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Increased plasma arginase activity in human sepsis: association with increased circulating neutrophils

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Abstract

Background—The pathophysiology of sepsis is incompletely understood. Impaired bioavailability of L-arginine, the substrate for NO synthesis, is linked to sepsis severity, and plasma arginase has been linked to hypoargininemia in other disease states. Circulating neutrophils are increased in sepsis and constitutively express arginase. We investigated whether plasma arginase activity is increased in human sepsis and whether this is associated with neutrophil numbers and activation.

Methods—We used HPLC and a radiometric assay to evaluate plasma amino acid concentrations and plasma arginase activity. The relationships between plasma arginase activity, neutrophil count, neutrophil activity and plasma L-arginine and arginine metabolites were evaluated in 44 sepsis patients and 25 controls.

Results—Plasma arginase activity was increased in sepsis patients, correlated with neutrophil count ($r=0.44$; $p=0.003$), but was independent of sepsis severity (SOFA or APACHE II score). Plasma HNP1-3 correlated with neutrophil count ($r=0.31$; $p=0.04$), was elevated in shock (median 180 ng/mL versus 83 ng/mL sepsis without shock, $p=0.0006$) and correlated with SOFA score. Sepsis patients with high neutrophil counts had significantly higher plasma HNP1-3 and arginase

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Author's contributions: Study design was performed by CJD, JSD, TW, YRM, TWY and NMA. Patient recruitment was carried out by JSD. Laboratory sample processing was performed by CJD, KAP and TW. HPLC assays were undertaken by YRM and CJD. Arginase activity assays were performed by YC with assistance from BJW. The data were analysed by CJD with help from JSD and NMA. The manuscript was drafted by CJD, JSD, TW and NMA. All authors had access to all data and contributed to the final draft of the paper. All authors read and approved the final manuscript.

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activity and lower plasma L-arginine concentrations than those with lower neutrophil counts and controls.

Conclusions—Plasma arginase activity, potentially derived in part from neutrophil activation, is elevated in sepsis, and may contribute to impaired bioavailability of L-arginine in sepsis.

Keywords

plasma arginase activity; L-arginine; sepsis; hypoargininemia

Background

Sepsis, a systemic inflammatory response to infection, is the most common reason for intensive care unit admission in the United States (1). Despite advances in management, severe sepsis still has a case-fatality rate of over 30% (1) and its pathophysiology is incompletely understood. Emerging data suggest that vascular dysfunction in severe sepsis is a state of endothelial nitric oxide (NO) deficiency (2, 3). L-Arginine is a precursor of NO, therefore there is renewed interest in hypoargininemia in sepsis (3, 4). L-arginine is essential for endothelial (5), microvascular (5), and immune (6) function. We have previously shown that the ratio of plasma L-arginine to asymmetric dimethylarginine (ADMA), an indicator of L-arginine bioavailability to nitric oxide synthase, correlates with disease severity and microvascular reactivity in sepsis (7). Plasma arginase activity has been linked to hypoargininemia and disease severity in other critical illnesses (8) and a recent study has demonstrated increased whole body arginase activity in sepsis (9).

Numbers of circulating activated neutrophils are increased in sepsis (10) and are a potential source of plasma arginase activity (11, 12). Human neutrophils constitutively express arginase I in gelatinase granules (11, 13). Arginase is released from granulocytes when granules fuse to the phagosome after phagocytosis, and degranulation or cell rupture releases arginase into the extra-cellular environment (11),(14). Arginase I is a trimer of identical subunits with a molecular weight of approximately 35 kDa (15), pI values of 9.25 – 9.35 (16), an optimum pH of 8.5 – 9.5 (16, 17) and an affinity coefficient for arginine of 2.3 mM at physiological pH (15). Although arginase activity is optimal in a strong alkaline environment, extra-cellular arginase functions at physiological pH when activated by factors stored in neutrophil azurophil granules (18, 19). Human neutrophil peptides (HNP1-3 or alpha defensins) are markers of azurophil granule release and HNP1-3 are elevated in adults with bacterial infection and sepsis (20, 21). Granulocytes have a short half life and intravascular death and granule release is a potential source of arginase activity in sepsis. Plasma arginase is reported to have a short half life of 10-15 minutes (22).

