

The Relationship of Kynurenine and Neopterin Levels and Their Association with a Selection of Other Immune Markers in Chronic Myeloid Leukaemia Patients

(CML / patients / kynurenine / neopterin / IDO / C-reactive protein / interleukin 6)

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Abstract. Among malignant diseases, chronic myeloid leukaemia (CML) is one of the best suited candidates for immunotherapy. For this purpose it is necessary to broaden the present knowledge on the immunology of this disease. As a part of such a project, the levels of kynurenine (KYN) and neopterin (NPT) were studied in 28 CML patients and in the same number of healthy subjects. At diagnosis, both KYN and NPT levels were found to be elevated in a significant portion of the patients and dependent on their leukocyte count. As in the case of KYN, increased NPT levels dropped after achieving remission. When correlating KYN and NPT levels with a selection of other markers tested, significant association was revealed only in the case of CRP and IL-6. However, there were several patients with increased KYN levels in whom NPT was not detected, and *vice versa*. The relapse of the disease observed in two pa-

tients was accompanied by an increased level of NPT in both cases, but by an increased level of KYN in only one of them. No significant correlation was found between KYN and NPT levels in sera taken at diagnosis. However, when the whole set of sera was taken into consideration, the association became statistically significant. Although the data obtained revealed a number of similarities between KYN and NPT production in CML patients, it also suggested a difference in the kinetics of these two biomarkers' production.

Introduction

Both kynurenine (KYN) and neopterin (NPT) are biomarkers induced by interferon γ (IFN- γ). KYN is a product of tryptophan metabolism induced by the indoleamine dioxygenase (IDO) family of enzymes, i.e., indoleamine 2,3-dioxygenase 1 (IDO-1), IDO-2, and tryptophan-2,3 dioxygenase (TDO), which are among the key immune regulators (van Baren and Van den Eynde, 2015). The ratio between KYN and TRY levels is generally accepted as the measure of their activity. NPT, a pyrazino-pyrimidine compound, is a metabolite of guanosine-triphosphate (GTP), which is degraded by GTP cyclohydrolase I (Murr et al., 2002). While a wide variety of cells are involved in KYN production, the major source of NPT are activated macrophages and dendritic cells. Its amount correlates with production of reactive oxygen species (Gostner et al., 2013), and the amount of NPT present serves as a measure of their production. Increased levels of KYN and NPT reflect cellular immune activation, associated with endogenous INF- γ production. *In vitro*, production of both KYN and NPT in peripheral blood mononuclear cells can be suppressed by a wide variety of immunosuppressive agents

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Abbreviations: CI – confidence interval, CML – chronic myeloid leukaemia, CRP – C-reactive protein, GTP – guanosine-triphosphate, HPLC – high-performance liquid chromatography, IDO – indoleamine 2,3-dioxygenase, IL – interleukin, IFN – interferon, KTI – kynurenine/tryptophan index, KYN – kynurenine, NPT – neopterin, TDO – tryptophan 2,3-dioxygenase, TKI – tyrosine-kinase inhibitors, TRY – tryptophan.

(Schroecksadel et al., 2011). In healthy subjects, both KYN and NPT levels correlate strongly with age (Bruunsgaard et al., 2001; Ledochowski et al., 2001; Vonka et al., 2015). Most likely, this is due to the age-dependent increase in INF- γ production that reflects a shift in the balance between pro- and anti-inflammatory states.

