Decreased glutathione and low catalase activity contribute to oxidative stress in children with α-1 antitrypsin deficiency

**ABSTRACT**

**Background** Recent investigations in animal models have revealed oxidative stress and oxidative damage in the pathogenesis of α-1 antitrypsin deficiency (AATD). However, no data are available on the oxidative stress status and antioxidant enzyme activity in these patients. This study was aimed to analyse the oxidative stress profile and enzymatic antioxidant defence mechanisms in children with AATD.

**Methods** Oxidative stress parameters and the activity of the main antioxidant enzymes were prospectively measured in serum of fifty-one children diagnosed with AATD and thirty-eight control individuals.

**Results** Oxidative stress was increased in the serum of children with intermediate- (MZ; SZ) and high-risk (ZZ) phenotypes for developing AATD-related emphysema and/or liver disease. When compared with the control group, intermediate- and high-risk groups showed significantly lower total glutathione and reduced glutathione levels, decreased catalase activity and increased glutathione peroxidase activity leading to an accumulation of hydrogen peroxide that would explain the significantly increased levels of oxidative stress biomarkers observed in these patients. No differences were observed between the control (MM) and the low-risk (MS; SS) groups. A gradation in oxidative stress parameters was observed when patients were compared among themselves, in that the expression of the Z allele produces a higher oxidative stress status in homozygous (ZZ) than in heterozygous (MZ; SZ) patients.

**Conclusions** Increased oxidative stress, together with reduced antioxidant defence are involved in the pathophysiology of AAT at early stages, opening up a new rationale for the use of antioxidant therapies in the treatment of the disease.

### Table 1: Biomarkers of oxidative stress in AATD and control children

<table>
<thead>
<tr>
<th>Oxidative stress biomarkers</th>
<th>Control (MM)</th>
<th>Low risk (MS; SS)</th>
<th>Intermediate risk (MZ; SZ)</th>
<th>High risk (ZZ)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSSG/GSH (%)</td>
<td>2.42 (1.97–3.57)</td>
<td>2.62 (1.07–3.77)</td>
<td>4.14 (2.60–6.32)</td>
<td>6.47 (4.56–9.50)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG (μM)</td>
<td>2990 (2592–3687)</td>
<td>2684 (2261–2821)</td>
<td>1818 (1474–2535)</td>
<td>1630 (1292–1800)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GSH (μM)</td>
<td>2864 (2532–3620)</td>
<td>2183 (1947–2622)</td>
<td>1741 (1464–3236)</td>
<td>1713 (1376–1937)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GSSG (μM)</td>
<td>72.98 (63.96–91.81)</td>
<td>85.86 (75.32–108.5)</td>
<td>64.00 (32.11–102.2)</td>
<td>75.60 (68.05–101.6)</td>
<td>0.254</td>
</tr>
<tr>
<td>MDA (pmol/mg)</td>
<td>0.85 (0.75–1.07)</td>
<td>0.72 (0.52–1.85)</td>
<td>1.15 (0.85–1.68)</td>
<td>1.94 (1.44–3.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PC (mmol/mg)</td>
<td>0.15 (0.12–0.20)</td>
<td>0.16 (0.13–0.23)</td>
<td>0.17 (0.16–0.18)</td>
<td>0.25 (0.19–0.26)</td>
<td>0.010</td>
</tr>
<tr>
<td>8-OHdG (ng/mL)</td>
<td>0.61 (0.54–0.71)</td>
<td>0.68 (0.60–0.82)</td>
<td>0.97 (0.68–1.11)</td>
<td>2.00 (1.16–2.54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SOD (U/ml)</td>
<td>5.38 (4.56–5.61)</td>
<td>5.90 (4.38–6.30)</td>
<td>6.16 (5.41–7.53)</td>
<td>5.76 (3.78–6.17)</td>
<td>0.080</td>
</tr>
<tr>
<td>CAT (mmol/min/mg)</td>
<td>37.66 (32.05–40.57)</td>
<td>36.95 (31.39–43.01)</td>
<td>26.92 (16.47–38.89)</td>
<td>17.67 (14.05–23.26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GPx (μmol/min/mg)</td>
<td>21.52 (18.32–23.18)</td>
<td>22.16 (20.79–24.48)</td>
<td>24.52 (19.37–26.79)</td>
<td>32.35 (21.96–37.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GRD (mmol/min/mg)</td>
<td>3.69 (3.41–4.38)</td>
<td>3.04 (2.79–4.97)</td>
<td>4.46 (2.67–5.22)</td>
<td>4.20 (2.86–4.87)</td>
<td>0.610</td>
</tr>
<tr>
<td>H₂O₂ (fluorescence units)</td>
<td>198.50 (138.3–333)</td>
<td>251.00 (212.0–335)</td>
<td>323.00 (258.5–350)</td>
<td>584.00 (369.0–601)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

