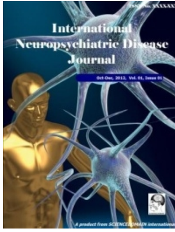

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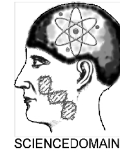

In vitro Measured Indoleamine 2, 3-dioxygenase Activity Correlates with Inflammation and Tryptophan Levels *In vivo*

Katrin Huesker[1*], Jana Schenk[1], Stefan Albrecht[1], Imad Lahdou[2] and Volker von Baehr[1]

[1]*Institute for Medical Diagnostics, Department of Immunology, Nicolaistr 22, 12247 Berlin, Germany.*
[2]*University of Heidelberg, Otto-Meyerhof-Center, Institute of Immunology, Department of Transplantation Immunology, Im Neuenheimer Feld 350, 69120 Heidelberg, Germany.*

Authors' contributions

This work was carried out in collaboration between all authors. Author KH designed the study, evaluated the data and wrote the manuscript. Author JS performed cell culture work and ELISA testing. Author SA extracted values from the clinical database. Author IL performed HPLC analyses and reviewed the manuscript. Author VVB originated the idea for this study and provided critical feedback. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To evaluate the diagnostic validity of an in vitro assay for indoleamine 2,3-dioxygenase (IDO) activity as indicator for biological alterations that are associated with depressive symptomatologies.
Study Design: Retrospective evaluation of a medical database.
Place and Duration of Study: Institute for Medical Diagnostics, Berlin, between April and July 2013.
Methodology: We compared values for $IDO_{in\ vitro}$, a so far unpublished assay for IDO activity that measures tryptophan degradation in peripheral blood monocytic cell (PBMC), with blood levels of tryptophan and tumor necrosis factor alpha (TNF-alpha). Values were derived from a clinical database comprising 441 patients who had received $IDO_{in\ vitro}$, tryptophan and/or TNF-alpha testing in the course of medical treatment between May 2011 and March 2013. All data was anonymized. Clinical significance of $IDO_{in\ vitro}$ was

**Corresponding author: Email: k.huesker@imd-berlin.de;*

evaluated by correlation of $IDO_{in\ vitro}$ to blood tryptophan and TNF-alpha α levels. Further, we challenged the validity of the $IDO_{in\ vitro}$ assay by investigating the influence of the PBMC proliferation rate as a potential confounding factor and comparing the responses to phythemagglutinin (PHA) and interferon-gamma (IFN-gamma), a recognized inducer of IDO activity.
Results: Low plasma tryptophan and high serum TNF-alpha are associated with increased $IDO_{in\ vitro}$ (2.8±3.3 vs. 2.2±1.1, P=0.04 and 2.7±3.5 vs. 2.1±1.6, P=0.05). Elevated $IDO_{in\ vitro}$ is not due to increased PBMC proliferation rates. PHA and IFN-gamma yield correlating $IDO_{in\ vitro}$ values (correlation coefficient=0.91).
Conclusions: $IDO_{in\ vitro}$ represents a valid diagnostic tool for IDO activity and correlates with inflammation and decreased availability of tryptophan in vivo. $IDO_{in\ vitro}$ may therefore serve as a biomarker to examine the interaction of inflammation and tryptophan metabolism that underlies a subgroup of depressive symptomatologies.

Keywords: Depression; inflammation; IDO; tryptophan; serotonin; TNF-alpha; cytokines.

1. INTRODUCTION

The essential amino acid tryptophan is required for the synthesis of peripheral and central serotonin, a neurotransmitter that has been implicated in depressive disorders by numerous studies [1]. Due to its function as a regulator of mood, appetite and sexual behavior, inhibition of serotonin biosynthesis by decreased availability of tryptophan is considered a risk factor that promotes the development of depressive disorders [2].This view is supported by the effects of acute tryptophan depletion in experimental settings, inflicting various aspects of depressive symptomatology [3,4]. Physiologically two enzymes have been shown to regulate tryptophan blood levels: (1) the liver enzyme tryptophan-2,3-dioxygenase (TDO), which acts in the homeostasis of tryptophan levels and is activated by high blood tryptophan [5], and (2) indoleamin-2,3-dioxygenase 1 (IDO1), whose expression is induced in immune cells by inflammatory cytokines such as interferon-gamma (IFN-gamma), tumor necrosis factor alpha (TNF-alpha), interleukin 1 (IL-1) and interleukin 6 (IL-6) [6,7]. A second mammalian IDO gene, termed IDO2, has been discovered more recently, presumably deriving from gene duplication during evolution [8]. While strong IDO2 expression was observed in the mouse kidney and placenta, available data does not rule out IDO2 expression in human PBMCs [9]. For clarity, this report will refer to IDO1 and IDO2 mediated tryptophan degradation as IDO activity.

