Usefulness of α7 Nicotinic Receptor Messenger RNA Levels in Peripheral Blood Mononuclear Cells as a Marker for Cholinergic Antiinflammatory Pathway Activity in Septic Patients: Results of a Pilot Study

José L. Cedillo,1 Francisco Arnalich,2 Carolina Martín-Sánchez,1 Angustias Quesada,2 Juan José Rios,2 María C. Malafitassi,1 Gema Atienza,1 Jaime Renart,2 Carmen Fernández-Capitán,2 Francisco García-Rio,3 Eduardo López-Collazo,4 and Carmen Montiel1

1Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma, 2Servicio de Medicina Interna, 3Servicio de Neumología, and 4Laboratory of Tumor Immunology, Unidad de Investigación, Hospital Universitario La Paz, and 5Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas–Universidad Autónoma, Instituto de Investigacion Sanitaria IdiPAZ, Madrid, Spain

Background. Stimulation of the vagus nerve in the so-called cholinergic antiinflammatory pathway (CAP) attenuates systemic inflammation, improving survival in animal sepsis models via α7 nicotinic acetylcholine receptors on immunocompetent cells. Because the relevance of this regulatory pathway is unknown in human sepsis, this pilot study assessed whether the α7 gene expression level in septic patients’ peripheral blood mononuclear cells (PBMC) might be used to assess CAP activity and clinical outcome.

Methods. The PBMCs α7 messenger RNA levels were determined by real-time quantitative reverse-transcription polymerase chain reaction in 33 controls and 33 patients at enrollment and after their hospital discharge. Data were analyzed to find significant associations between α7 level, vagally mediated heart rate variability as an indirect reflection of CAP activity, serum concentrations of different inflammation markers, and clinical course.

Results. Septic patients’ α7 levels were significantly increased and returned to control values after recovery. These α7 levels correlated directly with the vagal heart input and inversely with the magnitude of the patient’s inflammatory state, disease severity, and clinical outcome.

Conclusions. This study reveals that the PBMC α7 gene expression level is a clinically relevant marker for CAP activity in sepsis: the higher the α7 expression, the better the inflammation control and the prognosis.

Keywords. α7 nicotinic receptors; cholinergic antiinflammatory pathway; heart rate variability; sepsis; septic patients; vagus nerve.

Sepsis, the deleterious and nonresolving systemic inflammatory response to infection, is the leading cause of death in intensive care units (ICUs). Its pathogenesis is complex but is partly mediated by an imbalance between excessive inflammation and its compensatory antiinflammatory mechanisms [1, 2]. One of these mechanisms is the so-called cholinergic antiinflammatory pathway (CAP), part of a circuit physically linked to the immune system [3–6]. The vagus nerve afferent (sensory) fibers of this circuit detect peripheral molecular products from infection or injury and signal the brainstem nuclei to trigger the CAP response via vagus nerve efferent (motor) fibers (Supplementary Figure 1). This response culminates in T-cell release of acetylcholine (ACh), which interacts with α7 nicotinic ACh receptors (α7 nAChRs) on resident macrophages in the spleen to inhibit their proinflammatory cytokine production [7, 8].

Received 25 April 2014; accepted 22 July 2014; electronically published 4 August 2014.

Correspondence: Carmen Montiel, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain (carmen.montiel@uam.es).

The Journal of Infectious Diseases® 2015;211:146–55
© The Author 2014. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.

DOI: 10.1093/infdis/jiu425
Although the beneficial effect of CAP activation has been extensively reported in animal models of sepsis and other systemic inflammatory diseases [9–11], confirmation of this finding in humans has been impossible because no known marker accurately reflected the activity of the antiinflammatory pathway. Power spectral analysis of heart rate variability (HRV) in consecutive R-R intervals on an electrocardiogram is a reliable noninvasive method used in human diseases, including sepsis, for gathering quantitative information on the vagal-sympathetic tone in the sinoatrial cardiac node [12, 13]. Curiously, some vagally mediated HRV indices have been used for indirect measurement of CAP response to endotoxin administration in human experimental models [14].

