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MAJOR ARTICLE

Increased Indoleamine-2,3-Dioxygenase Activity Is Associated With Poor Clinical Outcome in Adults Hospitalized With Influenza in the INSIGHT FLU003Plus Study

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Background. Indoleamine-2,3-dioxygenase (IDO) mediated tryptophan (TRP) depletion has antimicrobial and immuno-regulatory effects. Increased kynurenine (KYN)-to-TRP (KT) ratios, reflecting increased IDO activity, have been associated with poorer outcomes from several infections.

Methods. We performed a case-control (1:2; age and sex matched) analysis of adults hospitalized with influenza A(H1N1) pdm09 with protocol-defined disease progression (died/transferred to ICU/mechanical ventilation) after enrollment (cases) or survived without progression (controls) over 60 days of follow-up. Conditional logistic regression was used to analyze the relationship between baseline KT ratio and other metabolites and disease progression.

Results. We included 32 cases and 64 controls with a median age of 52 years; 41% were female, and the median durations of influenza symptoms prior to hospitalization were 8 and 6 days for cases and controls, respectively ($P = .04$). Median baseline KT ratios were 2-fold higher in cases (0.24 mM/M; IQR, 0.13–0.40) than controls (0.12; IQR, 0.09–0.17; $P \leq .001$). When divided into tertiles, 59% of cases vs 20% of controls had KT ratios in the highest tertile (0.21–0.84 mM/M). When adjusted for symptom duration, the odds ratio for disease progression for those in the highest vs lowest tertiles of KT ratio was 9.94 (95% CI, 2.25–43.90).

Conclusions. High KT ratio was associated with poor outcome in adults hospitalized with influenza A(H1N1)pdm09. The clinical utility of this biomarker in this setting merits further exploration.

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Keywords. influenza; indoleamine-2,3-dioxygenase; kynurenine; outcome; tryptophan.

L-tryptophan (TRP) is an essential amino acid for many life forms including humans. Tryptophan is also an essential amino acid for protein synthesis for some bacteria, many fungi, and possibly some viruses. It is also the precursor molecule for several important neurotransmitters, that is, serotonin and melatonin. The role of tryptophan in the immune response to many pathogens (eg, fungi, tuberculosis, trypanosomiasis, chronic viral infections) is an area of increased interest [1–3]. There is also considerable interest in the central role of tryptophan in the immune response and/or surveillance of malignant cells/tumors, and inhibitors of tryptophan metabolism (indoleamine 2,3-dioxygenase [IDO] inhibitors) are being developed as adjunctive immunotherapeutics in the cancer setting [4].

With respect to the role in the immune response to some pathogens, tryptophan depletion through the catabolic enzyme, IDO, is thought to be a mechanism to "starve" pathogens of this essential amino acid, but in turn it is coupled with a damping down of the immune response, as downstream metabolites of tryptophan affect the host immune response as well. There appear to be 3 tryptophan-catabolizing enzymes [5], of which IDO1 appears to be most important in the immune response to pathogens. IDO1 catabolizes TRP to kynurenine (KYN), an aryl hydrocarbon receptor ligand; subsequent catabolism of

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*See the full listing of the INSIGHT FLU003 Plus Study Group after the References.

