Supplementary data

Supplementary data mentioned in the text is available to subscribers in Age and Ageing online.

**References**


**Age-related changes of serum  
N-acetyl-aspartate in healthy controls**

**Introduction**

N-acetyl-aspartate (NAA) is a significant indicator in the assessment of neuronal activities and abnormalities in the cerebral metabolism [1]; it is synthesised in neuronal...
mitochondria [2–4], then it is transported by a sodium/dicarboxylate molecule (NaC3) [5] to aspartoacylase-containing oligodendrocytes [6], which represent the NAA main catabolic route, and to astrocytes. Astrocytes play a role in the removal of NAA from extracellular space (ECS). Considering that astrocytes form a significant portion of the blood–brain barrier, it seems likely that these glial cells take up extracellular NAA and excrete it to the circulation. NAA turnover in the brain is very rapid, and if it was not for an equally rapid removal of NAA from ECS, this process could not continue and the function of the cycle would be compromised.

A continuous NAA efflux from the brain to the circulation and a clearance through the kidney, have both been demonstrated [7].

Many investigators have used magnetic resonance spectroscopy (1H-MRS) to study brain metabolism in vivo and the changes occurring in chemical components, such as human brain NAA, in normal and pathological states [8–13]. The vast majority of the studies have relied on the measurement of NAA change as a ratio between the metabolite of interest and some internal standard (IS) but this approach has recently been called into question [14]. Preliminary studies, using a gas-chromatography-mass-spectrometry (GC-MS) method, demonstrated the validity of NAA measurements in biological fluids, serum and cerebrospinal fluid (CSF), in patients with neurological diseases [15–17].

However, to the best of our knowledge, no report has described the changes according to gender and during physiological ageing of NAA serum levels in normal individuals.

In the present study, we aimed to evaluate the sex and age-related changes of serum NAA in healthy controls (HCs) by using a methodological approach of recent interest, the liquid chromatography-mass spectrometry (LC-MS) with stable isotope dilution.

**Methods**

**Population**

A total of 141 HCs divided according to age (4 months to 82 years) (Mean ± SD: 40.11 ± 23.05 years) were included in our study: individuals less than 15 years old (n = 30), young adults (15–39; n = 35), adults (40–59; n = 37) and elderly people (n = 39) with ≥60 years of age. These HCs had been previously recruited as controls in separate studies. The 30 younger people were included in paediatric studies on phenotypic and immunological evaluation in the Department of Evolutionary Age, University of Bari, Italy. The 35 young adults and the 37 adults between 40 and 59 years of age were from blood bank they attended as donors and enrolled in biomarker studies at the Department of Neurological and Psychiatric Sciences, University of Bari, Italy. Thirty-nine subjects between 61 and 82 years of age were recruited in the Department of Geriatrics, Center for Brain Aging, Memory Unit, University of Bari, Bari, Italy, as part of ongoing studies on Alzheimer’s disease. These subjects were examined by a geriatrician and underwent the standard battery of neuropsychological tests in order to judge if they were cognitively normal for their age and had no signs of mild cognitive impairment or early dementia.

All examinations were performed with the informed consent of the subjects or their relatives, and were in compliance with local institutional guidelines.

**Serum analysis and sample preparation**

Serum was removed after centrifugation and stored at −80°C. NAA as IS (Sigma, St Louis, MO, USA), formic acid (Aldrich Steinheim, Germany), ammonium acetate, HPLC grade acetonitrile and methanol were used (Fisher Scientific Fair Lawn, Fair Lawn, NJ, USA).

Quantification of NAA was achieved by the standard addition approach [18] and experiments were performed on six aliquots from each serum sample. A SpeedVac concentrator (Thermo Electron, Milford, MA, USA) was used and 2 μl of the residue were injected onto the LC-MS/MS (NaH2PO4 50 mM; pH corrected to 3.0 with H3PO4).

**LC-MS/MS system**

A reverse phase column (C18) (4.6 mm; 150 mm) was used for HPLC (Agilent Technologies, Palo Alto, CA, USA) on an isocratic gradient, with 0.1 M KH2PO4 and 0.025 M KCl at pH 4.5 at a flow rate of 1.5 ml/min monitored with UV detector. High-purity helium (35 p.s.i.; 1 p.s.i. i. 56894.76 Pa) and the API (atmospheric pressure ionization) source were used. The LC system was a Finnigan Mat (Spectra System P4000) with autosampler. Linearity and detection limits were determined by selected-ion monitoring and MS/MS (mass spectrometry/more selective) and the ion was used: m/z 260 (MH)+ fragmented with an energy of 29%. The linear dynamic range 5.7–57 μmol/l was explored. The instrumental precision was calculated by considering the repeatability of four measurements of chromatographic peak areas at two amount levels. Data were acquired by the Thermo Electron Corporation Excalibur software.

