Plasma choline depletion is associated with decreased peripheral blood leukocyte acetylcholine in children with cystic fibrosis\textsuperscript{1–3}

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ABSTRACT

Background: Choline is an important constituent of acetylcholine. Choline is needed for acetylcholine in the nonneuronal acetylcholine system that includes epithelial cells of the lung and intestine, endothelial cells, and immune cells. Plasma free choline concentrations are low in children with cystic fibrosis (CF), but the implications for acetylcholine are unknown.

Objective: We determined the relation between plasma free choline and related metabolites and leukocyte acetylcholine in children with CF and in a control group of healthy children without CF.

Design: This was a cross-sectional study in 34 children with CF who were pancreatic insufficient and taking pancreatic enzyme-replacement therapy and in 16 healthy children. Plasma free choline, betaine, dimethylglycine, methionine, homocysteine, and leukocyte acetylcholine concentrations were quantified by using isotope-dilution HPLC–tandem mass spectrometry.

Results: Mean (±SE) plasma free choline was 9.30 ± 0.37 and 6.54 ± 0.38 μmol/L (P < 0.05) and leukocyte acetylcholine was 1.21 ± 0.016 and 0.077 ± 0.011 pmol leukocyte acetylcholine/10\textsuperscript{6} cells (P < 0.05) in control children and children with CF, respectively. Leukocyte acetylcholine was positively correlated with plasma free choline concentration in children with CF (r = 0.412, P < 0.05) but not in control children. Plasma betaine, dimethylglycine, and methionine concentrations were also lower in children with CF than in control children (P < 0.05).

Conclusions: A low free choline and methyl status in children with CF is associated with reduced acetylcholine in leukocytes. Whether these changes are explained by a mutation in the CF transmembrane conductance regulator or disturbances in choline metabolism and the implications for immune function in CF are unknown. This trial was registered at clinicaltrials.gov as NCT01150136.


INTRODUCTION

Cystic fibrosis (CF) is the most common life-limiting autosomal condition in whites and is the result of mutations in the CF transmembrane conductance regulator (CFTR), which is a protein that spans the plasma membrane of epithelial cells and some intracellular membranes (1–3). The major and most extensively studied role of CFTR is as a cyclic AMP-regulated chloride channel located on the plasma membrane of epithelial cells (3, 4). CFTR is also present on other membranes and mediates the transport of several other organic ions, such as glutathione, in addition to regulating other ion channels, notably the epithelial Na\textsuperscript{+} and HCO\textsubscript{3}– channels (3–9). Mutations in CFTR lead to impaired Cl\textsuperscript{−} ion channel function, and this results in an increased viscosity of fluid secretions that lead to obstruction and fibrosis of affected organs such as the pancreas. However, many aspects of the pathogenesis of this progressive disease, including pulmonary, hepatobiliary, and immunologic complications, are incompletely understood. Furthermore, considerable differences in the phenotypic expression of the disease and its complications occur in individuals with the same mutation, thus implicating a modifying role of other genes or environmental variables, among which dietary factors could be important (10).

CFTR dysfunction causes exocrine pancreatic insufficiency in 85–90% of individuals with CF (11) Pancreatic insufficiency in addition to impaired functioning of the intestinal epithelium and hepatobiliary system leads to significant nutrient malabsorption, and this is substantially improved by clinical management with pancreatic enzyme replacement therapy (PERT) (12). Although PERT prevents the severe malnutrition that occurs in untreated individuals with CF who have pancreatic insufficiency, residual malabsorption of fat, including lysophosphatidylcholine, phosphatidylcholine, and sphingomyelin, persists (13, 14). Plasma free choline (not including lipid-bound forms) concentrations are low in children with CF (15) and in a range similar to concentrations in adults who consume a choline-deficient diet with hepatic and lymphocyte biomarkers of choline deficiency (16, 17). Choline plays critical roles as a component of phospholipids, as a methyl donor for the regeneration of methionine and the one-carbon folate pool, and as a component of acetylcholine (13, 18). Although usually considered in its role as a neurotransmitter, acetylcholine has important roles in many nonneuronal cells including in epithelial cells of the lung, gastrointestinal system, genito-urinary system, endothelial cells, smooth muscle cells, and the immune system (19–23). Recently, one report described reduced acetylcholine in the lung epithelium and leukocytes of adults with CF, although a possible relation to choline was not