To provide the first evidence of the relevance of this regulatory mechanism in human sepsis, we designed a pilot study in septic patients to evaluate whether differences in α7 messenger RNA (mRNA) expression levels in peripheral blood mononuclear cells (PBMCs) might reflect differences in CAP activity and, consequently, predict the net inflammatory response and clinical outcome.

METHODS

Study Population
This was a combined prospective pilot cohort and case-control study conducted in a 970-bed university-affiliated general hospital, with a single 78-bed internal medicine ward, that provides care for approximately 3500 hospitalized patients per year. The study was designed according to ethical principles for medical research in humans (Declaration of Helsinki, 2008) and approved by the Committee of Medical Research Ethics of University Hospital La Paz (Madrid, Spain). All participants signed informed consent forms prior to participation. The cohort group consisted of 33 white nonsmoking patients (13 males and 20 females; age range, 38–80 years [mean value ± SD], 60.3 ± 12.7 years), admitted to our wards from March to October 2012, who met the diagnostic criteria for sepsis according to the classification of the International Sepsis Consensus Conference [15]. All patients were treated according to the International Surviving Sepsis Campaign Guidelines [16]. Disease severity was assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) II score [17] determined on the day of diagnosis. The exclusion criteria for patients, the precautions taken to minimize the impact of confounding factors on HRV measurements, and the characteristics of the nonsmoking control group (n = 33) are detailed in the Supplementary Materials.

Clinical Design
We measured the PBMC α7 mRNA levels in control subjects at enrollment and in septic patients within the first 24 hours of diagnosing sepsis. Simultaneously, we also assessed serum concentrations of several inflammatory markers and autonomic cardiac function by time- and frequency-domain HRV spectral analysis on an electrocardiogram in all subjects. PBMC α7 mRNA levels and vagally mediated HRV indices were again measured in surviving patients 2 weeks after hospital discharge. Measurements for the sepsis group were noted and compared during the acute illness and after hospital discharge and also with respect to the control group. Comparisons were also made between patients who remained in the ward and the subgroup transferred to the ICU for treatment of severe sepsis and between survivors and nonsurvivors of sepsis. The primary independent variable was the α7 mRNA level in PBMCs. The primary outcome measurements were progression to severe sepsis and hospital mortality.

Blood Sampling and Processing
Techniques for PBMC isolation and RNA extraction, as well as for cytokine and acute-phase reactant protein serum level quantification, have been described elsewhere (Supplementary Materials) [18–21]. The serum levels for inflammatory markers in the control group and the lowest assay detection limits are shown in Supplementary Table 1.

Reverse Transcription of RNA and Quantitative Real-Time PCR (qPCR)
The α7 gene expression assay by qPCR from reverse-transcribed RNA, using the SYBR green-based assays (Bio-Rad, Hercules, CA) and the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA), has been described elsewhere [21]. Beta-2 microglobulin (B2M) and ubiquitin C (UBC) genes were selected for normalization of α7 gene expression, since their expression seems to be the most stable among housekeeping genes in human leukocytes [22]. The primers and cycling conditions of PCR are shown in the Supplementary Materials. Each single α7 value obtained by qPCR was normalized to each endogenous control (B2M and UBC), using the 2−ΔΔCt method. Data were calculated as relative α7 expression, using the same calibrator for all experiments (set to a value of 1); RNAs extracted from the PBMCs of 10 healthy control individuals were pooled for the calibrator.