Both KYN and NPT levels are enhanced in some types of cancer and can serve as prognostic markers (Melichar et al., 2006; Sucher et al., 2010; Pilotte et al., 2012). It has even been suggested that increased levels of KYN and NPT themselves may contribute to the development of cancer. Support for this connection has been provided by a recent prospective study, which has indicated an increased risk of overall cancer in subjects with the enhancement of their levels, even after adjustment for covariables (such as smoking) known to be risk factors for cancer (Zuo et al., 2014). In this respect, it may be of interest that inhibition of GTP cyclohydrolase I resulted in the inhibition of tumour growth (Pickert et al., 2013). However, this cannot be taken as a strong indication that NPT might play a role in tumour initiation and/or progression. Other GTP metabolites, such as tetrahydrobiopterin, which is an important cofactor of several enzymes, might be involved (Kim and Park, 2010). Both KYN and NPT may also play a role in the pathogenesis of heart disease (De Rosa et al., 2011). The association of increased KYN and NPT levels with autoimmune and neurodegenerative diseases, and some other conditions, has also been reported (Hamerlinck, 1999; Murr et al., 2002; Widner et al., 2002), and it has been speculated that they may play a role in the aetiology and course of these disorders.

In our previous paper (Vonka et al., 2015) we presented evidence that at diagnosis, i.e., prior to the start of any therapy, chronic myeloid leukaemia (CML) patients possessed significantly higher KYN levels than healthy control subjects, indicating that CML should be included into the group of malignancies with an increased activity of the IDO family of enzymes. In that paper KYN levels were presented in the form of a KYN/TRY index (KTI) calculated as follows: (KYN levels in $\mu\text{M/l}$ divided by TRY levels in $\mu\text{M/l}$) \times 1,000. The cut-off value of ≥ 40 was selected as an indicator of increased KYN level based on the results obtained in the healthy control subjects. It represented their mean KTI value + 2 SD. The difference between the patient groups and controls was highly significant; however, KTI levels of ≥ 40 were determined in less than half of the patients. Further analysis indicated a highly significant correlation between KTI values and leukocyte counts, but not between KTI values and age, a correlation which was highly significant in the healthy control subjects. In all of the patients with increased KYN levels, which were followed for a prolonged period of time, there was a drop to the norm after achieving haematological remission. On the other hand, all patients with normal KTI values at diagnosis, who were treated with INF- α , reacted with an increase of KTI, thus confirming previous

reporting that INF- α is also capable of activating IDO (Curreli et al., 2001). A similar phenomenon was only observed in one of eight patients treated exclusively with tyrosine-kinase inhibitors (TKI).

In this report concerning the same groups of subjects we present data on the correlation between KTI and some other selected immunological markers. A similar analysis was made for NPT. Finally, the results of testing the correlation between increased KYN levels and the presence of detectable amounts of NPT are presented.

Material and Methods

Patients and healthy control subjects

Patients and controls were the same as in our previous report (Vonka et al., 2015). In brief, a total of 28 patients (12 males and 16 females) whose median age was 44 years (range 24-72 years) and 28 healthy subjects (11 males and 17 females) whose median age was 46 years were enrolled. All patients were Philadelphia chromosome positive. Sera and leukocytes were collected from all patients at the time of diagnosis, i.e., prior to the start of any therapy. From 20 patients studied, additional materials were collected at different intervals (4 to 55 months) following the initial sampling. A total of 84 sera were collected; sera were stored frozen at -20°C and leukocyte suspensions in liquid nitrogen. Eight patients enrolled prior to 2004 were initially treated with IFN- $\alpha 2a$ (Pegasys, Roferon; F. Hoffmann-La Roche, Basel, Switzerland), and later on with tyrosine-kinase inhibitors. Patients enrolled after 2004 were treated with tyrosine-kinase inhibitors from the very beginning. In all patients, cytogenetic or molecular remission was achieved in the course of the observation period. As indicated in the previous paper (Humlova et al., 2006), statistically significant differences between the patients and healthy controls were found in the levels of IgA, C4 component complement, CRP, and IL-6, as well as in production of cytokines in stimulated CD3⁺ cells. A portion of these patients had been followed for several years, and in all of them the remission achieved tended to be associated with normalization of the immunological aberrations detected (Humlova et al., 2010).