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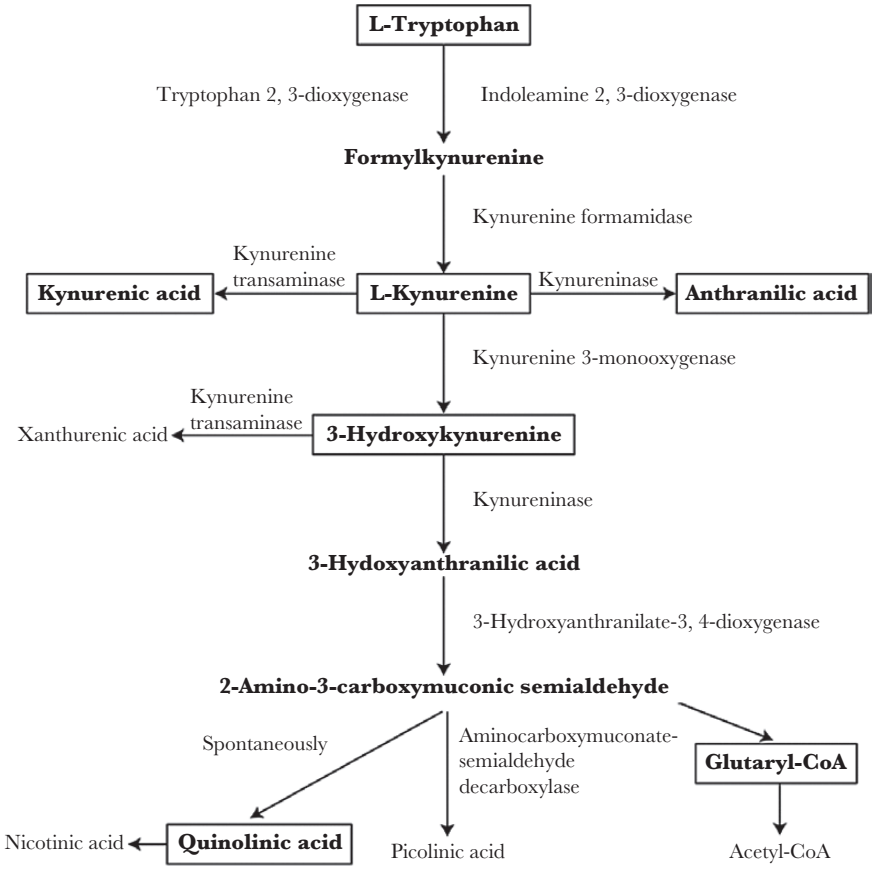

Figure 1. L-tryptophan metabolic pathway.

KYN leads to a number of KYN pathway metabolites (Figure 1). IDO1, found in the placenta, gut, and T cells, appears to play an important role in immune tolerance. IDO activity is induced by the immunomodulatory cytokine interferon-gamma (IFN-γ; which also appears to play an important role in mobilizing tryptophan into cells), other pro-inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), amyloid peptides, and lipopolysaccharides. Increased IDO activity ultimately leads, via the production of KYN (the "L-Kynurenine shunt"), to T-cell apoptosis, reduced T-cell proliferation, and an anergic state, with increased immunosuppressant, T-regulatory cells [6, 7]. The exact pathways for the interaction between antigen-presenting cells and the suppression of T-cell activity via IDO is not completely understood.

Increased plasma KYN levels and KYN-to-TRP (KT) ratios (as a measure of IDO activity) have been found in patients with systemic inflammatory response syndrome, sepsis, and septic shock, but as yet, it is unclear what the findings mean [8]. Suzuki and colleagues [9] explored the correlation between KT ratio and clinical outcome in patients hospitalized with community-acquired pneumonia (CAP). In this group of patients, there was a significant positive correlation between both KYN levels and KT ratio with severity of CAP. Moreover, when these CAP patients were divided into nonsepsis, sepsis, severe sepsis, and septic shock categories, there was a clear correlation between increasing sepsis severity and increasing levels of KYN, decreasing levels of TRP, and, as a result, increasing KT ratios indicative of IDO activation. Nonsurvivors also had significantly higher KT ratios than survivors [9].

To date, relatively little is known about the role of IDO in the human immune response to influenza, with most published data from murine influenza models. In the murine influenza model, influenza induced IDO activity in mouse lung tissue and draining lymph nodes [10]. Moreover, IDO knockout mice and mice treated with IDO inhibitors had better outcomes [11, 12].

In a multiplex biomarker analysis of patients enrolled with influenza A(H1N1)pdm09 virus in FLU003 Plus (see the Methods), Davey and colleagues [13] found several biomarkers that predicted poor clinical outcomes (defined as death, in-patient stay of >28 days, or intensive care unit [ICU] admission), including markers of macrophage activation/chemokines, T-cell activation, and acute phase reactants.

We therefore hypothesized that increased KT ratio would be associated with poor clinical outcomes (as defined by Davey and colleagues [13]) from influenza. In this analysis, we present novel data on the association of increased KT ratio and disease

progression and the association of KT ratio with the selected biomarkers identified by Davey and colleagues [13] and, additionally, interleukin-17 (IL-17) and interferon-gamma (IFN-γ), chosen specifically because of their role in regulating IDO activity.