HRV Analysis
Cardiac autonomic function was assessed by time- and frequency-domain HRV analysis for a 15-minute electrocardiogram, according to international standards (Supplementary Materials) [23]. The last 512 stationary R-R intervals were used to calculate the mean and root mean square of successive differences (RMSSD) of R-R intervals, using standard formulae [24, 25]; this value, in milliseconds, reflects all of the cyclic components responsible for variability. The areas under the spectral peaks within the ranges of 0.01 to 0.04, 0.04 to 0.15, 0.15 to 0.4, and 0.01 to 0.4 Hz were defined as the very low frequency (VLF) component, the low frequency (LF) component, the high-frequency fluctuation (HF) component, and total power (TP), respectively. The VLF
component of HRV was not considered because it reflects the renin-angiotensin-aldosterone modulation of the heart [24, 25]. The vagal regulation of the heart is a major contributor to RMSSD and HF fluctuation, whereas LF fluctuation is modulated by both vagal and sympathetic activities. The LF/HF ratio is used as an index of vagal-sympathetic balance in the heart [23, 24]. HF and LF can be expressed in milliseconds or in normalized units (nHF = HF/TP; nLF = LF/TP), as was done here.

**Statistical Analysis**

The Kruskal–Wallis test, followed by the Dunn post-hoc test, was used to analyze nonparametric data, and analysis of variance, followed by the Bonferroni post-hoc test, was used for parametric data. For the 2 blood samples collected from the same patient at different periods, the Wilcoxon paired test was used to assess differences between α7 mRNA levels, and the paired Student t test was used to assess differences between RMSSD and nHF values. Differences in α7 mRNA levels between patients with sepsis and those with severe sepsis and between surviving and nonsurviving patients were calculated by the Mann–Whitney test; differences in vagally mediated HRV indices were calculated using the unpaired Student t test. For serum concentrations of cytokines or acute reactant proteins, the Spearman correlation coefficient was used to analyze the correlations with α7 mRNA levels and the Pearson correlation coefficient was used to analyze the correlations with vagally mediated HRV indices. Data are reported as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), as indicated below. A P value of ≤.05 was considered statistically significant.

**RESULTS**

Table 1 shows clinical data for patients numbered according to the order of their enrollment in the study and listed in the table, after stratification into tertiles, in descending order of the magnitude of their normalized PBMC α7 mRNA levels. Pathogens were identified in 28 patients (78.3%), and 5 had negative blood culture results. The condition in 13 patients worsened and progressed to severe sepsis during the first 2 days; they were transferred to the ICU and are considered as a separate patient subgroup. Patients and control groups did not differ significantly with respect to age or sex distribution (see Methods).

**Differences in α7 mRNA Level in PBMCs From Septic Patients**

Normalized α7 mRNA expression in each control and patient at the time of their inclusion in the study are plotted in Figure 1A. Since α7 expression levels in PBMCs are upregulated in smokers [26], we measured this variable in a smoker group (Supplementary Materials) to confirm the sensitivity of the qPCR assay and validate our qPCR results. The mean α7 level (±SEM) was significantly higher in the patient group (9.53 ± 1.87; P ≤ .001) and smoker group (19.19 ± 3.28; P ≤ .01; data not shown) than in the control group (2.02 ± 0.22), which showed very little variability. In contrast, variability was so great among patients that we distributed them into tertiles according to their α7 expression levels: the first tertile was characterized by a high level of α7 expression, the second by a medium level, and the third by a low level. Figure 1B shows the mean α7 levels (±SEM) in patients grouped by tertiles; the differences in α7 expression between the first tertile (21.38 ± 3.38) and the second tertile (4.26 ± 0.29), but not the third tertile (2.40 ± 0.18), were significantly with respect to the control group.

**The α7 mRNA Level in PBMCs From Septic Patients Correlated Directly With the Vagally Mediated HRV Indices**

Our HRV data in the patient tertiles showed that the 2 indices that best reflect vagal heart input (RMSSD and nHF) were quite significantly higher in patients in the first tertile, compared with patients in the third tertile (Figure 1C); this also occurred with their α7 expression levels (Figure 1B). This coincidence suggested a direct relationship between the PBMC α7 mRNA level and vagal cardiac activity in septic patients, and this was confirmed. Thus, RMSSD and nHF values measured in 25 of the 33 septic patients at enrollment were directly correlated with their α7 mRNA levels (rho, 0.66 and 0.73, respectively); all correlations were strongly significant (P ≤ .001). Also, both the LF component of HRV and the LF/HF ratio in patients in the third tertile were significantly lower than those in the first; the LF/HF ratio was <1 in the third tertile (Figure 1C).