Methods

In addition to routine biochemical, haematological, cytogenetic, and molecular biologic tests, several immune markers were determined to construct an immunological profile of the CML patients. Some of the tests were performed immediately after the arrival of the respective materials at the laboratory; portions of the materials were preserved for further tests. The immunological investigations performed included measurement of levels of immunoglobulins (IgG total, IgG1, IgG2, IgG3, IgG4, IgA, IgM), complement components C3 and C4, C-reactive protein (CRP), interleukin 6 (IL-6), detection of autoantibodies, analysis of lymphocyte subpopulations, intracellular cytokine production by stimu-

Table 1. Correlation of KTI values with CRP and IL-6 levels in CML patients prior to the start of any treatment

Immune marker	N	r	CI	P
CRP	28	0.5194	0.1702–0.7527	0.0046**
IL-6	25	0.4847	0.09847–0.7440	0.0141*

Spearman correlation; N – number of patients; *P < 0.05; **P < 0.01

lated CD3⁺ cells, and the presence of antibodies to several herpes viruses and papillomaviruses. The methods used were described in detail in our previous publication (Humlova et al., 2006). The methods of KYN and NPT detection by high-performance liquid chromatography (HPLC) were described by Krcmova et al. (2011). KYN levels were expressed by a kynurenine/tryptophan index (KTI) calculated as (kynurenine levels in $\mu\text{M/l}$ /tryptophan levels in $\mu\text{M/l}$) \times 1,000. NPT levels were expressed in nM/l. In the present series of experiments, the sensitivity of NPT detection test was approximately 5 nM/l.

Statistical methods

For statistical analysis we used linear regression and Spearman correlation. To test the association in contingency tables, we used Fisher's exact test. The difference of continuous variables was tested using Mann-Whitney test. A significance level of $\alpha = 0.05$ was used for all statistical tests. Analysis was made using IBM SPSS and Graph Pad Prism software.

Results

First of all, we wanted to understand the correlation between KTI levels and the levels of the other immune markers tested. A significant correlation with KTI levels was revealed in the case of CRP and IL-6, as shown in Table 1, but not in any of the other immune markers tested (results not shown). We then wanted to find out whether there was a difference between the patients

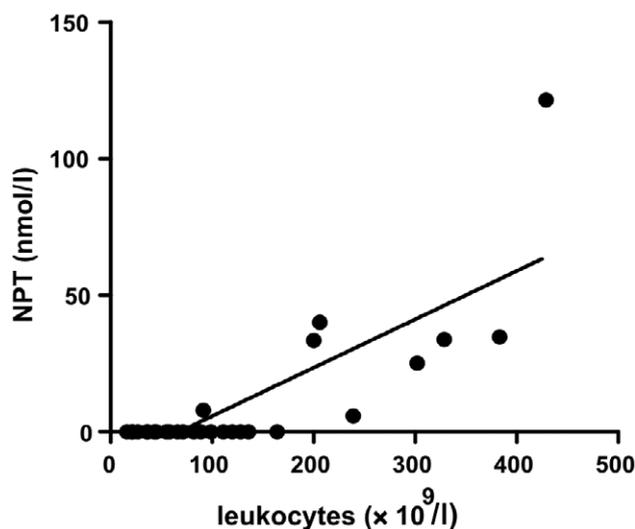


Fig. 1. Correlation between NPT levels and leukocyte count (linear regression, $P < 0.0001$; Spearman correlation, $r = 0.738$, $P < 0.0001$)

with increased (≥ 40) and normal (< 40) KTI levels and any aberrations of the tested immune markers. Although some differences were encountered, none were statistically significant (results not shown).