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addressed (24). The current study extends our work on choline in CF to address if a low plasma choline concentration in children with CF is accompanied by reduced leukocyte acetylcholine and the relation to other methionine-homocysteine and choline-dimethylglycine-betaine cycle metabolites.

SUBJECTS AND METHODS

Subjects

This study involved 35 children with CF who were outpatients of the CF Clinic at the British Columbia (BC) Children’s Hospital and 16 control children without CF or any other known health problems. Information on age and sex were recorded for each and 16 control children without CF or any other known health problems. Information on age and sex were recorded for each child, body weights and heights were measured, and z scores for weight and height were calculated (25). Venous blood was collected at the outpatient laboratory of the BC Children’s Hospital, and complete blood counts were measured by using routine clinical procedures in the hospital hematopathology laboratory. All children with CF were pancreatic insufficient and receiving PERT. Information on genotypes was collected for each child with CF from the medical records. Dietary intakes were measured with a food-frequency questionnaire, and choline intakes were measured with nutrient-analyses software (ESHA, version 10.6; Elizabeth Stewart Hands and Associates, Salem, OR) and the US Department of Agriculture database (release 2, 2008) for the choline content of foods as in previous studies (14). This study was approved by the University of British Columbia’s Clinical Screening Committee for Research and Other Studies Involving Human Subjects and the BC Children’s and Women’s Hospital Research Coordinating Committee. All parents and children provided written informed consent to participate in the study.

Blood collection

For this study, venous blood was collected from each child into two 6-mL tubes containing EDTA as the anticoagulant. One tube was immediately placed on ice, and plasma and red blood cells were separated by centrifugation and frozen at −70°C within 20 min of blood collection for later analysis of methyl metabolites (13, 26). The other tube at room temperature was used 20 min of blood collection for later analysis of methyl metabolites (13, 26). The other tube at room temperature was used immediately after collection to prepare peripheral blood leukocytes by centrifugation with Histopaque 1077 (Sigma-Aldrich, St Louis, MO) according to the manufacturer’s instructions.

Analytic methods

Isotope-dilution liquid chromatography-tandem mass spectrometry (MS) with stable isotope-labeled internal standards was used for analysis of plasma homocysteine, methionine, choline, betaine, and dimethylglycine, as previously described (15), and for the analysis of leukocyte acetylcholine. Choline in lysophosphatidylcholine, phosphatidylcholine, or other choline-containing lipids was not included. The MS is a Quattro Micro tandem MS configured with an electrospray source coupled to an Agilent HPLC equipped with a thermostatted autosampler (Waters Corp, Milford, MA). The internal standards used were homocysteine-d₄, methionine-d₅, choline-d₅, betaine-d₃, HCl, N-dimethylglycine-d₆, glycine HCl and acetylcholine d₈ (C/D/N Isotopes Inc, Pointe-Claire, Canada). For analysis of acetylcholine, leukocytes were freeze-dried, and 50 μL deionized H₂O/0.1% formic acid (vol:vol) and 10 μL acetylcholine d₉ was added as the internal standard. The solution was vortexed, 100 μL acetoniitrile was added, and the sample was vortexed again, centrifuged at 2600 × g at 5°C for 3 min, and transferred to an autosampler vial. For quantification of acetylcholine, the MS was operated with a positive electrospray in a selected reaction mode by using the transitions of m/z 146 → 87 for acetylcholine and m/z 155 → 87 for acetylcholine d₉ with the cone voltages set at 20 and 21 V, respectively, a collision voltage of 14 eV, and a collision gas pressure of 3.0 × 10⁻³ mbar. Chromatographic separation was achieved with an Rx-Sil 2.1 × 150-mm column and 2.1 × 12.5-mm precolumn, both of which were packed with 5 μm particles (Agilent Technologies, Mississauga, Canada) with a binary gradient of acetoniitrile (solvent A) and 15 mm ammonium formate and 0.04% formic acid (by volume) in deionized water (solvent B). The mobile phase flow was 0.50 ml solvent A/min with a gradient profile of 15% solvent B for 2.0 min and programmed to increase to 45% solvent B in 6 min and 65% solvent B at 7 min, and the gradient maintained to 7.5 min. The analysis was linear over the range of 25–200 nmol acetylcholine/L. Red cell and plasma folate and plasma vitamin B-12 were quantified by using a dual count radioimmunoassay (Inter-Medico, Markham, Canada) as previously described (26).