The aim of this study was to investigate whether plasma arginase activity is increased in sepsis and whether this is associated with circulating neutrophil numbers and activation markers. We hypothesised that compared to controls, sepsis patients would have increased plasma arginase activity and decreased plasma L-arginine concentrations in proportion to peripheral blood neutrophil counts.

Methods

Study participants

We studied a subset of forty-four patients with sepsis and twenty-five hospital controls selected from those previously enrolled in a study of endothelial function (23) according to prespecified criteria described below. Sepsis patients had suspected or proven infection and the presence of two or more criteria for the systemic inflammatory response syndrome (SIRS) on admission (24). Sepsis severity was estimated using the modified Sequential Organ Failure Assessment score (SOFA) or Acute Physiology and Chronic Health Evaluation (APACHE) II score. We enrolled patients within 24 hours of intensive care unit admission or within 36 hours of ward admission. Control subjects were recruited from hospital patients who had not met SIRS criteria within the last 30 days and who had no clinical or laboratory evidence of inflammation or infection. Written informed consent was obtained from all participants or next of kin. The Human Research Ethics Committee of Menzies School of Health Research and the Northern Territory Department of Health and Community Services approved the study.

Blood collection

Venous or arterial blood was collected into lithium heparin tubes using the BD Vacutainer system. Blood was transported at room temperature and plasma separated within 30 minutes and stored at -80°C (25). To exclude artefactual elevation of plasma arginase from ex-vivo red cell hemolysis post collection, we only included those patients and controls if plasma was separated and frozen within 30 minutes of collection and with no evidence of hemolysis. We have previously shown that this approach avoids artefactual arginase activity (23). Hemolysis was defined as visible red blood cell lysis or detectable plasma cell free hemoglobin $> 6 \mu\text{M}$ (26), measured by ELISA (Bethyl Laboratories). Of 55 sepsis patients with plasma processed within 30 minutes, we excluded 11 because of hemolysis. An automated counter (T890; Beckman Coulter) provided cell counts, and we prospectively divided the cohort into two groups based on neutrophil counts. High neutrophil counts were defined, a priori, as those greater than the median number of circulating neutrophils in the patients with sepsis; $14 \times 10^3/\mu\text{L}$; $n = 44$, a number approximately two times the normal range in healthy adults.

Amino acid and arginase activity measurements

We measured plasma L-arginine, citrulline and ornithine concentrations using High Pressure Liquid Chromatography (HPLC; Shimadzu, Kyoto, Japan) with UV (250 nm) and fluorescence (excitation 250 nm, emission 395 nm) detection (27). Plasma ADMA was measured by HPLC using a previously described method (28). Plasma arginase activity was measured using a radiometric assay, as previously described, and reported as micromoles of urea converted/milliliter/hour (29).

Plasma HNP1-3 and interleukin-6 measurement

The concentration of HNP1-3 in lithium heparin plasma was determined using ELISA (Hycult biotech, Hycult Biotechnology, The Netherlands) according to the manufacturer's

instructions. We determined interleukin-6 concentrations using a cytometric bead array (Human Th1/Th2 Cytokine Kit II, BD Biosciences Pharmingen, CA, USA) and a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, MA, USA), as previously reported (23). Results were analysed using FCAP array version 1.0.1 (Soft Flow Hungary for Becton Dickinson Biosciences). The lower limit of detection of the assay was 10 pg/mL. Values below the lower limit of detection were assigned a value halfway between zero and the lower limit of detection for statistical analysis.

Statistical analysis

Continuous parametric variables were compared using Student's t-test, and continuous non-parametric variables were compared using Mann-Whitney, Kruskal-Wallis or Wilcoxon tests as appropriate. Correlations were examined using Pearson's or Spearman's tests for parametric and non-parametric data respectively. A 2-sided p-value of <0.05 was considered significant. Analyses were performed using Prism version 6 (GraphPad Software, CA, USA) or Stata version 10.0 (Stata Corp, TX, USA). The relationship between plasma arginase activity and neutrophil count was examined using linear regression with log arginase activity as the outcome and controlling for SOFA score, components of the SOFA score, APACHE II score and interleukin-6 concentrations in multivariate analysis.

Results

Sepsis patients had significantly higher leukocyte counts compared to age, ethnicity and sex matched controls (Table 1). This difference was largely attributable to higher numbers of circulating neutrophils; however neutrophil count alone did not correlate with disease severity in sepsis.