METHODS

FLU003Plus is an ongoing, international observational study of adults hospitalized with influenza that began in 2009, following the emergence of the influenza A(H1N1)pdm09 virus [14]. Participants are eligible for enrollment in FLU003Plus if they have laboratory-confirmed influenza based on a local nucleic acid test (NAT) or influenza is suspected and a local NAT test has been performed. At enrollment, an upper respiratory tract swab is sent to a central laboratory for confirmation of influenza using polymerase chain reaction (PCR)–based NAT. FLU003Plus captures a wide range of clinical information, including the reasons for hospitalization, type of ward to which the patient was first admitted, and in subsequent visits at day 28 and day 60, clinical status including death. The primary end point of FLU003Plus is disease progression, defined as a composite of death, prolonged hospitalization >28 days, and postenrollment intensive care/mechanical ventilation/extracorporeal membrane oxygenation (ECMO) within 60 days of enrollment.

Ethics Statement

The FLU003Plus protocol and information statement and consent form were approved by both the local institutional ethics committees/review boards of the participant sites and the ethics committee of the sponsor of this study, the University of Minnesota. All participants or their representatives (when participants were unable to consent for themselves and where the ethics permission allowed for consent by a third party) provided written informed consent prior to their enrollment.

Study Design and Objectives

This was a matched case-control study. Cases were FLU003Plus patients with PCR-confirmed influenza A(H1N1)pdm09 virus with disease progression; controls had PCR-confirmed influenza A(H1N1)pdm09 virus and were matched on age (+/-4 years) and sex. Cases and controls were chosen from a subset of 209 FLU003Plus participants who were the focus of previous work on biomarkers [13]. Our primary objective was to explore the association of baseline (ie, the sample taken at the time of enrollment into FLU003Plus) KT ratio, as a marker of IDO activity, with disease progression. Key secondary objectives explored the association of baseline KT ratio with death, the multiplex panel of inflammatory biomarkers (as described by Davey et al. [13]), and baseline IFN-γ and IL-17. As described by Davey et al. [13], biomarkers were classified as belonging to 1 of 4 groups, that is, macrophage proinflammatory activation response, acute phase response, T-cell activation response, and macrophage chemokine response.

Laboratory Methods

Plasma Samples Preparation

Local labs at the sites stored plasma samples using the methodology described in the FLU003Plus laboratory manual. At study enrollment, blood was drawn into EDTA tubes and processed within 4 hours. All samples were centrifuged at room temperature at 1200 g × 15 minutes, and the plasma aliquoted. These aliquots were then either stored immediately at −70/−80°C or initially at −20°C for a maximum of 4 days before being moved (on dry ice) into a −70/−80°C freezer. All samples used in this analysis had undergone 1 freeze-thaw cycle.

Mass Spectrometry Analysis

Sample Preparation

The method used is as described by Gulcev and colleagues [15]. Aliquots of 100 µL plasma had a heavy standard of 3 µL of 100 µM of kynurenine D6 and 3 µL of 1 mM tryptophan 13C11 (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) added prior to any preparation. These aliquots were then mixed with 400 µL of ice-cold solvent (100% methanol), vortexed, and placed on ice for 10 minutes. Samples were then centrifuged at 13 000× g for 10 minutes at 4°C, and the supernatant was removed and transferred into a clean low-retention vial. This step was repeated once. Samples were concentrated using a vacuum centrifuge to ~50 µL. Formic acid was used to acidify the plasma samples that were added to the starting buffer used in ultraperformance liquid chromatography (5% acetonitrile, 95% water, 0.1% formic acid) to 100 µL.

Untargeted Mass Spectrometry Analysis

Undiluted sample (10 uL) was injected into a Thermo Q-Exactive liquid chromatography–mass spectrometry (LC-MS; ThermoFisher Scientific, Marietta, OH) at 40°C. The samples were subjected to a gradient going from Buffer A to Buffer B over 15 minutes, then flushing for 5 minutes. Buffer A consisted of 99.9% water with 0.1% formic acid, while Buffer B was 99.9% acetonitrile with 0.1% formic acid.

