located at the site of the tumor, but may also go on in the circulation when CTCs are detected by immunocompetent cells in the blood stream. This interaction could be of special relevance when T cells are depleted from the tumor microenvironment.

Acknowledgements

Financial support

The OVCAD study was funded by the European commission as a FP6 Specific Targeted Research and Innovation Project (contract no 018698).

Elena Ioana Braicu is a participant of the Charité Clinical Scientist Program funded by the Charité Universitätsmedizin Berlin and the Berlin Institute of Health. Johanna M. Gostner is a Hertha-Firnberg fellow funded by the FWF Austrian Science Fund.

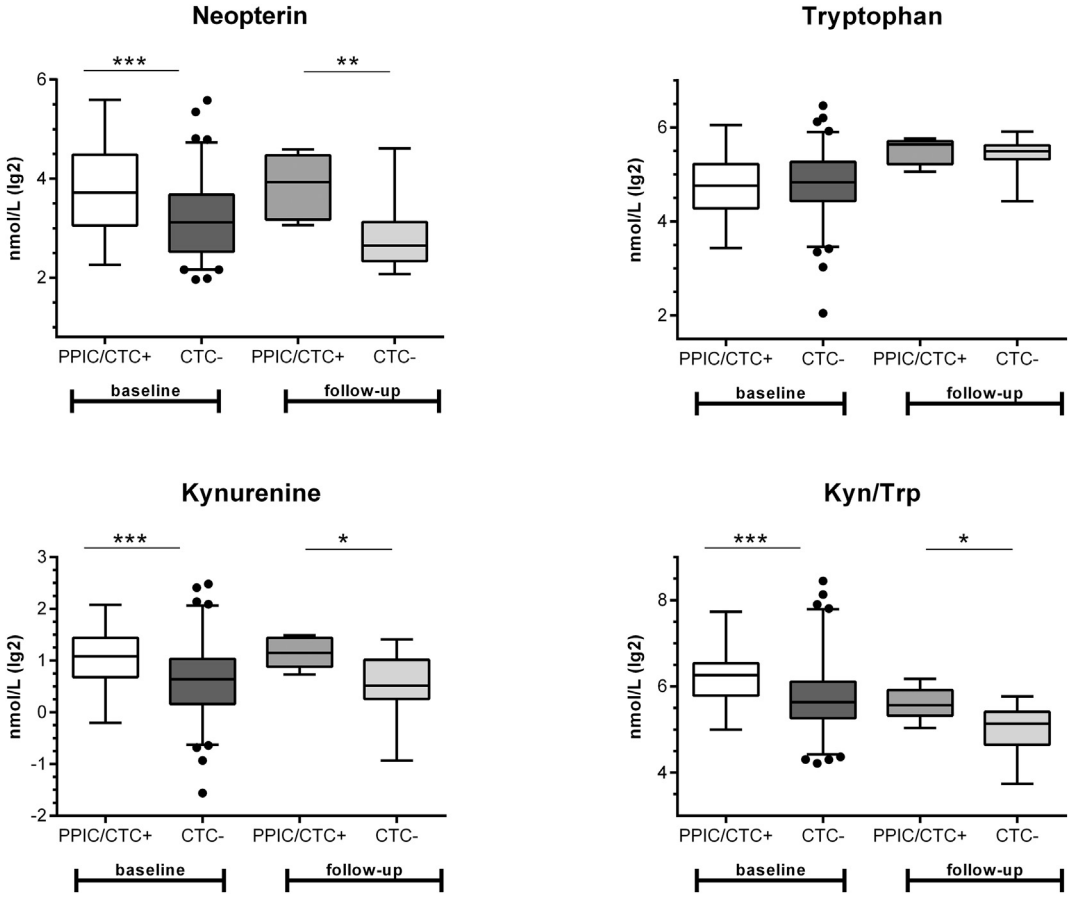

Fig. 3. Box plots showing the plasma concentrations of neopterin, tryptophan and kynurenine in ovarian cancer patients stratified by the presence of PPIC/CTCs. At baseline, 35 patients were classified as PPIC/CTC-positive and 172 as CTC-negative. At follow-up only platinum-sensitive patients were included (6 PPIC/CTC-positive and 29 CTC-negative patients). Likewise, the ratio of kynurenine to tryptophan (Kyn/Trp) as a measure of tryptophan metabolism is shown. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers indicate the 2.5th and 97.5th percentiles, outliers are represented by dots. *p*-Values were calculated using the two-sided t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Conflict of interest

Robert Zeillinger is shareholder and CEO of OncoLab Diagnostics GmbH that holds patents EP2686439 and EP2309273.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2018.02.020.

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