Plasma arginase activity was increased, albeit not statistically significantly, in sepsis patients (median 0.17, IQR [0.09 – 0.23]) compared with controls (median 0.13, IQR [0.05 – 0.16], $p=0.07$, Table 1). Furthermore, sepsis patients with neutrophil counts above the group median ($14 \times 10^3/\mu\text{L}$) had significantly higher plasma arginase activity (median 0.21, IQR [0.14 – 0.26]), than those with neutrophil counts equal to or below the median (0.10 [0.05 – 0.18]; $p=0.001$) and controls ($p=0.004$), Figure 1A. Conversely, plasma L-arginine concentration was decreased in sepsis patients (median 33 μM , IQR [27 – 47]) compared with controls (median 81 μM , IQR [69 – 91], $p<0.0001$). Sepsis patients with neutrophil counts above the median had lower concentrations of plasma L-arginine (median 30 μM , IQR [20 – 41]) than sepsis patients with less circulating neutrophils (39 μM [30 – 53]; $p=0.02$) and hospital controls ($p < 0.001$); Figure 1B. In addition, sepsis patients with neutrophil counts above the group median had significantly elevated plasma HNP1-3 (median 115 ng/mL, IQR [85 – 201]) compared with those with neutrophil counts equal to or below the median (78 ng/mL [37 – 115]; $p=0.03$) and hospital controls (27 [17 – 83]; $p < 0.001$), Figure 1C.

In sepsis patients, neutrophil count positively correlated with plasma arginase activity ($r = 0.44$, $p = 0.003$; Figure 2A), and inversely correlated with plasma L-arginine concentration ($r = -0.32$, $p = 0.04$, Figure 2B). However, no association was found between plasma arginase activity and plasma arginine concentration in all sepsis patients or sepsis patients

with neutrophil counts above the group median. Neutrophil count was positively correlated with plasma concentration of HNP1-3 ($r = 0.31$, $p = 0.04$; Figure 2C). These associations were independent of disease severity and inflammation, and persisted on multivariate analysis controlling for SOFA score, APACHE II score and plasma interleukin-6 concentrations. No other leukocytes, including monocytes or lymphocytes, significantly correlated with plasma arginase activity, L-arginine or HNP1-3. There was also no association between cell free haemoglobin and plasma arginase activity or plasma L-arginine.

Both HNP1-3 and the arginine/ADMA ratio correlated with disease severity in sepsis patients. Of the 44 sepsis patients, 15 had septic shock and 29 had sepsis without shock. There was no significant difference in the age or gender of the septic shock, sepsis without shock and control groups. HNP1-3 was higher in septic shock patients (median 180 ng/mL, IQR [101 – 249]) than patients without shock (median 83 ng/mL, IQR [34 – 108], $p=0.0006$) and controls (median 27 ng/mL, IQR [17 – 83], $p<0.0001$) (Figure 3A) and correlated with SOFA score ($r_s=0.34$, $p=0.02$). The arginine/ADMA ratio was lower in septic shock patients (median 45.1, IQR [33.6 – 73.3]) than patients without shock (median 92.3, IQR [57.4 – 108.7], $p=0.0001$) and controls (median 144.3, IQR [125 – 167], $p<0.0001$) (Figure 3B) and correlated with SOFA score ($r_s=-0.54$, $p=0.0002$). The Global Arginine Bioavailability Ratio (GABR=arginine/[ornithine+citrulline]) was lower in septic shock patients (median 0.6, IQR [0.39 – 0.72]) compared to sepsis patients without shock (median 0.74, IQR [0.51 – 0.93], $p=0.04$) (Figure 3C) but it did not correlate with SOFA score. There was no significant difference between the plasma arginase activity, circulating neutrophil count, plasma ornithine concentration or plasma citrulline concentration in sepsis patients with and without shock (data not shown). In all sepsis patients, or those with only shock, plasma arginase activity did not directly correlate with the arginine/ADMA ratio or GABR.

Discussion

Plasma arginase activity is increased in patients with sepsis, and correlates with the number of circulating neutrophils. HNP1-3 is a marker of azurophil granule release which allows arginase to function at physiological pH; the correlation of neutrophil count both with HNP1-3 and with plasma arginase activity, suggests that neutrophil-derived arginase may contribute to elevated plasma arginase activity and decreased plasma arginine in severe sepsis. Activated neutrophils release arginase into the extra-cellular environment via degranulation (14), a process known to occur in response to endotoxin (30). Since neutrophils are a source of arginase, they may play multiple roles in sepsis. Activated neutrophils can be beneficial by clearing pathogens by phagocytosis, but they may also be potentially deleterious by releasing arginase, depleting extra-cellular L-arginine and NO bioavailability, potentially contributing to microvascular dysfunction and diminished NO-mediated microbial killing.