XCMS Processing

The RAW files from the Q-exactive were converted into mzXML using MSconvert [16] and processed using XCMS online under the Q-Exactive parameters. The resulting file was used to extract intensities of a specific tryptophan and kynurenine metabolite's m/z and RT.

Liquid Chromatography–Tandem Mass Spectrometry Selective Reaction Monitoring Analysis of Tryptophan and Kynurenine

Diluted (1:1000 tryptophan and 1:100 for kynurenine) samples (20 µL) were subjected to injection using an Agilent autosampler liquid chromatography–tandem mass spectrometry (LC-MS/MS) with an analytical Waters Symmetry C18, 3.5-µm column connected to the 5500 iontrap (Sciex, Framingham, MA) fitted with a turbo V electrospray source. The samples were subjected

to a linear gradient of 2% acetonitrile, 0.1% formic acid to 98% acetonitrile 0.1% formic acid for 10 minutes at a column flow rate of 250 μL/min. Transitions monitored are in Table S1. The data were analyzed using MultiQuant (Applied Biosystems, Foster City, CA), which provided the peak area for the transitions. A standard curve was constructed using concentration ratios of heavy tryptophan/tryptophan and heavy kynurenine/kynurenine from fentomole to nanomole in 20 μL.

Measurement of IL-17 and IFN-γ

IL-17 and IFN-γ were measured using the Luminex platform at the University of Minnesota Cytokine Reference Laboratory (CLIA'88 licensed). Following the manufacturer's instructions, fluorescent magnetic beads (R&D Systems, Minneapolis, MN) coated with IL-17 and IFN-γ antibodies were added to each sample. After incubation and washing, biotinylated detection antibody was added, followed by phycoerythrin-conjugated streptavidin. The beads were read on a dual-laser fluidics–based Luminex instrument (Bioplex 200) that determines the analyte being detected via color coding; the other measures the magnitude of the PE signal from the detection antibody, which is proportional to the amount of analyte bound to the bead. Samples were run in duplicate, and values were interpolated from 5-parameter fitted standard curves.

Measurement of Other Inflammatory Biomarkers

Other inflammatory markers (see Table 1) were previously published [13] and were used in this current analysis.

Statistical Methods

With 96 samples, power was 80% to detect a difference of 0.089 mM in KT ratios between cases and controls. Descriptive statistics were used to summarize the baseline characteristics. Spearman rank correlation coefficients were used to explore associations of baseline biomarkers with KT ratio. Conditional logistic regression was used to summarize the association of

Table 1. Clinical Characteristics, Kynurenine, Tryptophan, and KT Ratio, Multiplex Panel of Inflammatory Biomarkers of Cases and Controls Hospitalized With Influenza A(H1N1)pdm09

	Case (n = 32)	Control (n = 64)	
	No. (%) or Median (25th, 75th %)	No. (%) or Median (25th, 75th %)	*P* Value[a]
Female[b]	13 (41)	26 (41)	-
Age[b]	52 (41, 60)	53 (40, 60)	-
Nonwhite race	7 (22)	13 (20)	.84
Smoker	10 (36)	22 (34)	.86
Days since onset of influenza symptoms	8 (6, 10)	6 (4, 7)	.04
Asthma or chronic obstructive pulmonary disease	6 (19)	14 (22)	.70
Immune suppressive condition/treatment	8 (25)	7 (11)	.33
Cardiovascular or chronic liver/renal disease	8 (25)	11 (17)	.10
KYN μM	6.1 (3.9, 12.2)	3.9 (3.0, 5.9)	.003
TRP μM	32.4 (23.7, 40.7)	37.0 (27.1, 44.9)	.10
KT ratio	0.24 (0.13, 0.40)	0.12 (0.09, 0.17)	<.001
Macrophage proinflammatory activation response biomarkers			
IL-6, pg/mL	17.2 (12.9, 24.5)	10.7 (4.2, 19.0)	.01
TNA-α, pg/mL	14.4 (11.2, 18.4)	12.4 (10.3, 16.1)	.02
CD163, ng/mL	1608 (963, 2629)	710 (516, 1078)	<.001
sICAM-1, ng/mL	526 (294, 791)	237 (95.7, 404)	.002
IL-8, pg/mL	50.4 (26.0, 77.3)	23.5 (13.7, 44.7)	.004
Acute phase response biomarkers			
D-dimer, μg/mL	3.55 (1.4, 5.0)	1.04 (0.6, 1.7)	<.001
LBP, μg/mL	35.4 (14.1, 57.3)	18.2 (9.7, 45.0)	.07
sVCAM-1, ng/mL	715 (527, 970)	392 (177, 667)	.002
T-cell activation response biomarkers			
IL-2, pg/mL	3.67 (2.4, 7.9)	2.08 (1.2, 4.2)	.009
IL-10, pg/mL	26.7 (11.6, 95.5)	10.8 (6.7, 19.4)	.003
Macrophage chemokine response biomarkers			
MCP-1, pg/mL	1164 (539, 2440)	585 (379, 930)	.001
IP-10, pg/mL	3160 (919, 7308)	1068 (518, 2453)	.009