TDO and IDO degrade tryptophan at the expense of serotonin synthesis, activating the kynurenine pathway. Several kynurenine metabolites have been shown to enter the CNS and exert additional depressiogenic effects [10,11]. Decreasing the availability of tryptophan for protein synthesis during inflammation, IDO impedes the proliferation of pathogens. The concomitant reduction of serotonin synthesis promotes the lassitude commonly referred to as “sickness behavior” in animal models [12]. In conditions of chronic, sterile and/or inadequate inflammation however, enhanced IDO mediated tryptophan degradation is inefficient in immune defence but may contribute to depressive symptomatology by inhibition of serotonin synthesis [13,14].

Owing to its recognized significance in immune defence and depressive disorders, knowledge of individual IDO activity may yield valuable information for diagnosis and open alternative therapeutic pathways. IDO enzymatic activity has been quantified as

kynurenine/tryptophan ratio in cultures of stimulated PBMCs [15]. These studies demonstrate that IDO activity is increased due to immune activation in vitro. Furthermore, several IDO inhibitors with recognized antidepressant, antioxidant or antiinflammatory properties have been identified, such as St. John's wort, resveratrol, aspirin and cacao [16,17,18,19].

This paper represents a retrospective evaluation of laboratory parameters requested for medical purposes by several independent physicians since 2011. We present a modified protocol to measure IDO activity in vitro and compare IDO activity to blood levels of tryptophan and TNF-alpha. This is to our knowledge the first study to assess the physiological significance of an in vitro assay for IDO activity.

2. MATERIALS AND METHODS

2.1 Subjects

The retrospective data evaluation was based on a patient database comprising a total of 441 individuals (265 females) aged 9 to 83 years (mean 51.5 years) who had requested $IDO_{in\ vitro}$, tryptophan or TNF-alpha analysis upon consultation of several independent physicians in Germany between May 2011 and March 2013. The laboratory tests were performed solely in the context of medical diagnostics, as part of medical treatment schemes. According to the Helsinki declaration all patients gave written informed consent. An ethical approval was not required. All data was anonymized upon extraction from the database. Comparison of $IDO_{in\ vitro}$, tryptophan and TNF-alpha was carried out retrospectively between April and July 2013 in order to challenge the clinical significance of $IDO_{in\ vitro}$.

2.2 $IDO_{in\ vitro}$

PBMCs were isolated from 10 ml heparinized whole blood by ficoll density gradient centrifugation as previously described [20] and resuspended in RPMI (Biochrom) to a concentration of $1x10^6$ cells/ml. 2 x 1ml cell suspension from each patient were cultured in presence or absence of 10µg/ml PHA (Sigma Aldrich) in a 48-well flat bottom cell culture plate (Becton Dickinson) for 48 hours at 37ºC. Stimulation with IFN-gamma was performed by identical conditions replacing PHA by 2.5µg/ml Imukin (Boehringer Ingelheim Pharma). Supernatants were then collected after brief centrifugation (5 min, 1200rpm) and stored at -20ºC for further analysis. For $IDO_{in\ vitro,}$ analysis of tryptophan concentrations in PBMC supernatants was performed by ELISA (Labor Diagnostika Nord) following a modified version of the manufacturer's protocol, by omitting the precipitation step for both standard, controls and supernatants to increase sensitivity. Standard and controls were diluted 1:12.5 in phosphate buffered saline (PBS, PAA). Supernatants were not diluted. In the final calculation of tryptophan concentrations the supernatant values were divided by 12.5. $IDO_{in\ vitro}$ was calculated as the ratio of tryptophan in unstimulated versus PHA stimulated PBMC cultures. PHA stimulated proliferation of PBMCs was measured by lymphocyte transformation test (LTT) as previously described [20] and quantified as stimulation index (SI) accordingly.