Statistical analyses

Subject characteristics were analyzed by using descriptive statistics. One-factor analysis of variance was used to detect significant differences in metabolic concentrations between children with CF and control children. Pearson’s correlation coefficients were calculated to determine associations between plasma choline and leukocyte acetylcholine. Results are presented as means ± SEMs unless otherwise stated. All data were analyzed with the Statistical Package for Social Sciences software (SPSS for Windows, version 15.0, SPSS, Chicago, IL). P < 0.05 was considered significant.

RESULTS

Characteristics of the children with CF (n = 34) and control children (n = 16) are shown in Table 1. Briefly, the age (mean, range) of children with CF (10.4 y, 4–17 y) was not different from that of the control children (10.9 y, 5–16 y), respectively (P > 0.05). Of the 34 children with CF, 22 children were homozygous, and 9 children were heterozygous, for the Δ508 mutation, and 3 children had other mutations. Children with CF

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CF (n = 34)</th>
<th>Control (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>10.4 ± 0.82²</td>
<td>10.9 ± 0.79</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>21/13</td>
<td>8/8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>32.5 ± 6.1²</td>
<td>30.1 ± 4.9²</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>146 ± 1.2</td>
<td>145 ± 1.2</td>
</tr>
<tr>
<td>Leukocytes (×10⁸/L)</td>
<td>8.93 ± 0.63²</td>
<td>8.03 ± 0.26</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>136 ± 2.31</td>
<td>128 ± 1.80</td>
</tr>
</tbody>
</table>

² Mean ± SEM (all such values).

Table 1

Characteristics of children with cystic fibrosis (CF) and children without CF
had lower weight and height z scores than did control children ($P < 0.05$; Table 1). Leukocyte counts were also higher, although hemoglobin concentrations were not different, in children with CF compared with control children. The dietary analysis showed a choline intake of $447 \pm 180$ and $382 \pm 172$ mg choline/day for children with CF and for control children, respectively. Higher intakes of choline by children with CF were due to higher intakes of milk and other dairy products, which reflected recommendations for higher fat intakes in this patient population ($P < 0.05$; Table 2).

The mean leukocyte acetylcholine concentration was significantly lower in children with CF than in control children ($P < 0.05$; Table 2). The 25th–75th range of leukocyte acetylcholine in control children was $0.054–0.175$ pmol acetylcholine/10$^6$ cells, and 17 out of 34 (50%) children with CF had a leukocyte acetylcholine concentration below the 25th percentile, and 26 of 34 had a leukocyte acetylcholine concentration below the mean. Plasma concentrations of choline, betaine, dimethylglycine, and methionine were also significantly lower in children with CF than in control children ($P < 0.05$; Table 2). The separation of results by sex did not change the interpretation. CF ($n = 21$) and control ($n = 8$) boys had plasma choline concentrations of $6.27 \pm 0.56$ and $6.54 \pm 0.61$ mmol/L, betaine concentrations of $32.8 \pm 1.78$ and $31.5 \pm 2.52$ mmol/L, dimethylglycine concentrations of $2.70 \pm 3.63$ and $2.66 \pm 2.90$ mmol/L, and methionine concentrations of $3.91 \pm 4.56$ and $3.19 \pm 4.56$ mmol/L, respectively. Similarly, CF ($n = 13$) and control ($n = 8$) girls had plasma choline concentrations of $6.97 \pm 8.11$ and $9.36 \pm 0.51$ mmol/L, betaine concentrations of $32.8 \pm 9.62$ and $45.2 \pm 3.02$ mmol/L, dimethylglycine concentrations of $2.62 \pm 2.11$ and $3.18 \pm 0.76$ mmol/L, and methionine concentrations of $26.0 \pm 3.30$ and $35.6 \pm 4.56$ mmol/L, respectively.