**Differences in PBMC α7 Level and Cardiac Vagal Tone Influence the Net Inflammatory State of Septic Patients**

Figure 2 shows serum concentrations of cytokines and acute-phase reactant proteins in patients grouped by tertiles. For patients in the first tertile (high α7 expression), the levels of C-reactive protein (CRP), fibrinogen, serum amyloid A (SAA), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 10 (IL-10) were significantly lower than in patients from the third tertile. There was a significant inverse correlation between α7 mRNA level and most of the inflammatory markers in the set of all patients: for CRP, rho = −0.76 (P ≤ .001); for fibrinogen, rho = −0.76 (P ≤ .001); for SAA, rho = −0.65 (P ≤ .001); for IL-6, rho = −0.67 (P ≤ .001); for TNF-α, rho = −0.46 (P ≤ .01); for IL-1β, rho = −0.73 (P ≤ .001); and for IL-10, rho = −0.76 (P ≤ .001). Interestingly, a significant inverse correlation was also noted between RMSSD and nHF and most of the above inflammatory markers: for CRP, r = −0.53 (P ≤ .01) and −0.45 (P ≤ .05), respectively; for fibrinogen, r = −0.52 (P ≤ .01) and −0.44 (P ≤ .05), respectively; for SAA, r = −0.57 (P ≤ .01) and −0.58 (P ≤ .01), respectively; for IL-6, r = −0.55 (P ≤ .01) and −0.53 (P ≤ .01), respectively; for IL-1β, r = −0.57 (P ≤ .01) and −0.42 (P ≤ .05), respectively; and for IL-10, r = −0.61 (P ≤ .01) and −0.53 (P ≤ .01), respectively.
Table 1. Demographic and Clinical Characteristics of Patients Stratified by Their α7 Messenger RNA Expression Levels Into Tertiles