2.3 Supernatant Tryptophan and Kynurenine by RP-HPLC

High pressure liquid chromatography (RP-HPLC) of tryptophan and kynurenine in supernatants was performed as described previously [21,22]. In brief, the supernatants were deproteinized with 2.4 M perchloric acid for 5 min. After centrifugation (5830 rpm for 15 min at 4ºC), supernatants were transferred into new tubes. The pH value adjusted to 7.0, and the 100 µl filtered supernatant was injected into a C-18 column (Supelco). Samples were eluted with PBS over 30 min. Tryptophan was monitored by means of its native fluorescence at the excitation wavelength of 285 nm and emission wavelength of 360 nm whereas kynurenine was detected simultaneously by ultraviolet (UV) absorption at the wavelength of 230 nm. The peaks of kynurenine and tryptophan were identified following their comparison with the retention time of previously determined standard compounds. Quantification was based on the ratios of the peak areas of the compound to the internal standard [21,22].

2.4 Blood Tryptophan and TNF-alpha by ELISA

Plasma tryptophan concentrations were analyzed by ELISA (Labor Diagnostika Nord) according to the manufacturer's instructions. In brief, samples, standards and controls were precipitated and subsequently derivatized according to the manufacturer`s manual. Incubation with the tryptophan antiserum was carried out over night at 4°C. Upon secondary antibody incubation and enzymatic colour reaction the signal intensities were measured using a Tecan reader at 450 nm. Serum TNF-alpha was analyzed by an automated immunoassay analyzer (Immulite™ 1000, Siemens) following the manufacturer's protocol.

2.5 Statistics

Association of $IDO_{in\ vitro}$ with plasma tryptophan and serum TNF-alpha was assessed by unpaired t test (GraphPad Software). Correlation coefficients were analyzed by Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1 Tryptophan Degradation in Stimulated PBMCs is an Indicator for IDO Activity

We modified the experimental setup by Schröcksnadel and coworkers [17]. For IDO induction, PBMCs were cultured for 48 hours in the presence of PHA. Following previous reports, IDO activity was measured as the ratio of concentrations of tryptophan (Trp_{stim}) and its metabolite kynurenin (Kyn_{stim}) in cell culture supernatants [15,17]. Comparison of Kyn_{stim}/ Trp_{stim} ratios with ratios of tryptophan in supernatants of unstimulated versus stimulated PBMC cultures (Trp_{basal}/Trp_{stim}) revealed a close correlation (correlation coefficient=0.99; Table 1). This close correlation argues that both Kyn_{stim}/ Trp_{stim} and Trp_{basal}/Trp_{stim} represent equally valid measures to quantify IDO activity. For clarity, we designated the ratio TRP_{basal}/TRP_{stim} as $IDO_{in\ vitro}$.

3.2 $IDO_{in\ vitro}$ is Not Determined by Cell Proliferation

In order to further validate $IDO_{in\ vitro}$ as a measure for IDO activity, we set out to evaluate the influence of cell proliferation both on tryptophan levels and $IDO_{in\ vitro}$. Cell proliferation was

measured in PHA stimulated PBMCs by thymidine incorporation in a lymphocyte transformation test (LTT) and quantified as stimulation index (SI) [20].There was no association between SI and $IDO_{in\ vitro}$ (correlation coefficient= -0.63; Fig. 1).