For biomarkers, values are $\log_{10}$ transformed for significance testing.

Abbreviations: IL, interleukin; KT, kynurenine-to-tryptophan ratio; KYN, kynurenine; LBP, lipopolysaccharide-binding protein; TRP, tryptophan.

[a]Univariate conditional logistic.

[b]Matching factor.

the baseline KT ratio at enrollment with disease progression. Odd ratios (ORs) for upper and middle vs lower tertiles of the KT ratios are provided with the 95% confidence intervals and *P* values. This analysis was repeated with adjustment for duration of symptoms at enrollment, with and without additional adjustment for $\log_{10}$ transformed biomarkers IL-10, sVCAM1, IL-2, and MCP-1. These particular biomarkers were chosen as they represented the biomarkers most strongly related to disease progression in the categories of macrophage proinflammatory activation response, acute phase response, T-cell activation response, and macrophage chemokine response, respectively. *P* values were not adjusted for these multiple comparisons.

RESULTS

Thirty-two participants met our case definition; 22 of these died. Two controls were available for all cases. Cases had been symptomatic for a median of 8 days, whereas controls had been symptomatic for a median of 6 days ($P = .04$ for the difference) (Table 1). Median baseline KT ratios were 2-fold higher for cases (0.24 mM/M; 25th, 75th percentiles, 0.13, 0.40) than controls (0.12 mM/M; 25th, 75th percentiles, 0.09, 0.17; $P \leq .001$). All 12 inflammatory biomarkers except lipopolysaccharide-binding protein were significantly elevated in cases compared with controls (Table 1). The correlation of KYN, TRP, and KT ratios with each of the 12 biomarkers is described in Table 2; all 12 of these biomarkers correlated with the KT ratio.

When KT ratios were divided into tertiles (Table 3), 60% of cases vs 20% of controls had a KT ratio in the highest tertile (0.21–0.84 mM/M), and 16% of cases vs 39% of controls had a ratio in the lowest tertile (0.04–0.10 mM/M). Table 3 shows the unadjusted and adjusted (for duration of symptoms at the time of enrollment) conditional logistic analyses. The unadjusted odds ratio (cases vs controls) was 5.94 (95% CI, 1.7–20.3) for those in the highest tertile of KT ratio as compared with the lowest tertile. When adjusted for symptom duration alone, the OR for disease progression (ie, case vs control status) for those in the highest vs lowest KT ratio tertile was 9.94 (95% CI, 2.25–43.90; $P \leq .001$). When restricted to the cases who died (n = 22), the OR for death (cases vs controls, and adjusted for symptom duration) for those in the highest vs lowest KT ratio tertile was 12.14 (95% CI, 1.69–87.25; $P = .004$; data not shown).

The analysis was repeated adjusting for duration of symptoms at enrollment and $\log_{10}$ transformed biomarkers IL-10, sVCAM1, IL-2, and MCP-1. With this adjustment, the predictive value of the highest KT ratio tertile vs the lowest tertile for disease progression, was attenuated to 3.34 (95% CI, 0.55–20.33; $P = .06$; data not shown).

In addition, we explored the relationship of poor outcome with downstream metabolites of L-kynurenine (Figure 1). We found clear associations between poor outcomes and higher levels of downstream KYN metabolites that included kynurenic acid, anthranilic acid, 3-hydroxykynurenine, and quinolinic acid (Table 4). One downstream metabolite, glutaryl-CoA, was decreased in cases compared with controls.