<table>
<thead>
<tr>
<th>Tertile, Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Infection Site</th>
<th>Clinical Isolate Source(s)</th>
<th>Microorganism</th>
<th>APACHE II Score</th>
<th>Developed Severe Sepsis</th>
<th>Hospitalization Duration, d</th>
<th>Died</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First tertile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>65</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>11</td>
<td>No</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>Patient 27</td>
<td>45</td>
<td>M</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>None</td>
<td>16</td>
<td>No</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>Patient 1</td>
<td>62</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>None</td>
<td>12</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Patient 18</td>
<td>57</td>
<td>F</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>14</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Patient 26</td>
<td>68</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>13</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Patient 13</td>
<td>52</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>12</td>
<td>No</td>
<td>9</td>
<td>No</td>
</tr>
<tr>
<td>Patient 31</td>
<td>64</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>14</td>
<td>No</td>
<td>11</td>
<td>No</td>
</tr>
<tr>
<td>Patient 6</td>
<td>75</td>
<td>F</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>13</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Patient 33</td>
<td>44</td>
<td>M</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>None</td>
<td>15</td>
<td>No</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>Patient 2</td>
<td>77</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>14</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Patient 32</td>
<td>62</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>None</td>
<td>16</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td><strong>Overall, mean ± SD</strong></td>
<td>61.0 ± 10.8</td>
<td>13.6 ± 1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Second tertile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>48</td>
<td>F</td>
<td>Abdominal abscess</td>
<td>Blood culture</td>
<td>P. mirabilis</td>
<td>14</td>
<td>Yes</td>
<td>13</td>
<td>No</td>
</tr>
<tr>
<td>Patient 22</td>
<td>62</td>
<td>M</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>18</td>
<td>Yes</td>
<td>15</td>
<td>No</td>
</tr>
<tr>
<td>Patient 16</td>
<td>41</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>None</td>
<td>20</td>
<td>Yes</td>
<td>19</td>
<td>No</td>
</tr>
<tr>
<td>Patient 20</td>
<td>62</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>17</td>
<td>No</td>
<td>21</td>
<td>No</td>
</tr>
<tr>
<td>Patient 17</td>
<td>49</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>18</td>
<td>Yes</td>
<td>21</td>
<td>No</td>
</tr>
<tr>
<td>Patient 7</td>
<td>78</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>14</td>
<td>No</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Patient 25</td>
<td>41</td>
<td>F</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>12</td>
<td>No</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>Patient 21</td>
<td>67</td>
<td>F</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>K. pneumoniae</td>
<td>19</td>
<td>No</td>
<td>19</td>
<td>No</td>
</tr>
<tr>
<td>Patient 15</td>
<td>67</td>
<td>F</td>
<td>Lung</td>
<td>Blood culture</td>
<td>S. pneumoniae</td>
<td>18</td>
<td>No</td>
<td>18</td>
<td>No</td>
</tr>
<tr>
<td>Patient 23</td>
<td>80</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>16</td>
<td>No</td>
<td>9</td>
<td>No</td>
</tr>
<tr>
<td>Patient 29</td>
<td>38</td>
<td>M</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>18</td>
<td>Yes</td>
<td>9</td>
<td>No</td>
</tr>
<tr>
<td><strong>Overall, mean ± SD</strong></td>
<td>57.5 ± 14.9</td>
<td>16.7 ± 2.4a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Third tertile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 10</td>
<td>67</td>
<td>F</td>
<td>Upper urinary tract</td>
<td>Urine culture</td>
<td>E. coli</td>
<td>17</td>
<td>Yes</td>
<td>11</td>
<td>No</td>
</tr>
<tr>
<td>Patient 9</td>
<td>71</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>15</td>
<td>No</td>
<td>12</td>
<td>No</td>
</tr>
<tr>
<td>Patient 28</td>
<td>45</td>
<td>M</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>15</td>
<td>No</td>
<td>9</td>
<td>No</td>
</tr>
<tr>
<td>Patient 5</td>
<td>52</td>
<td>F</td>
<td>Lung</td>
<td>Blood culture</td>
<td>S. pneumoniae</td>
<td>18</td>
<td>Yes</td>
<td>14</td>
<td>Yes</td>
</tr>
<tr>
<td>Patient 24</td>
<td>75</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>20</td>
<td>Yes</td>
<td>18</td>
<td>Yes</td>
</tr>
<tr>
<td>Patient 11</td>
<td>52</td>
<td>F</td>
<td>Abdominal abscess</td>
<td>Blood culture</td>
<td>E. coli</td>
<td>20</td>
<td>Yes</td>
<td>18</td>
<td>Yes</td>
</tr>
<tr>
<td>Patient 30</td>
<td>56</td>
<td>M</td>
<td>Upper urinary tract</td>
<td>Urine, blood culture</td>
<td>E. coli</td>
<td>21</td>
<td>Yes</td>
<td>17</td>
<td>Yes</td>
</tr>
<tr>
<td>Patient 14</td>
<td>51</td>
<td>F</td>
<td>Abdominal abscess</td>
<td>Blood culture</td>
<td>P. mirabilis</td>
<td>16</td>
<td>Yes</td>
<td>16</td>
<td>No</td>
</tr>
<tr>
<td>Patient 8</td>
<td>80</td>
<td>F</td>
<td>Lung</td>
<td>Sputum, urine</td>
<td>S. pneumoniae</td>
<td>15</td>
<td>Yes</td>
<td>12</td>
<td>No</td>
</tr>
</tbody>
</table>
To evaluate whether the worsening or resolution of sepsis affects CAP activity as deduced from the PBMC $\alpha_7$ mRNA level and the 2 indices for vagally mediated input to the heart, all measurements were repeated in survivors 15 days after discharge. The results reveal that CAP responded dynamically to the inflammatory challenge in sepsis (Figure 3A). Thus, $\alpha_7$ levels were high during acute illness and dropped to the levels of the control group once the patient recovered. In contrast, the RMSSD and nHF indices were low during the septic process and approached control values after its resolution, a finding in line with previous studies reporting that reduced vagally mediated HRV indices are common features in both experimental human endotoxemia [27] and sepsis [12, 28]. However, irrespective of the direction of the $\alpha_7$ levels or cardiac vagal tone during sepsis, our data clearly indicate that the higher the CAP activity in patients, the better their clinical course and prognosis. In fact, PBMC $\alpha_7$ level and RMSSD and nHF values were inversely correlated with APACHE II scores (rho = $-0.73$ [$P \leq .001$], $-0.68$ [$P \leq .001$], and $-0.71$ [$P \leq .001$], respectively) and inversely associated with disease severity (Figure 3B) and mortality (Figure 3C). To assess the usefulness of the PBMC $\alpha_7$ mRNA level for predicting mortality, we used a cutoff of 3, the highest $\alpha_7$ level in the third tertile (range, 1.32–3.0), which contained all of the deceased patients (Table 1). There were no deaths among patients with $\alpha_7$ mRNA levels of $\geq 3$ (first tertile; range, 9.0–31.7) and second tertile (range, 3.1–6.1). Meanwhile, in the group with $\alpha_7$ levels of $\leq 3$ (third tertile), 60% of patients died (P < .001). Accordingly, 85.2% of the survivors (95% confidence interval, 70.7%–99.7%) had $\alpha_7$ mRNA levels of $>3$, and all of the patients who died had levels of $<3$.