Table 1. Correlation between the ratios Kyn_{stim}/Trp_{stim} and Trp_{basal}/Trp_{stim} (“$IDO_{in\ vitro}$”)

	Trp_{basal} (µM)	Trp_{stim} (µM)	Kyn_{stim} (µM)	Kyn_{stim}/Trp_{stim}	Trp_{basal}/Trp_{stim} (“$IDO_{in\ vitro}$”)
1	15,06	3,02	3,17	1,05	4,99
2	14,22	8,85	< 0,5	< 0,06	1,61
3	14,42	6,53	1,84	0,28	2,21
4	14,68	6,44	< 0,5	< 0,78	2,28
5	16,59	0,61	4,1	6,72	27,20
6	15,53	1,89	4,46	2,36	8,22

Correlation coefficient =0.99

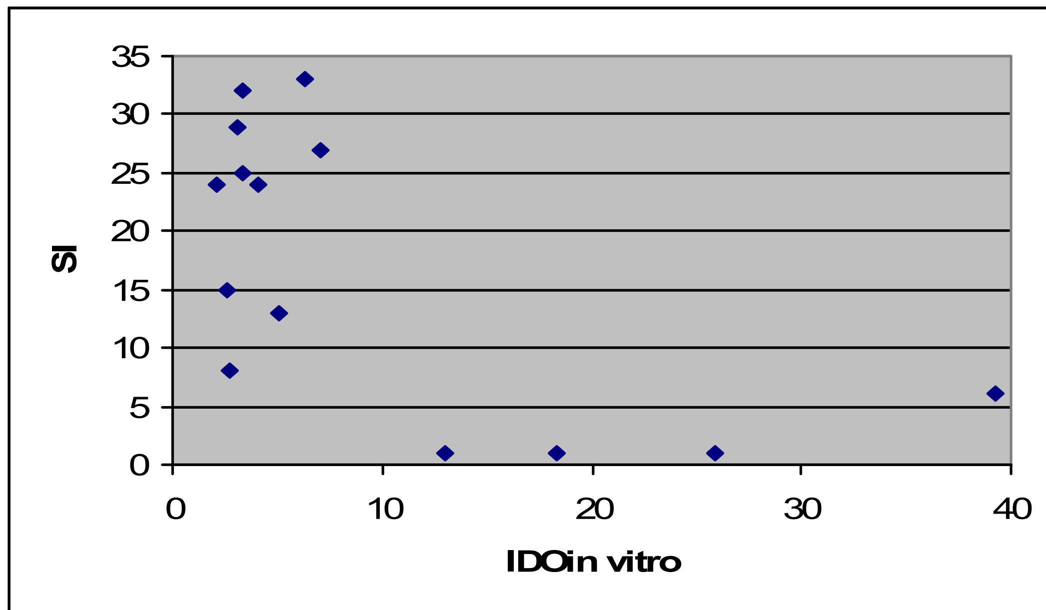

Fig. 1. $Ido_{in\ vitro}$ and cell proliferation measured by LTT (stimulation index, SI) did not correlate
correlation coefficient= -0.63

3.3 IFN-gamma and PHA Induce Similar Effects on $IDO_{in\ vitro}$

As IFN-gamma represents a well characterized inductor of IDO activity, we compared IFN-gamma and PHA mediated stimulation of $IDO_{in\ vitro}$. To this end, we cultured PBMCs in presence of 10µg/ml PHA or 2.5 µg/ml IFN-gamma. Comparison of the resulting $IDO_{in\ vitro}$ values showed a strong correlation between the PHA and IFN-gamma induced $IDO_{in\ vitro}$ values, albeit with a lower amplitude of IFN-gamma induced ratios (correlation coefficient= 0.91; Fig. 2).