Previous reports have described increased plasma concentrations of molecules liberated by neutrophils (31-34). Plasma HNP1-3 are elevated in patients with sepsis, non-bacterial infection and tuberculosis and plasma levels correlate weakly with peripheral blood neutrophil counts (21). Our data support these findings and further demonstrate an

association between elevated plasma HNP1-3 and sepsis disease severity. HNP1-3 are mainly produced by neutrophils (35), suggesting that activation, degranulation, leakage, or cell rupture can release azurophilic granules, the contents of which may then activate arginase also liberated by neutrophils (18). We have shown that sepsis patients with increased neutrophil count have the highest concentration of HNP1-3, the highest arginase activity and the lowest arginine concentration, with relationships independent of disease severity. Furthermore, HNP1-3 correlates with the neutrophil count, but not monocyte count, in sepsis. Therefore, neutrophil-derived plasma arginase may contribute to hypoarginemia in sepsis.

Arginase released from damaged or lysed erythrocytes, hepatocytes, monocytes, endothelial cells, and smooth muscle cells (36) may also have contributed to the plasma arginase activity we measured in sepsis patients and altered hepatic and/or renal function may have impacted on the clearance of arginase. However, patients with detectable haemolysis were excluded so lysed erythrocytes are not a major source of arginase activity in this study. Furthermore, the lack of association between plasma arginase activity and total SOFA score, the renal and hepatic components of the SOFA score or APACHE II score do not support a major contribution of arginase from hepatic sources or altered clearance. Finally, the lack of association between plasma arginase activity and plasma interleukin-6 levels suggests that arginase activity is not merely a reflection of generalised inflammation.

Monocytes are also a potential source of plasma arginase activity in sepsis. In humans, arginase has been detected in the peripheral blood mononuclear cell (PBMC) fraction after injury (37), and in patients with active pulmonary tuberculosis (38), in inflammatory synovial fluid macrophages of patients with arthritis (39), inflammatory cells of bronchoalveolar lavage fluid of asthmatic patients(40), psoriatic lesions (41) and in activated monocytes of patients with autoimmune diseases (42). However, as neutrophils are the predominant leukocyte source of arginase in humans (11) and as plasma arginase activity correlates with the neutrophil count but not the monocyte count in sepsis, circulating neutrophils are a more likely source of plasma arginase activity in sepsis than monocytes.

Hypoargininemia in sepsis is probably multi-factorial, with several mechanisms potentially contributing. These include decreased intestinal absorption, increased protein synthesis and increased arginase activity (reviewed in (4)). The absence of a direct correlation between plasma arginase activity and plasma arginine in sepsis is in accord with malaria studies (8) and is consistent with multiple factors contributing to reduced plasma arginine in sepsis. Nevertheless, recent stable-isotope infusion study demonstrated that sepsis patients have a higher conversion of whole-body L-arginine to urea compared to controls, indicating increased total body arginase activity (9). This supports our finding of increased arginase activity in plasma from sepsis patients.

There was no association between the concentration of plasma arginase activity or neutrophil count and sepsis severity (SOFA or APACHE II score) in our study. This emphasizes the heterogeneity of sepsis, a condition that can include either high or low neutrophil counts (43). Severity of sepsis may be related to the degree of neutrophil activation rather than neutrophil counts alone (44) as suggested by our HNP1-3 data.

Similarly, although plasma L-arginine concentration alone is not associated with sepsis severity (23), the ratio of plasma L-arginine to asymmetric dimethylarginine significantly correlates with endothelial NO bioavailability and sepsis severity (7). Thus the lack of a direct relationship between plasma arginase activity or neutrophil count and sepsis severity may reflect the complexity of sepsis pathophysiology.

Conclusions

Increasing data suggest that vascular dysfunction in severe sepsis is a state of NO deficiency. As a result there is renewed interest in hypoargininemia in sepsis, and in the potential for adjunctive treatments targeting the L-arginine-NO pathway to improve NO bioavailability (3). Results from our study suggest that neutrophil-derived arginase may contribute to increased plasma arginase activity in sepsis and suggests new potential targets for adjunctive treatment.