IFN-γ and IL-17 were measured in cases and controls; 83% of the IFN-γ levels were below the lower detection limit (data not shown), and therefore we could not analyze relationships between IFN-γ and outcomes. IL-17 levels (Table 5) were categorized as below the lower detection limit, and approximate median levels in those with detectable results. Equivalent numbers of cases and controls had IL-17 levels below the detection limit at study enrollment.

Table 2. Correlation of Baseline Biomarkers With KYN, TRP, and the KT Ratio

	KYN		TRP		KT Ratio	
	Coeff	*P* Value	Coeff	*P* Value	Coeff	*P* Value
Macrophage proinflammatory activation response biomarkers						
IL-6, pg/mL	0.44	<.001	–0.06	.55	0.40	<.001
TNA-α, pg/mL	0.53	<.001	–0.04	.69	0.47	<.001
CD163, ng/mL	0.51	<.001	–0.13	.20	0.50	<.001
sICAM-1, ng/mL	0.20	.05	–0.12	.25	0.26	.010
IL-8, pg/mL	0.62	<.001	–0.08	.45	0.55	<.001
Acute phase response biomarkers						
D-dimer, µg/mL	0.42	<.001	–0.09	.36	0.45	<.001
LBP, µg/mL	0.38	<.001	–0.05	.66	0.35	<.001
sVCAM-1, ng/mL	0.24	.02	–0.08	.44	0.28	.005
T-cell activation response biomarkers						
IL-2, pg/mL	0.54	<.001	–0.05	.64	0.49	<.001
IL-10, pg/mL	0.51	<.001	–0.13	.19	0.53	<.001
Macrophage chemokine response biomarkers						
MCP-1, pg/mL	0.56	<.001	–0.19	.06	0.58	<.001
IP-10, pg/mL	0.63	<.001	–0.08	.45	0.56	<.001

P Values are from a univariate conditional logistic model.
Abbreviations: IL, interleukin; KT, kynurenine-to-tryptophan ratio; KYN, kynurenine; LBP, lipopolysaccharide-binding protein; TRP, tryptophan.

DISCUSSION

Despite the relatively small cohort of patients studied, our data reveal a strong association between high KT ratio and poor clinical outcome in adults hospitalized with influenza.

The high levels of metabolites of the tryptophan metabolic pathway also strengthen the evidence for IDO activation in this setting. IDO is induced by pro-inflammatory cytokines such as TNF-α and IFN-γ following viral infection. In murine models, influenza induces IDO expression in lung tissue and lymph nodes [10, 11], and IDO inhibitors improve T-cell responses toward the virus [12]. Most of the effects of tryptophan catabolism come from accumulation of its active downstream metabolites, many of which modulate the inflammatory state. Kynurenic acid is a potent agonist of the orphan G-protein-coupled receptor GPR35, whose expression in T cells leads to an immunosuppressive phenotype [17]. In monocytes and macrophages, the interaction of GPR35 with kynurenic acid downregulates the pro-inflammatory effects of bacterial lipoloysaccharide

Table 3. Odds Ratios for Tertiles of KYN, TRP, and KT Ratio

	Case (n = 32)		Control (n = 64)		Unadjusted[a]		Adjusted[a]	
Tertiles	No.	Pct.	No.	Pct.	OR[a]	95% CI	OR[a]	95% CI
Kynurenine, μM								
2.00–3.67	6	18.8	26	40.6	ref.		ref.	
3.68–5.89	9	28.1	23	35.9	1.55	0.51–4.70	2.61	0.64–7.28
5.90–33.9	17	53.1	15	23.4	4.25	1.39–12.98	5.95	1.60–22.08
P value, trend					.003		.005	
Tryptophan, μM								
9.7–28.3	13	40.6	19	29.7	2.46	0.80–7.58	3.73	1.07–13.01
28.4–41.0	12	37.5	20	31.3	2.10	0.70–6.27	3.11	0.90–10.67
41.1–62.1	7	21.9	25	39.1	ref.		ref.	
P value, trend					.10		.06	
KT ratio, mM/M								
0.04–0.10	5	15.6	25	39.1	ref.		ref.	
0.11–0.20	8	25.0	26	40.6	1.50	0.40–5.64	1.96	0.44–8.79
0.21–0.84	19	59.4	13	20.3	5.94	1.74–20.33	9.94	2.25–43.90
P value					<.001		<.001	

Abbreviations: KT, kynurenine-to-tryptophan ratio; KYN, kynurenine; OR, odds ratio; TRP, tryptophan.