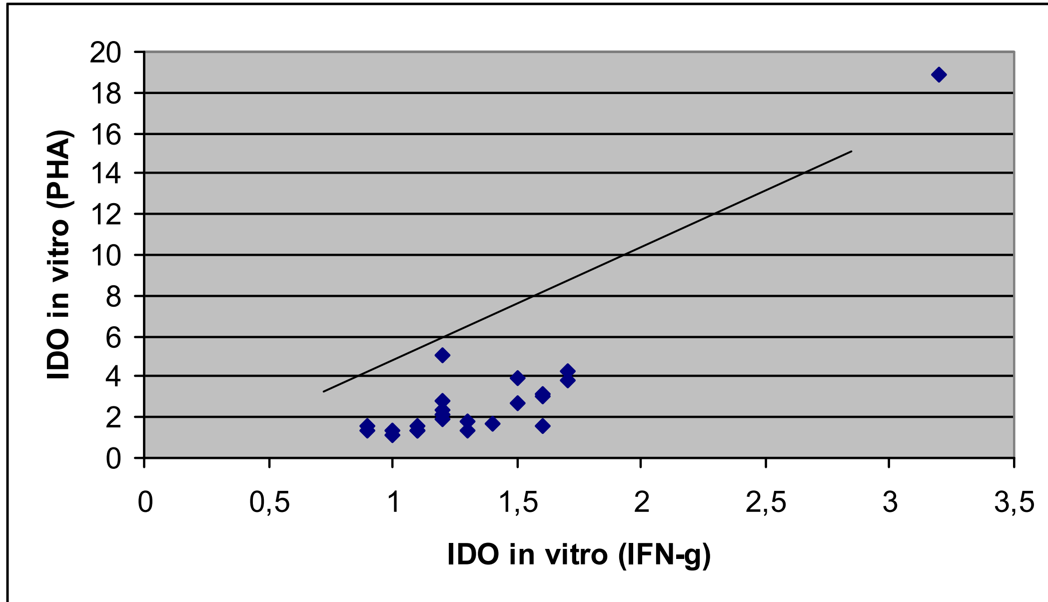

Fig. 2. PHA and IFN-gamma stimulated PBMC cultures yielded correlating $IDO_{in\ vitro}$ values
correlation coefficient =0.91

3.4 Low Plasma Tryptophan is Associated With High $IDO_{in\ vitro}$

In a next step, we set out to assess the physiological significance of our in vitro assay for IDO activity in vivo. As IDO has been shown to degrade tryptophan, we investigated whether high $IDO_{in\ vitro}$ correlated with decreased plasma tryptophan. Indeed, retrospective evaluation of clinical data from 398 patients revealed that $IDO_{in\ vitro}$ in patients with low (<0.9 mg/dl; n=84) tryptophan was significantly higher than in patients with high (≥0.9 mg/dl; n=314) tryptophan levels ($IDO_{in\ vitro}$=2.8±3.3 vs. 2.2±1.1; *P*=0.04 unpaired t test; Fig. 3).

3.5 Inflammation is Associated with Elevated $IDO_{in\ vitro}$

As inflammatory cytokines induce IDO expression in vivo, we next asked whether increased serum levels of the inflammatory cytokine TNF-alpha were associated with high $IDO_{in\ vitro}$. Retrospective evaluation of clinical data from 276 patients showed that increased serum levels of TNF-alpha α (≥8.3pg/ml, n=103) indeed correlated with higher $IDO_{in\ vitro}$ when compared to $IDO_{in\ vitro}$ in presence of normal TNF-alpha α levels (<8.3pg/ml, n=173; $IDO_{in\ vitro}$=2.7±3.5 vs. 2.1±1.6, *P*=0.05, unpaired t test; Fig. 4).