**DISCUSSION**

This study represents the first experimental evidence of the importance of CAP in restraining excessive inflammatory response in septic patients. Moreover, our data also show that the effectiveness of this antiinflammatory mechanism in a given patient may be deduced from the $\alpha_7$ gene expression level determined in his/her PBMCs.

The selection of $\alpha_7$ mRNA level in patients’ PBMCs as an indicator of CAP activity was based on the following reasons: (1) $\alpha_7$ nAChR is the primary receptor mediating the cholinergic antiinflammatory response, (2) it is expressed in many types of cytokine-producing cells [11], and (3) it is easily studied with a minimally invasive procedure. The methodological difficulty with this approach is the partial duplication of the $\alpha_7$ subunit in humans (referred to as dup$\alpha_7$), which we and others have found sequences as a negative regulator of $\alpha_7$ nAChR activity in vitro [21, 29]. The nucleotide sequences of $\alpha_7$ and dup$\alpha_7$ mRNAs are highly homologous (>99%), and both
transcripts have a similar distribution pattern in brain and immune cells; consequently, both isoforms can be amplified at the same time by qPCR, and if care is not taken to design primers specifically for the α7 gene (CHRNA7) and not for the dupα7 gene (CHRFAM7A), they could be misidentified, as has already happened. We circumvented this difficulty by using primers targeting the divergent N-terminal region of the α7 subunit, which spans exons 1–4 of the CHRNA7 gene.

Three of our findings in relation to the α7 mRNA level in PBMCs should be highlighted: (1) the level was significantly higher in most septic patients (first and second tertiles) than in controls (Figure 1A and 1B), (2) it responded dynamically to the inflammatory challenge in sepsis (Figure 3A), and (3) there is a subgroup of patients (third tertile) with low levels who have a defective response to infection and the worst outcome. All of these results are perfectly compatible with the behavior of a typical biomarker.

The report that vagus nerve control of HRV indirectly reflects CAP response to endotoxin administration in human experimental models [14] has been confirmed by clinical studies showing that decreased vagal cardiac activity, measured through the RMSDD and nHF indices, is an early signal of deterioration and mortality in septic patients [30]. Moreover, pharmacological activation of CAP in human endotoxemia is associated with increased vagal cardiac activity, as measured by changes in HRV [31]. Since we found that PBMC α7 mRNA levels are directly correlated with RMSDD and nHF values in septic patients, we suspected that α7 levels could be a marker for CAP activity in sepsis since their upregulation in immune cells would enhance the antiinflammatory potential of endogenously released ACh.