**Method validation**

The intraday and interday imprecision, expressed as coefficient of variation (CV%), was assessed using a pooled serum sample repeatedly analysed (10 times on the same day and for 10 consecutive days) by a standard addition. The recovery was so calculated: [recovery (%) = 100 × (measured concentration–endogenous concentration)/ added concentration]. Accuracy was evaluated by comparing the results obtained in this study with those measured independently by GC-MS in the same laboratory.
Statistical analysis

NAA serum levels were represented as mean ± standard deviation, median and 25th–75th percentiles. Multivariate analysis of variance was used to assess the effects of age and gender on the metabolite serum levels. In order to respect the assumption of homoscedasticity, the NAA values were log transformed (Ln NAA*100). The statistical inferences due to multiple comparisons among age categories and gender were adjusted according to Bonferroni inequality. The level of significance was set at $P$-value $= 0.05$.

Results

The intraday and the interday CV of the LC/MS assay resulted 2.1 and 6.2%, respectively, and the recovery was evaluated of about 87%.

NAA values stratified by age and gender categories are shown in Table 1. NAA serum levels were significantly different with advancing age ($F = 11.09$; df = 3, 133; $P < 0.01$), whereas there was not a significant difference between genders ($F = 0.30$; df = 1, 133; $P = 0.58$), also when gender was considered across categories of age ($F = 0.11$; df = 3, 133; $P = 0.95$).

NAA serum levels in older subjects ($\geq 60$ years) were significantly lower ($0.06 \pm 0.03$ mM/l) than in other younger groups ($\leq 14$ years: $0.12 \pm 0.08$ mM/l, Bonferroni $P$-values $< 0.01$; 15–40 years: $0.10 \pm 0.09$ mM/l, Bonferroni $P$-values $< 0.01$; 40–59 years: $0.14 \pm 0.08$ mM/l, Bonferroni $P$-values $< 0.01$). In Figure 1, the mean log-transformed NAA serum levels ($\pm$SE) (mM/l) are graphically represented by age categories.

Discussion

NAA is regarded as the most important marker of neuronal activities but, until now, its concentration in normal brain is the result of 1H-MRS acquisition only with limitations [19], so the task of validating more accurate methodologies is mandatory. Our study demonstrated that the LC/MS assay may be a valid and reproducible (CV $< 10\%$) diagnostic tool for analysing (recovery $= 87\%$) NAA in biological fluids.

We demonstrated that NAA serum levels did not differ between genders, whereas they were significantly lower in older subjects ($> 60$ years) than those in younger groups. There are many 1H-MRS reports on HCs on decreased NAA, NAA/Gr or NAA/Cho ratio levels with advancing ages in different brain regions [14, 19]. Our results confirm the brain 1H-MRS changes of NAA, suggesting that NAA serum levels could be considered a significant systemic indicator of neuronal activities in HCs as well as in patients with neurological disorders [20–23].

### Table 1. N-acetyl aspartate (NAA) serum levels [Mean ± SD and Median (25th–75th percentiles) values] (mM/l) by gender and age categories

<table>
<thead>
<tr>
<th>N-acetyl aspartate</th>
<th>Total Mean ± DS</th>
<th>Men Mean ± DS</th>
<th>Women Mean ± DS</th>
<th>$P$-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (25th–75th percentiles)</td>
<td>Median (25th–75th percentiles)</td>
<td>Median (25th–75th percentiles)</td>
<td></td>
</tr>
<tr>
<td>All ages</td>
<td>n = 141</td>
<td>0.11 ± 0.07</td>
<td>0.11 ± 0.06</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>Men (%)</td>
<td>(53.2)</td>
<td>0.09 (0.05–0.14)</td>
<td>0.09 (0.06–0.15)</td>
<td>0.05 (0.07–0.13)</td>
</tr>
<tr>
<td>$\leq 15$ years</td>
<td>n = 30</td>
<td>0.12 ± 0.08</td>
<td>0.12 ± 0.06</td>
<td>0.13 ± 0.09</td>
</tr>
<tr>
<td>Men (%)</td>
<td>(53.3)</td>
<td>0.11 (0.07–0.14)</td>
<td>0.11 (0.07–0.14)</td>
<td>0.09 (0.07–0.15)</td>
</tr>
<tr>
<td>15–39 years</td>
<td>n = 35</td>
<td>0.10 ± 0.09</td>
<td>0.11 ± 0.06</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Men (%)</td>
<td>(51.4)</td>
<td>0.10 (0.05–0.15)</td>
<td>0.11 (0.05–0.17)</td>
<td>0.10 (0.05–0.13)</td>
</tr>
<tr>
<td>40–59 years</td>
<td>n = 37</td>
<td>0.14 ± 0.08</td>
<td>0.13 ± 0.07</td>
<td>0.14 ± 0.09</td>
</tr>
<tr>
<td>Men (%)</td>
<td>(67.6)</td>
<td>0.13 (0.08–0.18)</td>
<td>0.13 (0.09–0.17)</td>
<td>0.13 (0.05–0.21)</td>
</tr>
<tr>
<td>$\geq 60$ years</td>
<td>n = 39</td>
<td>0.06 ± 0.03</td>
<td>0.07 ± 0.04</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>Men (%)</td>
<td>(41)</td>
<td>0.05 (0.04–0.07)</td>
<td>0.06 (0.04–0.09)</td>
<td>0.05 (0.04–0.07)</td>
</tr>
</tbody>
</table>