[a]Conditional logistic. Adjusted model contains covariates for duration of symptoms at time of enrollment. *P* value shown is for lab result as a continuous variable.

[18–20]. Quinolinic acid is an end-product of L-kynurenine metabolism and is a known agonist to N-methyl-D-aspartate receptors in nerve cells. Quinolinic acid also generates reactive oxygen species capable of inducing the secretion of potent chemokines and pro-inflammatory cytokines [21, 22]. The metabolite 3-hydroxykynurenine (3-HK) is a redox active

Table 4. Odds Ratios for Tertiles of KYN Metabolites

	Case (n = 32)		Control (n = 64)		Unadjusted[a]		Adjusted[a]	
Tertiles (log_{10})	No.	Pct.	No.	Pct.	OR[a]	95% CI	OR[a]	95% CI
Kynurenic acid								
6.54–6.93	8	25.0	24	37.5	ref.		ref.	
6.93–7.13	9	28.1	23	35.9	1.01	0.29–3.46	0.88	0.24–3.17
7.14–8.99	15	46.9	17	26.6	2.86	0.94–8.70	2.34	0.72–7.62
P value, trend					.002		.004	
Anthranilic acid								
6.34–6.71	6	18.8	26	40.6	ref.		ref.	
6.72–6.90	9	28.1	23	35.9	1.84	0.52–6.44	1.88	0.50–7.00
6.91–7.72	17	53.1	15	23.4	5.45	1.52–19.44	5.59	1.43–21.84
P value, trend					<.001		.002	
3-hydroxykynurenine								
6.26–6.72	6	18.8	26	40.6	ref.		ref.	
6.73–7.09	8	25.0	24	37.5	1.10	0.32–3.78	1.49	0.38–5.90
7.10–8.25	18	56.3	14	21.9	3.98	1.39–11.36	5.18	1.54–17.42
P value, trend					.003		.003	
Quinolinic acid								
5.66–6.58	7	21.9	25	39.1	ref.		ref.	
6.59–6.92	7	21.9	25	39.1	1.09	0.31–3.92	1.59	0.38–6.60
6.93–8.32	18	56.3	14	21.9	4.01	1.28–12.54	5.21	1.42–19.10
P value, trend					.001		.001	
Glutaryl-CoA								
5.03–6.59	17	53.1	15	23.4	37.52	4.19–336.1	27.39	3.01–249.4
6.60–6.92	13	40.6	19	29.7	16.96	2.16–133.4	16.07	2.02–127.8
7.08–7.72	2	6.3	30	46.9	ref.		ref.	
P value, trend					.003		.009	

Abbreviations: KYN, kynurenine; OR, odds ratio.

[a]Conditional logistic. Adjusted model contains a covariate for duration of symptoms at time of enrollment. *P* value shown is for lab result as a continuous variable.

Table 5. Odds Ratios for Categories of Baseline IL-17 Levels

	Case (n = 32)		Control (n = 64)		Unadjusted[a]		Adjusted[a]	
IL-17 Levels	No.	Pct.	No.	Pct.	OR[a]	95% CI	OR[a]	95% CI
Undetectable	15	46.9	30	46.9	ref.		ref.	
0.16–0.59 pg/mL	6	18.8	18	28.1	0.61	0.18–2.06	0.33	0.08–1.33
0.60–15.5 pg/mL	11	34.4	16	25.0	1.30	0.49–3.43	0.98	0.34–2.78

Abbreviation: OR, odds ratio.

[a]Conditional logistic. Adjusted model contains covariate for duration of symptoms at time of enrollment.

compound that regulates the local oxidative status. In a pro-oxidative state, 3-HK demonstrates cellular toxicity [23].