![Figure 1. Analysis of α7 messenger RNA (mRNA) expression levels in peripheral blood mononuclear cells and heart rate variability (HRV) parameters in the study subjects. A, Normalized α7 expression determined by quantitative polymerase chain reaction in individual patients and nonsmoking healthy volunteers (controls). Each value was obtained in triplicate and represents an average of 3–5 separate determinations. The horizontal bars show the mean value for the group. B, Comparison of α7 levels in controls and patients distributed into tertiles according to their α7 expression values (first tertile, high level; second tertile, medium level; third tertile, low level). $t^*P<.001$ and $t^*P<.01$, after comparing the indicated tertile with the control group; $***P<.001$, after comparing the first and third tertiles. C, Vagal-sympathetic activity measured by changes in HRV indices in septic patients stratified into the tertiles described above. $P<.05$, $**P<.01$, and $***P<.001$, after comparing the indicated tertiles. Abbreviations: LF/HF, low frequency/high frequency ratio; nHF, high-frequency power component after normalization; nLF, low-frequency power component after normalization; RMSSD, root mean square successive differences of R-R intervals.](#)
upon CAP stimulation. Although this causal relationship between α7 levels and CAP activity needs to be confirmed in larger longitudinal studies with adequate power, there are many experimental findings supporting this proposal. For instance, nicotine-mediated upregulation of α7 mRNA expression in THP-1 monocytes leads to an enhanced antiinflammatory potential for α7 nAChR agonists (as measured by the reduction of TNF-α levels) [26], and α7-deficient macrophages are refractory to the antiinflammatory effect of cholinergic agonists. Meanwhile, mice lacking α7 nAChRs are more susceptible to systemic inflammation and lethal endotoxemia than wild-type mice [7, 8].

The above proposal regarding the usefulness of PBMC α7 mRNA levels as a CAP activity marker was reinforced in the present study by the significant inverse correlation between these levels and the serum concentrations of all the proinflammatory cytokines (IL-1β, TNF-α, and IL-6) and the acute-phase reactant proteins. The inverse correlation between the levels of α7mRNA (reflecting CAP activity) and the antiinflammatory cytokine IL-10 seems a bit surprising, but it could be explained by the higher plasma concentrations of 2 antiinflammatory cytokines, IL-10 and IL-1 receptor agonist (IL-1RA), reported in young healthy volunteers receiving a recombinant human IL-6 infusion to induce plasma levels of this cytokine characteristic of low-grade inflammation [32]. Although there is no real evidence that this also occurs in our septic patients, it is highly probable since our patients’ IL-6 levels are significantly higher than those recorded in the volunteers of the above study. Thus, it is likely that the high IL-10 serum levels in our patients with poor CAP activity could be related to their high IL-6 concentrations, reflecting a local feedback loop that would limit proinflammatory response. The small sample size probably precluded our statistical confirmation of an inverse correlation between α7 mRNA levels and IL-1RA.

Interestingly, the PBMC α7 level and the RMSSD and nHF values were inversely correlated with the APACHE II scores, as well as negatively associated with disease severity (Figure 3B) and mortality (Figure 3C). These results, aside from the better control of inflammation described above, possibly explain why patients in the first tertile (high CAP activity) had a better outcome. In fact, none of the first tertile patients developed severe sepsis, while most of the patients (70%) whose condition deteriorated to severe sepsis and all of those who eventually died were in the third tertile (Table 1). Moreover, the high sensitivity and specificity of the septic patients’ α7 levels (with a cutoff of 3) in relation to mortality suggests that these levels may have a predictive value for mortality in sepsis. Nevertheless, further studies with larger numbers of patients are needed to confirm this preliminary finding.