Leukocyte acetylcholine concentrations showed a significant positive linear relation to plasma choline concentrations in children with CF ($r = 0.412$, $P = 0.016$) but not to leukocyte counts ($r = 0.22$, $n = 34 P > 0.05$). In contrast, leukocyte acetylcholine concentrations showed no relation to plasma choline concentrations control children without CF ($r = 0.020$, $P > 0.05$, $n = 16$). Linear relations between an essential nutrient and functions dependent on that nutrient are expected only in the presence of deficiency but not when the nutrient supply is above needs. Analyses of red blood cell and plasma folate and plasma vitamin B-12 showed no evidence of lower folate or vitamin B-12 status in children with CF; instead, red blood cell and plasma folate and plasma vitamin B-12 concentrations were all higher in children with CF than in control children ($P < 0.05$; Table 2).

To explore if a low leukocyte acetylcholine concentration, which was arbitrarily defined as a leukocyte acetylcholine concentration below the control group mean value, was associated with differences in plasma free choline or other methyl metabolite concentrations, the children were grouped on the basis of a leukocyte acetylcholine amount $<0.121$ or $\geq 0.121$ pmol leukocyte acetylcholine/10$^6$ cells, which was the mean value for children in the control group (Figure 1 and Table 2). Plasma metabolite concentrations in children in the CF group were compared with the respective metabolite concentrations in control children with the same stratification by leukocyte acetylcholine amounts. In the absence of a relation, it was expected that plasma choline, methionine, and their metabolite concentrations would not differ in children stratified by leukocyte acetylcholine amounts. Leukocyte acetylcholine concentrations in children with $<0.121$ pmol leukocyte acetylcholine/10$^6$ cells were $0.07 \pm 0.017$ pmol/10$^6$ cells ($n = 8$) and $0.048 \pm 0.006$ pmol/10$^6$ cells ($n = 26$) for control children and children with CF, respectively, and their plasma choline concentrations were $9.59 \pm 0.78$ and $5.84 \pm 0.35$ mmol/L, respectively ($P < 0.01$). In contrast, leukocyte acetylcholine concentrations in control children and children with CF with $\geq 0.121$ pmol leukocyte acetylcholine/10$^6$ cells was $0.161 \pm 0.034$ pmol/10$^6$ cells ($n = 8$) and $0.162 \pm 0.015$ pmol/10$^6$ cells ($n = 26$), respectively, with corresponding plasma choline concentrations of $9.07 \pm 0.38$ and $8.63 \pm 0.78$ mmol/L, respectively. Plasma methionine concentrations showed a similar pattern; children with CF and control children with $\geq 0.121$ pmol leukocyte acetylcholine/10$^6$ cells had plasma methionine concentrations of $33.7 \pm 3.91$ and $31.5 \pm 4.24$ mmol/L, respectively ($P > 0.05$). In contrast, children with CF having $<0.121$ pmol leukocyte acetylcholine/10$^6$ cells had plasma methionine concentrations of $21.1 \pm 2.91$ and $23.7 \pm 3.91$ mmol/L, respectively ($P < 0.05$).