Next, we determined the presence and levels of NPT in the patients and healthy controls. As indicated in Table 2, prior to treatment, detectable levels of NPT were revealed in eight patients but not in a single healthy control, which makes the difference between the two groups highly significant ($P = 0.0044$). Similarly as in the case of KYN (Vonka et al., 2015), NPT levels correlated significantly with the leukocyte count (Fig. 1), but not with age (results not shown). As seen in Table 3, in all seven NPT-positive patients from whom additional sera were available, a decrease in the leukocyte count was associated with the drop of NPT to undetectable levels. This resembles what we have observed in patients with elevated KYN levels in pretreatment sera

Table 2. Presence of detectable amounts of NPT in CML patients prior to the start of any treatment and in healthy control subjects

Group	N	NPT-	NPT levels (nM/l)				NPT+	P
			< 5	5–10	11–50	51–100		
Patients	28	20	2	5	0	1	8	0.0044**
Controls	28	28	0	0	0	0	0	

Fisher's exact test; N – number of patients; NPT+ – NPT detected; NPT- – NPT not detected; ** $P < 0.01$

Table 3. Drop of NPT levels in association with a decrease of leukocyte count in the course of treatment

Time of sampling	Parameter followed	Patient No.						
		1	2	3	4	5	6	7
At diagnosis	NPT level ⁽¹⁾	5.90	25.12	121.42	40.09	7.94	33.84	33.51
	leukocytes ⁽²⁾	239	302	429	206	91	329	200
After treatment	NPT level	< 5	< 5	< 5	< 5	< 5	< 5	< 5
	leukocytes ⁽³⁾	16.5(10)	7.4(27)	4.4(30)	16.0(3)	6.4(9)	3.0(7)	28.0(22)

⁽¹⁾ – nM/l; ⁽²⁾ – $\times 10^9/l$; ⁽³⁾ – in brackets number of months after the start of the treatment

Table 4. Comparison of NPT presence with CRP and IL-6 levels in CML patients prior to the start of treatment

Group	N	CRP Mean	P	N	IL-6 Mean	P
NPT +	8	26.38	0.0048**	8	10.59	0.0003***
NPT –	20	9.35		17	4.15	

Mann-Whitney test; N – number of patients; NPT+ – NPT detected; NPT– – NPT not detected; *** – $P < 0.001$

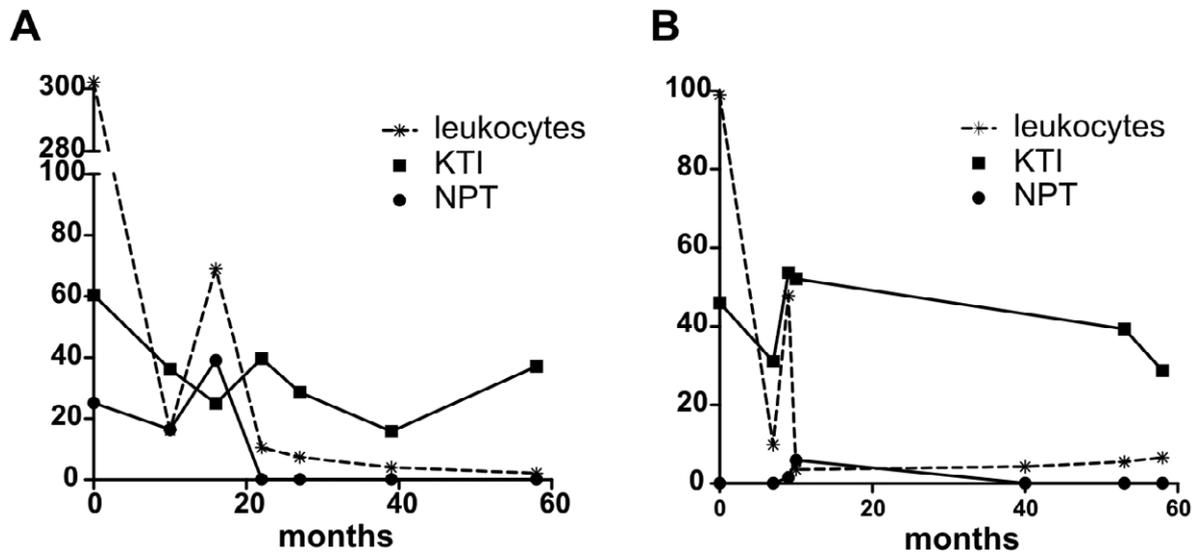


Fig. 2. Leukocyte counts and NPT and KTI levels in two patients who relapsed after IFN- α treatment; leukocyte counts: $\times 10^9/l$; kynurenine expressed as KTI, NPT in nM/l; (A) patient 12, (B) patient 24