Our study also provides relevant new findings on the involvement of CAP in human sepsis. To date, only 2 preliminary

---

**Figure 2.** Inflammatory state of septic patients grouped into tertiles according to their α7 messenger RNA expression levels. Dot plots represent the serum or plasma concentrations of acute-phase reactant proteins and proinflammatory and antiinflammatory cytokines in each patient. *P < .05, **P < .01, and ***P < .001, after comparing the indicated tertiles. Abbreviations: CRP, C-reactive protein; IL-1RA, interleukin 1 receptor antagonist; IL-1β, interleukin 1β; IL-6, interleukin 6; IL-10, interleukin 10; SAA, serum amyloid A; TNF-α, tumor necrosis factor α.
studies using a human endotoxemia model have focused on this issue, obtaining limited beneficial effects from CAP stimulation with α7 nAChR agonists [33, 34]. The discrepancy with our results may lie in the sterile inflammation model they used, which cannot reproduce what happens in actual human sepsis. The mechanism behind differential α7 gene expression in septic patients is still unknown. Age and sex were not related (Table 1), leaving 2 possible explanations based on a genetic predisposition and a third nongenetic explanation for this differential expression. Polymorphisms in the promoter and/or enhancers of the gene might alter transcriptional efficiency [35], or there could be some sort of microRNA-dependent regulation [36]. The third possibility is based on the identification of a subpopulation of regulatory lymphocytes (CD3⁺CD4⁺CD25⁻) that, through their α7 nAChRs, appear to be essential for vagus nerve control of systemic inflammation in experimental sepsis in mice [37]. Since a massive and persistent apoptosis of this subpopulation of lymphocytes has been reported in non-surviving septic patients [38], this mechanism might contribute to the depletion of α7 levels found in our patients with the worst outcomes. Clarifying the involvement of each of these mechanisms requires further studies.

As stated above, an inherent limitation of the pilot-study design is the relatively small sample size and the impossibility of establishing causality. Another shortcoming is the presence of confounders that might influence HRV measurements, which

![Figure 3. Regulation of cholinergic antiinflammatory pathway (CAP) activity in septic patients and its relationship with the severity and clinical outcome. CAP activity was estimated by 2 routes: the α7 messenger RNA (mRNA) level in peripheral blood mononuclear cells and indirectly through the vagally mediated heart rate variability indices (root mean square successive differences of R-R intervals [RMSSD] and high-frequency power component after normalization [nHF]). A, CAP activity is dynamically regulated during acute septic illness and returns to control values after its resolution. B and C, Patients with higher CAP activity have a better clinical course and prognosis. **P < .01 and ***P < .001, after comparing the indicated data.](image-url)
we tried to minimize here. In contrast, some noteworthy strengths are (1) the highly specific qPCR assay of \( \alpha_7 \) mRNA levels, (2) the positive and highly significant association between PBMC \( \alpha_7 \) levels and vagal HRV indices measured simultaneously in each subject, (3) the strongly significant negative correlation between these CAP activity markers and the patient’s net inflammatory state, (4) the use of 3 different body sources to determine CAP activity (circulating PBMCs, heart, and blood serum), and (5) the measurement of PBMC \( \alpha_7 \) levels and vagal cardiac HRV indices at 2 time points in each patient, at acute illness and after recovery, allowing each patient to be an internal control for her/himself.

In summary, our results are consistent with the view that a poor cholinergic antiinflammatory response to systemic inflammation could be at the root of the poor prognosis for patients with sepsis. Consequently, our study raises the possibility that activating this pathway in high-risk septic patients has therapeutic potential: this activation could be used as an adjunctive therapy to enhance the therapeutic efficacy of standard treatments in sepsis.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the patients and healthy volunteers, for their participation; and the internal medicine service and ICU nurses, residents, and senior staff attending physicians of the University Hospital La Paz, for their cooperation in making this study possible.

Financial support. This work was supported by the Spanish Ministerio de Ciencia e Innovación (grant SAF2011-23575 to C. M. and F. A.); the Fundación Mutua Madrileña Investigación Biomédica (grant FMM2011 to C. M. and F. A.); Spanish Ministerio de Educación, Cultura y Deporte (Fellowship of Formación de Personal Universitario to J. L. C.); Spanish Ministerio de Economía y Competitividad (Fellowship of Formación Personal Investigador to C. M. S.); and Chilean Ministerio de Educación (Chile Fellowship to M. C. M.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References