![FIGURE 1. Mean (±SE) concentrations of plasma choline, betaine, dimethylglycine, methionine, and homocysteine in children grouped by leukocyte acetylcholine (ACH) concentration $<0.121$ or $\geq 0.121$ pmol/10$^6$ cells for children without cystic fibrosis (CF) (control; 2 groups, $n = 8$ each) and children with CF (2 groups, $n = 26$ and 8), respectively. The concentration of each metabolite was compared between children with CF and control children in the same leukocyte ACH concentration range. *Difference between control and CF groups, $P < 0.05$ (ANOVA).](image-url)

### TABLE 2
Peripheral blood mononuclear cell acetylcholine and plasma choline, betaine, dimethylglycine, homocysteine, and methionine concentrations and red blood cell folate in children with cystic fibrosis (CF) and control children

<table>
<thead>
<tr>
<th></th>
<th>CF ($n = 34$)</th>
<th>Control ($n = 16$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine (pmol/10$^6$ cells)</td>
<td>0.077 ± 0.011$^{1,2}$</td>
<td>0.121 ± 0.016</td>
</tr>
<tr>
<td>Choline (μmol/L)</td>
<td>6.54 ± 0.38$^2$</td>
<td>9.30 ± 0.37</td>
</tr>
<tr>
<td>Betaine (μmol/L)</td>
<td>30.1 ± 1.95$^2$</td>
<td>43.0 ± 2.20</td>
</tr>
<tr>
<td>Dimethylglycine (μmol/L)</td>
<td>2.66 ± 0.14$^2$</td>
<td>3.62 ± 0.39</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>25.3 ± 1.78</td>
<td>32.3 ± 2.52</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>6.24 ± 0.41</td>
<td>5.64 ± 0.34</td>
</tr>
<tr>
<td>Red blood cell folate (ng/mL)</td>
<td>737 ± 51.8$^2$</td>
<td>532 ± 37.2</td>
</tr>
<tr>
<td>Plasma folate (ng/mL)</td>
<td>25.7 ± 1.41$^2$</td>
<td>18.7 ± 1.02</td>
</tr>
<tr>
<td>Plasma vitamin B-12 (pg/mL)</td>
<td>1212 ± 107$^2$</td>
<td>832 ± 135</td>
</tr>
</tbody>
</table>

$^1$ Mean ± SEM (all such values).

$^2$ Significantly different from the control group, $P < 0.05$ (ANOVA).

*Difference between control and CF groups, $P < 0.05$ (ANOVA).
children with CF and control children with <0.121 pmol leukocyte acetylcholine/10^6 cells had plasma methionine concentrations of 23.4 ± 1.62 and 34.4 ± 4.74 μmol/L, respectively (P < 0.05). Of the 8 children with CF with ≥0.121 pmol leukocyte acetylcholine/10^6 cells and higher plasma choline and methionine concentrations, 4 children were homozygote for ΔF508, 2 of 9 were heterozygote for ΔF508 (one child had each of ΔF508/G542X and ΔF508/38S0–3T/G85R75Q), and 2 of 3 had other mutations (one each of G85E/G85E and W1282X/W1282X).

DISCUSSION

This study shows that leukocyte acetylcholine concentrations are lower with a significant association between decreased plasma free choline and leukocyte acetylcholine concentrations in children with CF. In addition to choline, plasma concentrations of betaine, dimethylglycine, and methionine were lower in children with CF than in healthy children without CF, as shown in previous studies by us in children with CF (15). Leukocyte acetylcholine concentrations showed no association to plasma choline concentrations in children without CF, which suggest that plasma choline does not limit choline uptake or acetylcholine synthesis in healthy children. In contrast, the significant positive association between plasma free choline and leukocyte acetylcholine concentrations in children with CF provided new evidence, to our knowledge, that disturbances in choline metabolism in this disorder extended to leukocytes. However, because a direct measurement of leukocyte choline was not made, factors other than reduced plasma choline may be involved in the decreased leukocyte acetylcholine observed in children with CF.