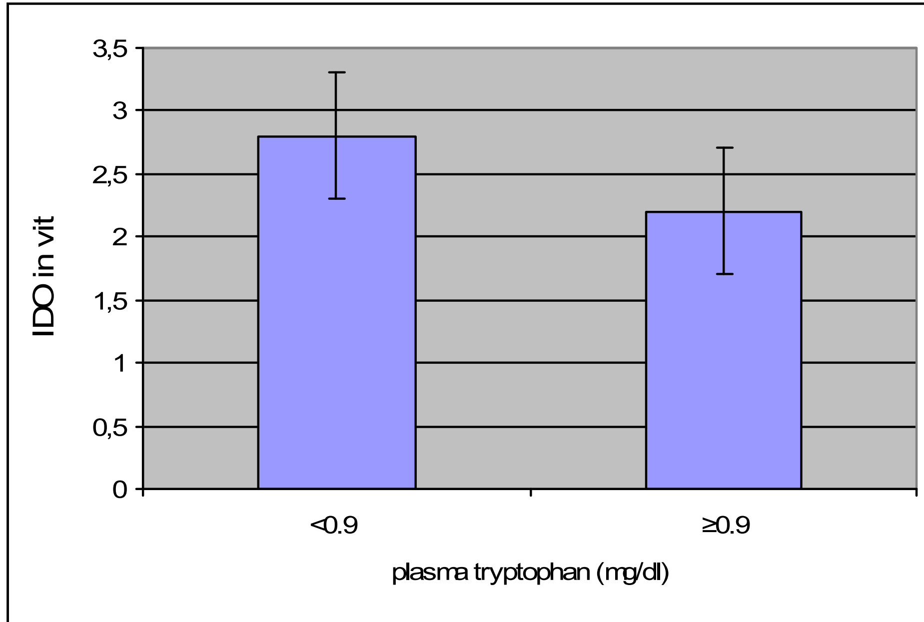

Fig. 3. Low (<0,9 mg/dl) plasma tryptophan is associated with significantly higher $IDO_{in\ vitro}$ than high (≥0,9 mg/dl) plasma tryptophan
(2.8±3.3 vs. 2.2±1.1; P=0,04 unpaired t test)

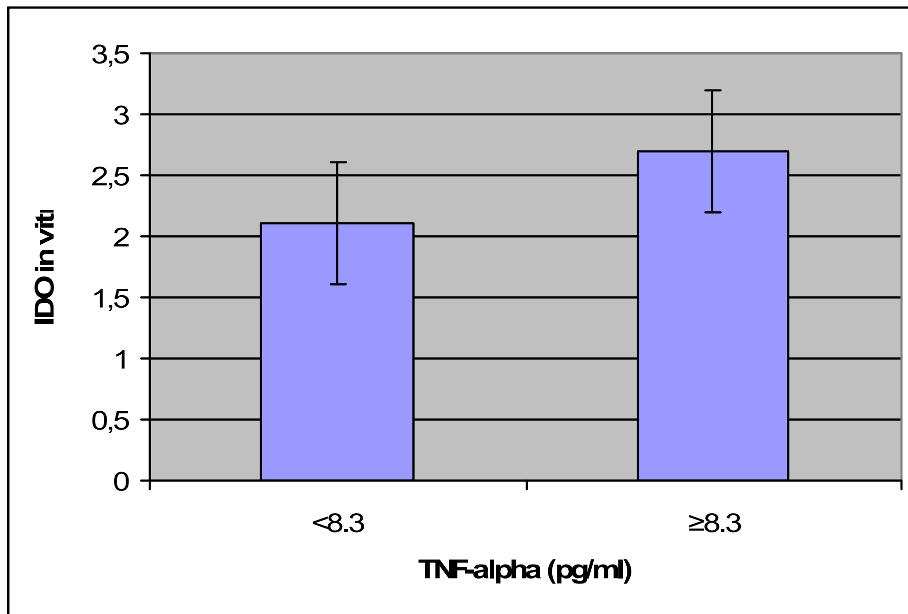

Fig. 4. High (≥8.3 pg/ml) serum TNF-alpha (α) is associated with significantly higher $IDO_{in\ vitro}$ than normal (<8.3pg/ml) serum TNF-alpha
(2.7±3.5 vs. 2.1±1.6; P=0.05 unpaired t test)

3.6 $IDO_{in\ vitro}$ Varies between Individuals

Our patient sample showed considerable variation of $IDO_{in\ vitro}$, ranging from 0,9 to 39 (Table 2 and data not shown). To investigate whether elevation of $IDO_{in\ vitro}$ was transient or constitutive we reviewed the data obtained from patients who had received repeated $IDO_{in\ vitro}$ measurements between May 2011 and March 2013. The majority of patients with increased $IDO_{in\ vitro}$ showed normal values at other time points (15/17 = 88%; Table 3). However, 2/17 patients (12%) repeatedly showed high $IDO_{in\ vitro}$, potentially indicating chronically elevated IDO activities in these individuals.