Although the effect of downstream metabolites was diluted somewhat when we controlled for baseline levels of other cytokines, there was still a trend to significance for the highest KT ratio tertile compared with the lowest as a predictor of poor clinical outcome. It is important to note that we did not set out to show that KT ratio performed better than the multiplex panel of inflammatory cytokines/vascular markers. Rather, our aim in exploring this association was to try and understand the biological relationship between these inflammatory markers and the IDO pathway in this setting. Our data suggest that activation of the IDO pathway might contribute to poor outcomes, independent of other inflammatory pathways.

We were unable to quantify IFN-γ levels in the majority of patients in this subanalysis, which we suspect was due to a technical issue related to the samples utilized having already undergone 1 freeze-thaw cycle [24] and this cytokine being vulnerable to a freeze-thaw cycle in a way that assessment of amino acids and their metabolite measured using LC-MS/MS are not. Another reason for this may have been the prolonged interval from symptom onset to sampling or inadequate responses [25]. IL-17 appears to have a dual role both as a pro- and anti-inflammatory cytokine, especially when produced by regulatory T cells [26–29]. With respect to IL-17, there was no clear difference in the baseline levels between cases and controls, and as such, in this analysis at least, we have no evidence that IL-17 was contributing to the cytokine storm or, conversely, and reflecting its dual role, contributing to an immunosuppressive state.

In this analysis, we chose death and/or mechanical ventilation as the end points of interest, as both are clinically important, robust, and verifiable. While sites were required to report suspected/confirmed infections with other pathogens including bacteria, the study did not require the submission of supporting documentation. As such, we felt we could not verify these sepsis/bacterial infection events with sufficient certainty to include them as either an end point or covariate in the analysis. Our findings, however, may still represent the net effect of influenza plus secondary bacterial (and/or other pathogen) infections in both cases and controls, especially as they are similar to the findings revealed by other groups that have demonstrated a strong association between elevated IDO activity and poor clinical outcomes in those with CAP +/- sepsis and septic shock [3, 9] or sepsis alone [30]. Moreover, in these other studies, IDO activity was predictive of mortality, unlike the respiratory rate or the C-reactive protein level [9]; in 1 study of sepsis, decreases in the median KT ratio over time correlated with decreases in the Sequential Organ Failure Assessment score, an objective assessment of improving clinical outcome [30]. While tryptophan deficiency alone can lead to cell dysfunction or death, most of the effects of tryptophan catabolism come from accumulation of its active downstream metabolites, such as kynurenine [31, 32], which inhibits the clonal expansion of $CD4^+$ T cells, leading to increased tolerance and immunosuppression. Quinolinic acid, another downstream metabolite, in nanamolar concentrations, is metabolized to nicotinic acid mononucleotide and the nicotinamide adenine dinucleotide via a salvage pathway, but in high concentration, it is directly toxic to cells [22, 32].

It will be important to expand our understanding of the role of this immunoregulatory metabolic pathway in all types of influenza (and other concurrent infections) and the relative balance of immune activation vs immunosuppression. These data will help discern whether the many IDO inhibitors currently in development as adjunctive therapies for cancer may have potential clinical utility as therapeutic agents in severe influenza and/or other infections. However, clinicians considering the use of IDO inhibition in this setting should proceed with caution, as drugs inhibiting 1 part of an interlinked and complex metabolic pathway may have a detrimental effect via the upregulation of compensatory pro-inflammatory and/or immunosuppressive pathways.

Conclusion and Future Directions

In summary, in this small case-control study, we have shown that adults hospitalized with influenza A(H1N1)pdm09 and high KT ratio have greater odds of progression to death or mechanical ventilation. Measurement of this biomarker can be performed on plasma or serum, utilizing a standard tandem mass spectrometry approach available in most analytical laboratories; moreover, the assay is robust and reproducible across different laboratories. While this initial study reveals IDO activity as a putative biomarker to identify those at risk of deterioration, our findings need to be confirmed in further studies of all types/subtypes of influenza, preferably with longitudinal

sampling, in which clinical outcomes are rigorously captured. Lastly, future studies in which peripheral blood mononuclear cells are collected and isolated would potentially identify the cellular production of IDO and give insight into the relationship of this metabolic pathway with the innate immune response.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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