Acetylcholine is known to be present in many cells outside of the nervous system including in epithelial cells of the airway, intestine, kidney, skin, placenta, and leukocytes, alveolar macrophages, lung smooth muscle, and several other cells where it functions in paracrine and autocrine signaling via nicotinic and muscarinic acetylcholine receptors (19–23, 28, 29). Choline uptake is mediated by several mechanisms that include low-affinity facilitated diffusion, intermediate-affinity Na⁺-independent transporters that include organic cation transporters, and high-affinity Na⁺-dependent choline transporters (21). Although high-affinity transporters are abundant in neurons and function almost exclusively in supplying choline for neuronal acetylcholine synthesis, polyspecific organic cation transporters are involved in choline transport across the plasma membrane in other cell types (21, 29). This involvement raises the possibility that defective CFTR function might contribute to altered choline transport in nonneuronal cells through interference with the organic cation transporters.

Choline has numerous metabolic and physiologic roles that span from its role as a component of plasma lipoprotein, cell membrane, and biliary and surfactant lipids to providing methyl groups for the methionine-homocysteine cycle and one-carbon folate pool, in addition to its role in acetylcholine (13, 18). Betaine is formed from choline via choline oxidase and betaine aldehyde dehydrogenase, whereas dimethylglycine transfers methyl groups from betaine to homocysteine to generate methionine (13). The pattern of reduced plasma choline, betaine, dimethylglycine, and methionine in children with CF compared with in control children (P < 0.05) suggests a depletion of methyl groups. The reason for low choline could include impaired digestion and absorption of choline-containing lipids, an increased turnover to supply methyl groups and glycine, or the inhibition of endogenous synthesis via phosphatidylethanolamine-N-methyltransferase (13, 14, 16, 31). In contrast, low methionine could also result from decreased regeneration from homocysteine or increased metabolism of homocysteine via cystathione β-synthase to generate cysteine for synthesis of glutathione or further metabolism to taurine.

Although nonneuronal acetylcholine is present in many organs and cells that are affected in CF, including the airways, intestines, and immune system, almost nothing is known about acetylcholine in CF. One previous study described a 70% reduction in acetylcholine in bronchi and lung parenchyma and in peripheral blood leukocytes of 13 patients with CF compared with in 9 healthy control subjects (24). The lymphocytic cholinergic system plays an important role in the regulation of immune function with evidence that impaired lymphocytic cholinergic activity is related to immune dysfunction and up-regulated inflammatory cytokine production (29). Persistent and up-regulated inflammatory responses and elevated inflammatory cytokines are present in patients with CF (32–35). Furthermore, choline deficiency in adult humans results in DNA damage and apoptosis in peripheral blood lymphocytes (17). Acetylcholine from nonneuronal cells, including the airway surface epithelium, is also a major regulator of airway function, including the maintenance of cell-cell contact, stimulation of fluid secretion, ciliary beat frequency, and mucociliary clearance, all of which are relevant to CF (23, 28, 30), and decreased acetylcholine has been reported for bronchi and lung parenchyma of CF patients (24).

In conclusion, to our knowledge, this is the first report to link low plasma free choline to reduced leukocyte acetylcholine in humans, specifically in children with CF. The extent to which decreased choline status and decreased acetylcholine in peripheral blood leukocytes contributes to the severity of clinical manifestations in CF, including the chronic up-regulated inflammatory state and cytokine production, deserves more detailed study. The link between choline and acetylcholine shown in the current report also extended the importance of this essential tertiary amine beyond its role in lipid metabolism, the methylolation of homocysteine, and as a classic neurotransmitter.

We thank the parents and children who participated in this study and the staff of the CF Clinic at the BC Children’s Hospital and Child and Family Research Institute, Nutrition and Metabolism research cluster for facilitating this study.

The authors’ responsibilities were as follows—SMI: conceived the study; AGFD: was the clinician lead; SMI and AGFD: designed and organized the study and prepared the manuscript; BNB and PJS: enrolled the subjects and conducted the study; and DH: developed the acetylcholine methodology and conducted sample analyses. None of the authors had a conflict of interest.

REFERENCES