Table 2. Comparison of $IDO_{in\ vitro}$ and cell proliferation (stimulation index = SI) revealed no association

Sample	Viability [%]	$IDO_{in\ vitro}$	SI
1	97,5	5,1	13
2	96,6	4,1	24
3	97,4	2,1	24
4	92,4	2,7	8
5	97,6	3,3	25
6	99,0	3,1	29
7	98,0	2,6	15
8	90,4	6,3	33
9	91,6	7,1	27
10	89,1	13,0	1
11	91,4	3,3	32
12	92,8	39,2	6
13	89,7	18,3	1
14	89,0	25,9	1

correlation coefficient= -0.63

Table 3. Ranges of $Ido_{in\ vitro}$ values in patients with repeated analyses

$IDO_{in\ vitro}$	n
<3	19
<3 and ≥3	15
≥3	2

3.7 Discussion

Both the Kyn/Trp ratio in stimulated PBMCs published by Schroecksnadel and colleages and the $IDO_{in\ vitro}$ value presented here measure IDO activity based on tryptophan levels in PBMC cultures. For both protocols, a central question regards the extent to which mechanisms other than IDO mediated degradation may account for tryptophan consumption. A major influence of other metabolic pathways however seems unlikely for several reasons: (1) Expression of TDO, the other known tryptophan degrading enzyme, is restricted to the liver and therefore not present in PBMCs [5]; (2) although serotonin synthesis takes place in mononuclear cells upon PHA induction [23], this pathway usually consumes less than 5% of the available tryptophan [24]. Therefore we expect its effect on $IDO_{in\ vitro}$ (as well as on Kyn/Trp) to be rather small.

Importantly, tryptophan consumption by protein synthesis inevitably takes place in dividing cells and will lower tryptophan levels in PBMC cultures, introducing errors to Kyn/Trp as well as $IDO_{in\ vitro}$. It is therefore important that the comparison of $IDO_{in\ vitro}$ to both proliferation rates and IFN-gamma mediated stimulation argue against cell proliferation as a major determinant of $IDO_{in\ vitro}$, suggesting that tryptophan consumption by protein synthesis is low compared to tryptophan degradation by increased IDO activity.

The retrospective analysis of our clinical database shows that increased $IDO_{in\ vitro}$ values correlate with lower blood tryptophan levels as well as increased prevalence of high serum levels of TNF-alpha. This association with tryptophan metabolism and inflammation in vivo further supports the validity of $IDO_{in\ vitro}$ as a measure for systemic IDO activity. In fact, the observed correlations may even be stronger due to the presence of confounding factors that retrospectively could be excluded: (1) For our patients there is no data on conditions that reduce blood tryptophan independent of IDO, such as high TDO activity due to psychosocial stress [25] or decreased tryptophan resorption due to fructose malabsorption [26]. (2) Data on inflammatory cytokines other than TNF-alpha are not available for the majority of these patients. It is thus conceivable that some patients with low TNF-alpha will in fact show elevated Interleukin-1 or Interleukin-6 secretion, both recognized inducers of IDO activity [7]. An intriguing observation is that $IDO_{in\ vitro}$ varies considerably between different patients both upon PHA and IFN-gamma mediated induction, albeit less pronounced for the latter. Consistent with the concept of enhanced IDO activity during acute inflammation, most patients with high $IDO_{in\ vitro}$ showed normal values at other time points. Still, we observed repeatedly high $IDO_{in\ vitro}$ in a minority of patients. While the underlying mechanism cannot be investigated further in this study due its retrospective nature, we perceive three likely alternatives: (1) Repeatedly high $IDO_{in\ vitro}$ may be caused by repeated episodes of acute inflammation; (2) $IDO_{in\ vitro}$ may be chronically elevated due to either chronic inflammation or (3) genetically enhanced IDO activity. Indeed, genetic influences on IDO activity have been reported for polymorphisms in IDO itself [27,28] well as in factors interacting with IDO such as IFNG [29].

4. CONCLUSION

Taken together, the current report presents a modified cell based in vitro assay for IDO activity and evaluates its significance in a clinical context. Our data indicate that $IDO_{in\ vitro}$ may serve as a biomarker and diagnostic tool to characterize the individual's influence of inflammation on tryptophan metabolism. Furthermore, $IDO_{in\ vitro}$ may gain importance as a clue for potential therapeutic effects of tryptophan supplementation, indicating whether or not supplemental tryptophan may further increase any accumulation of kynurenine metabolites. Future studies will be necessary to further explore the regulation of IDO activity in chronic inflammatory disorders and to evaluate the possibilities of therapeutic intervention.

CONSENT

All patients gave written informed consent before blood collection.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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