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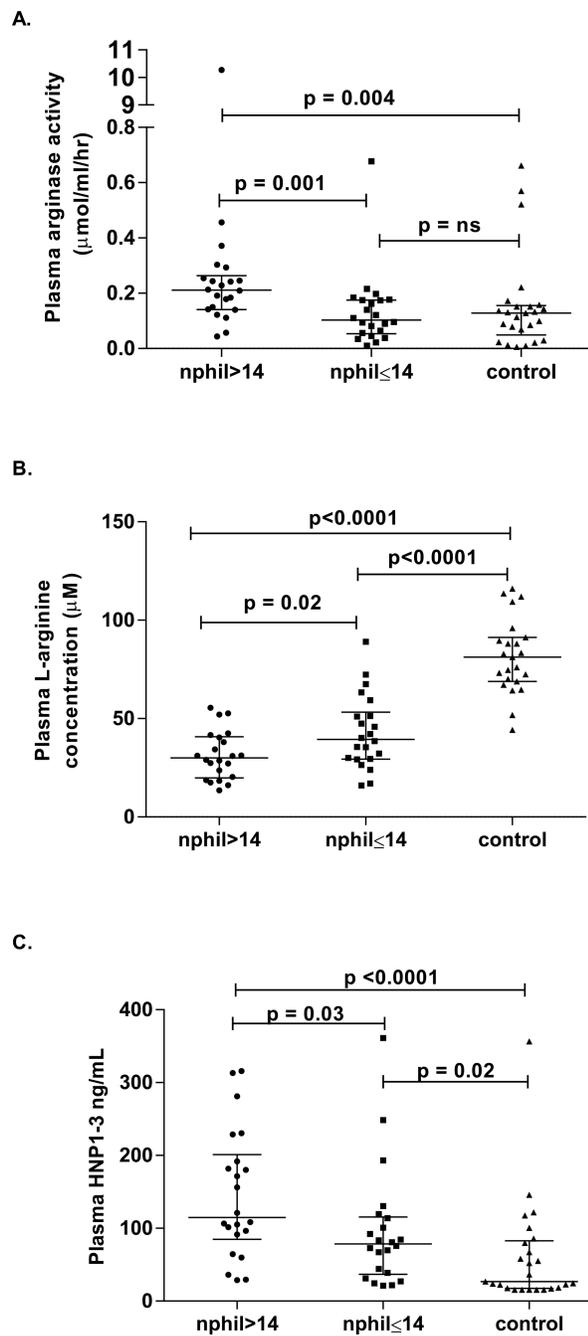


Figure 1.

Plasma arginase activity (A), plasma L-arginine concentration (B) and plasma HNP 1-3 concentration (C), in sepsis patients with circulating neutrophil counts greater than the median ($>14 \times 10^3/\mu\text{L}$, $n=22$), less than the median ($\leq 14 \times 10^3/\mu\text{L}$, $n=22$) and hospital controls ($n=25$). Bars represent the median and inter-quartile range.