(Vonka et al., 2015). We next tried to correlate the presence of detectable amounts of NPT with the levels of the other immune markers tested. The results presented in Table 4 indicate that a significant difference between the two groups, originally NPT-positive or NPT-negative patients, was found in the case of CRP and IL-6, but not in any of the other immune markers tested (results not shown). At odds with the findings concerning the KYN levels that increased above the cut-off value in all IFN- α -treated patients (Vonka et al., 2015), no detectable increase of NPT levels was observed following IFN- α treatment (results not shown). The findings of two patients originally treated with IFN- α and in whom disease relapse occurred were atypical and may be of interest. As shown in Fig. 2, for the first patient (Fig. 2A), in whom haematological remission was only achieved after a prolonged period of time, detectable amounts of NPT persisted for at least 16 months, having been de-

Table 5. Presence of NPT in patients with increased KTI values in sera taken prior to the start of any treatment as determined in those sera and in all sera collected

Sera	KTI	N	NPT +	NPT –	P
Prior to treatment	≥ 40	13	5	8	0.4097
	< 40	15	3	12	
All sera	≥ 40	27	7	20	0.0483*
	< 40	57	5	52	

Fisher's exact test; N – number of patients; NPT+ – NPT detected; NPT– – NPT not detected; * – $P < 0.05$

tected in three successive samples. A leukocyte count increase was associated with an increase of NPT level, but with a slight decrease of KTI value. In the second patient (Fig. 2B), a simultaneous increase of KTI and the appearance of detectable NPT were observed nine months later, when disease relapse was detected.

Finally, we tested the association of KYN levels with the presence of NPT. As indicated in Table 5, in sera taken at diagnosis no significant association between increased KYN levels ($KTI \geq 40$) and the presence of detectable amounts of NPT was found. However, when all sera (including those taken in the course of treatment) were taken into consideration, the association became statistically significant.

Discussion

It was the main purpose of the present study to complement our previously established results on KYN/CML association with similar data concerning NPT. It was also our intention to compare KYN and NPT levels at the time of diagnosis and during the course of treatment, and to define their association with a selection of several other immune markers tested. The analysis was complicated by the fact that KYN levels were detected in all patients as well as in all healthy control subjects (Vonka et al., 2015), while NPT, detectable by the method employed, was revealed only in a portion of the patients and not once in the control subjects. This was due to using a test for NPT determination that was less sensi-

tive than some of the ELISA-based tests capable of detecting low levels of NPT. In fact, most recently, when using an ELISA kit for testing a few of the sera, we revealed markedly increased levels of this biomarker in comparison with the HPLC technique (results not shown). In any case, the levels of NPT were higher in CML patients than in the matched control subjects. They correlated with the leukocyte cell count and tended to normalize in the course of therapy.