* $P$-value evaluated on log transformation of NAA serum levels by age categories.

**Figure 1.** Mean log-transformed N-acetyl aspartate (NAA) serum levels ($\pm$SE) (mM/l) by age categories. *Difference in log-transformed NAA serum levels between $\geq 60$ years old individuals and each other age category.*
Our data, at this moment, could only generate hypotheses. A defective activity of NAA transporters from neurons to oligodendrocytes and/or a compromised NAA astrocyte uptake during ageing, might explain the lower NAA serum levels observed in older rather than in younger subjects, but also exhausted mechanisms of neurodegeneration in the latest decades of life in HCs cannot be excluded. A recent paper [24] demonstrated that the rates of brain atrophy were greater in younger than in older individuals with amnestic mild cognitive impairment who did not progress to Alzheimer disease, and that they did not change, significantly, with age in oldest (70–90 year old) cognitively normal subjects. Another longitudinal study [25] in a cohort of healthy, non-demented elderly who underwent annually quantitative volumetric MRI, found that after age 65 there was minimal brain volume loss and that the brain volume tended to be more similar in the middle-old (mean age, 81 years) and oldest-old (mean age, 87 years) groups than in the young-old group (mean age, 70 years).

Finally, it cannot be excluded that serum NAA changes are merely explained by alterations in peripheral NAA physiology (metabolism, excretion, protein binding) due to ageing.

Future studies will need to confirm our findings in larger cohorts of healthy individuals who would possibly undergo a contemporary evaluation of NAA levels in brain by 1HMRS.

Nevertheless the availability of age-related serum NAA values, by means of a validated diagnostic tool for analysing NAA in biological fluids, can serve as norms for comparison with NAA changes associated with neurological diseases.

Key points

- NAA is the most important marker of neuronal activities.
- LC/MS may be considered a valid and reproducible diagnostic tool for measuring NAA in biological fluids.
- Low NAA serum levels are associated with ageing (>60 years) in HCs.
- The availability of age-related serum NAA values in HCs can serve as norms for comparison with NAA changes in neurological diseases.

Conflicts of interest

There are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organisations within 3 years of beginning the work submitted that could inappropriately influence authors work. There are no institutions that have contracts relating to this research. There are no agreements of authors or institutions that could be seen as involving a financial interest in this work. There are no sources of financial support related to the manuscript being submitted. The data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere while under consideration at Age and Ageing. Appropriate approval and procedures were used concerning human subjects in our study. All authors have reviewed the contents of the manuscript being submitted, have approved its contents and validated the accuracy of the data. All participants provided informed consent and the study was approved by the ethical local committee.

Funding

This work was supported by Progetto Strategico Regione Puglia “Neurobiotech” PS 124.

References

Planning trials in older patients with stroke: data from the International Stroke Trial

SIR—Older people have been substantially under-represented in randomised trials of treatments for acute stroke and stroke prevention, so there is a need to gather good quality evidence from randomised trials on the effects of treatments in older people [1, 2].

We aimed to demonstrate that it is feasible to perform trials which recruit large numbers of very old stroke patients [3] and to describe the frequency of important outcomes in a cohort of patients with acute ischaemic stroke aged over 80, as an aid to planning future studies which seek to include older stroke subjects.

Methods

The methods used in International Stroke Trial (IST) have been described in detail elsewhere, but briefly 19,435 patients were randomised, in whom the final diagnosis was a confirmed ischaemic stroke in 17,370, clinically definite stroke of unknown pathological type in 992 and haemorrhagic stroke in 599 patients [3]. We present data for those with confirmed ischaemic stroke.

We chose ‘over 80 years at stroke onset’ as our definition of ‘oldest old’ as this cut-off had been used in a number of earlier publications on the topic and in several randomised trials of treatments for acute stroke and for stroke prevention [4–10].

We extracted the baseline data and the frequency of outcome events within 14 days and final outcome at 6 months.

Sources of funding

Details of the funding sources for IST were given in the main publication [3].

Results

Of the ischaemic stroke patients 4,425/17,370 (25.5%) were aged 80 years or older. The countries with the highest proportion of older patients were Sweden, Switzerland and