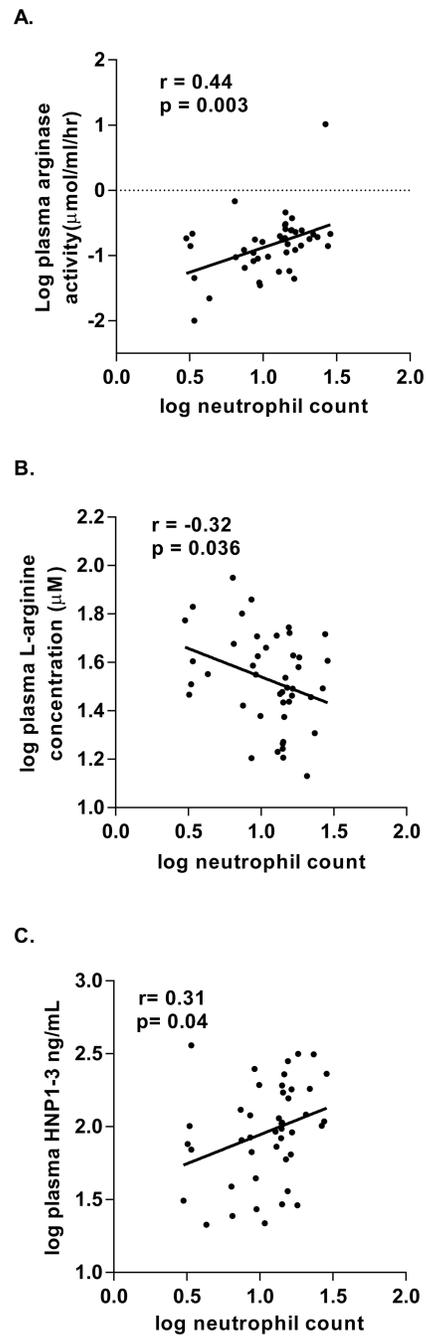


Figure 2. The relationship between neutrophil count and plasma arginase activity (A), plasma L-arginine concentration (B), and plasma HNP 1-3 in the blood of 44 sepsis patients.

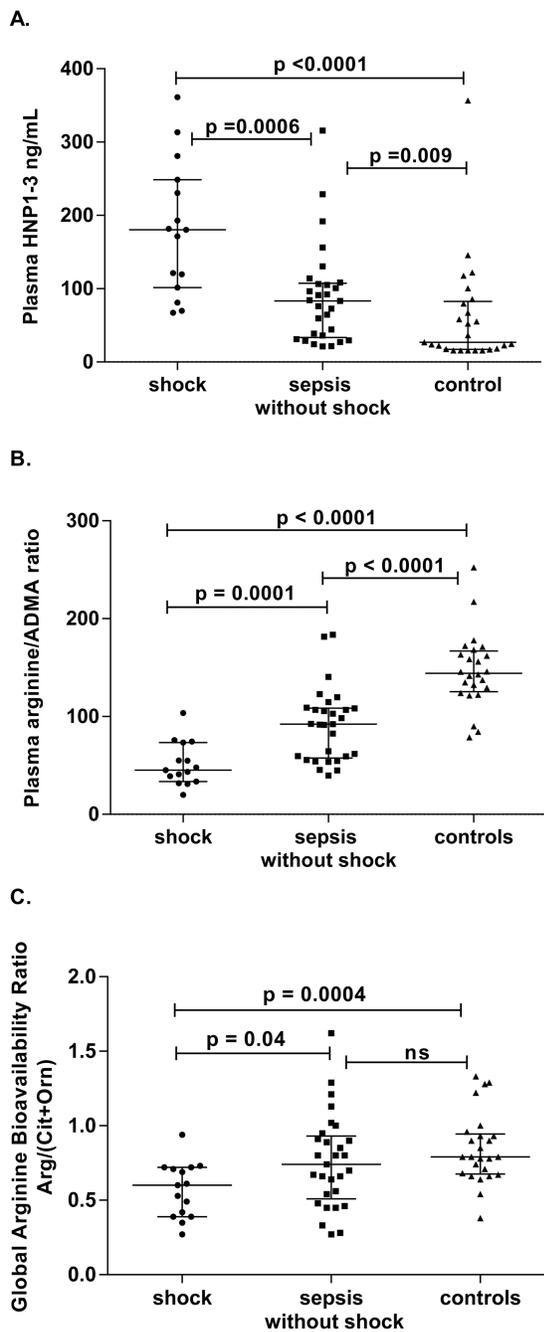


Figure 3. Association between disease severity and plasma HNP 1-3 (A), plasma arginase activity (B) and the Global Arginine Bioavailability Ratio (GABA), in 15 patients with septic shock, 29 sepsis patients without shock and 25 hospital controls.