Thus, from the data obtained it seems clear that similarly to the case of KYN, CML is associated with induction of NPT in a significant segment of CML patients. These data seem to be in accord with previous observations of the increased concentrations of NPT in haematological malignancies (Hausen et al., 1982; Reibnegger et al., 1991). However, very few studies have been completed on the nature of the association of NPT with CML. The respective findings have regularly been presented only as side observations. Thus, it has been reported that both IFN- α and IFN- β are capable of inducing NPT production in CML patients nearly at the same rate, but the latter has been inefficient for the treatment of CML (Aulitzky et al., 1993). This seems to suggest that NPT does not play a significant role in the course of the disease. In another study it has been shown that after bone marrow transplantation to a group of patients suffering from haematological malignancies, including two CML patients, the appearance of IL-10 correlated with a poor prognosis and also with elevated NPT levels (Hempel et al., 1997). There was no clear indication, however, that NPT could be involved in the outcome of the treatment. NPT levels have also been followed in CML patients treated with different doses of pegylated IFN- α 2a. Although NPT levels had increased and a correlation was revealed between the IFN- α dose administered and the rate of enhancement of NPT production, no data have been provided suggesting a relationship between NPT levels and the treatment outcome (Talpez et al., 2005).

Similarly as in the case of KYN, we found a very strong correlation of NPT levels with leukocyte counts, but not with the patient age. As in the case of KYN, remission induced by the therapy was associated with a drop in NPT levels. There was a significant correlation of the levels of both KYN and NPT with the levels of CRP and IL-6, which was certainly not unexpected. It should be noted that a significant correlation between NPT with CRP and IL-6 has recently been reported in chronic kidney disorders (Yadav et al., 2012). When correlating KYN and NPT levels in the sera taken at diagnosis, some correlation was observed, but not reaching statistical significance. However, with the results obtained in all sera, including those that were collected in the course of treatment, the correlation was significant.

The data presented indicated similarities between the production of KYN and NPT in CML patients; still, some differences were apparent. Not all sera with increased KTI values possessed detectable amounts of NPT, and *vice versa*. This was especially apparent in the

patients with a normal KTI who had been treated with IFN- α 2a. In all of these, at least a transitory enhancement of KTI was revealed (Vonka et al., 2015). However, this did not occur in the case of NPT. These observations seem to indicate that the mechanisms involved in inducing these two biomarkers, although similar, are not identical.

The fact that, with the achievement of disease remission, KYN and NPT tended to normalize seems to go against the conclusions drawn from the prior prospective study (Zuo et al., 2014) that their enhanced levels increase the risk of cancer. Should it be a constitutive marker of such subjects, one might expect their enhanced levels to persist even after achieving remission; however, this was not the case. It is therefore more likely, as the authors of that particular study have admitted, that the aberrations of KYN and NPT observed prior to cancer detection may have been due to the presence of an occult cancer, not yet clinically manifest.

We have shown elsewhere that there are many aberrations of the immune system in CML patients and that there is a general tendency to their normalization when the remission is achieved (Humlova et al., 2010; Vonka and Petrackova, 2015). The results of our present study extend the validity of this observation to other immune markers. Although it is likely that most of these aberrations are the consequence of the disease, some may contribute to tumour progression. For example, this is highly probable in the case of KYN, which has a direct immunosuppressive effect (Mellor et al., 2003; Prendergast, 2008), and, in addition, the activation of the IDO family of enzymes results in depletion of tryptophan, an essential amino acid playing an important role in immune reactions. The role of NPT is less clear. Its strong association with cancer and some other diseases could indicate its involvement in their pathogenesis. However, as already mentioned, to our knowledge no convincing data on its role in the initiation or progression of cancer are yet available. In general, it is not easy to evaluate aberrant immune reactions observed in cancer patients in terms of cause and effect. However, the lack of such evidence does not diminish the repeatedly reported diagnostic value of enhanced NPT levels in cancer, including CML. Future research will hopefully reveal whether it plays some role in the pathogenesis of the above-mentioned diseases or whether its presence is simply a marker of other conditions responsible for their aetiology and/or progression.

To summarize, the present data indicate that in CML patients with increased levels of either KYN or NPT, achieving remission is associated with their normalization. This suggests that both these biomarkers can be used for monitoring the outcome of the therapy. Hopefully, in the future they may also be useful for monitoring the efficacy of therapeutic vaccines.

Disclosure of conflict of interest

None of the authors have conflicts of interest to report.

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