Table 1

Characteristics of study patients

	All sepsis	Sepsis Nphil>14	Sepsis Nphil 14	Control	All sepsis vs control	Sepsis nphil>14 vs control	sepsis nphil 14 vs control	Sepsis nphil>14 vs nphil 14
Subjects, n	44	22	22	25	ns	ns	ns	ns
Age *	50 (46 - 55)	50 (44 - 55)	51 (44 - 58)	45 (40 - 50)	ns	ns	ns	ns
Male, n (%)	26 (59%)	11 (50%)	15 (68%)	17 (68%)	ns	ns	ns	ns
ATSI, n (%)	26 (59%)	14 (64%)	12 (55%)	13 (52%)	ns	ns	ns	ns
Pneumonia n (%)	22 (50%)	11 (50%)	11 (50%)	N/A	N/A	N/A	N/A	ns
Gram negative organism	11 (25%)	7 (14%)	4 (18%)	N/A	N/A	N/A	N/A	ns
Required vasopressors	12 (27%)	6 (27%)	6 (27%)	N/A	N/A	N/A	N/A	ns
SOFA score	3 (1 - 8)	2 (1 - 7)	5 (1 - 9)	N/A	N/A	N/A	N/A	ns
SOFA hepatic component	0 (0 - 0.75)	0 (0 - 0.25)	0 (0 - 1)	N/A	N/A	N/A	N/A	ns
SOFA renal component	0 (0 - 1)	0 (0 - 1.25)	0.5 (0 - 1.25)	N/A	N/A	N/A	N/A	ns
APACHE II score [‡]	15 (8 - 20)	13.5 (8 - 20.5)	16 (5.5 - 18.5)	N/A	N/A	N/A	N/A	ns
White blood cell × 10 ³ /μL [‡]	15 (10 - 18)	18 (17 - 25)	11 (7 - 14)	8 (6 - 10) [#]	0.001	<0.0001	ns	<0.0001
Neutrophil × 10 ³ /μL [‡]	14 (9 - 16)	16 (14 - 21)	9 (4 - 10)	5 (3 - 6) [#]	0.0002	<0.0001	ns	<0.0001
Monocyte × 10 ³ /μL [‡]	0.6 (0.4 - 1.1)	0.95 (0.5 - 1.2)	0.5 (0.3 - 0.6)	0.5 (0.5 - 0.6) [#]	ns	0.02	ns	0.006
Lymphocyte × 10 ³ /μL [‡]	0.9 (0.50 - 1.3)	1.1 (0.8 - 1.7)	0.8 (0.5 - 1.1)	2.2 (1.9 - 2.2) [#]	0.002	0.04	0.004	0.07
Immature granulocyte × 10 ³ /μL [‡]	0 [0 - 6.7]	0 [0 - 6.7]	0 [0 - 3.1]	0 [0 - 0] [#]	ns	ns	ns	ns
Plasma interleukin 6 (pg/mL)	267 (76 - 563)	277 (105 - 832)	267 (63 - 428)	5 (5 - 5)	<0.0001	<0.0001	<0.0001	ns
Plasma cell free hemoglobin (μM) [‡]	0.74 (0.56 - 1.2)	0.88 (0.52 - 1.32)	0.68 (0.56 - 1.1)	0.66 (0.43 - 1.2)	ns	ns	ns	ns
Plasma arginase activity μmol/mL/hr [‡]	0.17 (0.09 - 0.23)	0.21 (0.14 - 0.26)	0.10 (0.05 - 0.18)	0.13 (0.05 - 0.16)	0.07	0.004	ns	0.0009
Plasma L-arginine (μM) [‡]	33 (27 - 47)	30 (20 - 41)	39 (30 - 53)	81 (69 - 91)	<0.0001	<0.0001	<0.0001	0.02
Plasma ADMA (μM) [‡]	0.48 (0.39 - 0.58)	0.44 (0.39 - 0.57)	0.54 (0.41 - 0.61)	0.57 (0.47 - 0.62) ^{##}	0.02	0.005	ns	ns
Plasma L-arginine/ADMA ratio [‡]	69.0 (46.0 - 105.1)	61.0 (44.5 - 104.4)	79.2 (47.3 - 105.9)	144.3 (125.3 - 167.0) ^{##}	<0.0001	<0.0001	<0.0001	ns
Plasma citrulline (μM) [‡]	12 (9 - 18)	12 (10 - 19)	11 (8 - 17)	26 (20 - 45)	<0.0001	<0.0001	<0.0001	ns
Plasma ornithine (μM) [‡]	41 (30 - 50)	38 (27 - 46)	43 (34 - 58)	70 (57 - 84)	<0.0001	<0.0001	0.0002	ns

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	All sepsis	Sepsis Nphil>14	Sepsis Nphil 14	Control	All sepsis vs control	Sepsis nphil>14 vs control	sepsis nphil 14 vs control	Sepsis nphil>14 vs nphil 14
Global Arginine Bioavailability Ratio (arginine/(citrulline+ornithine))[‡]	0.68 (0.47 – 0.88)	0.59 (0.44 – 0.80)	0.72 (0.59 – 0.93)	0.79 (0.68 – 0.95)	0.02	0.004	ns	ns

ATSI = Aboriginal or Torres Strait Islander,

* Mean (95% confidence interval),

[‡]Median (interquartile range) or [range],

n = 12,

n = 24