All values are expressed as median (IQR). Statistic significance after application of Kruskal-Wallis test. p Values lower than 0.05 were statistically significant (labelled in bold). AATD: α-1 antitrypsin deficiency; 8-OHdG; 8-hydroxydeoxyguanosine; CAT, catalase; GPx, glutathione peroxidase; GRD, glutathione reductase; GSH, reduced glutathione; GSSG, oxidised glutathione; MDA, malondialdehyde; PC, protein carbonyl; SOD, superoxide dismutase; TG, total glutathione.
for results and discussion on multiple hypothesis testing).

In conclusion, our study supports the hypothesis that OS is a feature of AATD at early stages, and is associated with the presence of AAT Z protein. An increased chronic oxidative status could contribute to the higher risk of lung and liver damage observed in these patients, suggesting a rationale for the use of antioxidant therapies in the treatment of the disease.5

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Collaborators Maria Mercedes Navarro-García.

Contributors Study design: AE, FS, PC-F and FD. Patient recruitment, anamnesis and physical examination: AE, MA, SC, FS and PC-F. Measurement of oxidative stress parameters: SP and FD. Statistical analysis: SP and FD. Article writing: AE, FS, PC-F and FD.

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Patient consent Obtained.

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References
ONLINE DATA SUPPLEMENT

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Author names: Amparo Escribano, Mónica Amor, Sara Pastor, Silvia Castillo, Francisco Sanz, Pilar Codoñer-Franch, Francisco Dasí

Table S1. Demographic and clinical characteristics in AATD and control children.

Figure E1. Glutathione levels in AATD patients and control individuals. High (ZZ) and intermediate-risk (MZ; SZ) patients showed significantly lower TG levels than control individuals. No significant differences were observed between low-risk and control individuals (A). GSH levels were significantly decreased in serum of AATD patients compared to control individuals (B) whereas no significant differences were observed in the GSSG levels (C). Cellular oxidative status as determined by the GSSG/GSH ratio showed a significant imbalance towards increased oxidative status in high and intermediate-risk patients (D). Abbreviations are found in the text. Asterisks indicate levels of statistical significance with respect to the control group (**p<0.001; ***p<0.0001).

Figure E2. Oxidative stress biomarkers are increased in serum of AATD patients. High (ZZ) and intermediate-risk (MZ; SZ) patients showed significantly higher levels of malonyldialdehyde (A), 8-hydroxydeoxyguanosine (B) and protein carbonylation (C) than control individuals (MM). No significant differences were observed between the low-risk (MS; SS) and the control group. Abbreviations are
found in the text. Asterisks indicate levels of statistical significance with respect to the control group (*p<0.01; **p<0.001; ***p<0.0001).

**Figure E3. Antioxidant enzyme capacity in AATD patients and control individuals.** High (ZZ) and intermediate-risk (MZ; SZ) patients showed significantly higher CAT (B) and GPx (C) activities than the control group (MM) whereas no differences in these enzymatic activities were observed between low-risk (MS; SS) and control groups. No significant differences were observed in SOD (A) and GRd activities (D) in any of the groups. Abbreviations are found in the text. Asterisks indicate levels of statistical significance with respect to the control group (**p<0.001; ***p<0.0001).

**Figure E4. Hydrogen peroxide accumulates in leukocytes of AATD patients.** High (ZZ) and intermediate-risk (MZ; SZ) patients showed significantly higher basal concentration of hydrogen peroxide determined by 2’,7’ dichlorofluorescein diacetate (DCFH) fluorescence than control individuals (MM). No significant differences were observed between the low-risk (MS; SS) and the control group. Asterisks indicate levels of statistical significance with respect to the control group (*p<0.01; **p<0.001).

**Figure E5. Figure 1. Schematic overview of glutathione metabolism and enzymatic antioxidant defence mechanisms in patients with AATD as compared to control individuals. Left:** Eukaryotic cells possess antioxidant enzymes that are responsible for neutralising reactive oxygen species, which may oxidize nucleic acids, lipids and proteins leading to cell malfunction if they
accumulate. Superoxide dismutase detoxifies superoxide anion ($O_2^-$), which is converted to hydrogen peroxide ($H_2O_2$). Reaction of $O_2^-$ and $H_2O_2$ in the presence of ferrous iron ($Fe^{++}$) produces hydroxyl radicals ($\cdot$OH). Superoxide anion is able to react with nitric oxygen (NO) to form the much more powerful oxidant peroxynitrite ($ONOO^-$). In the presence of neutrophil myeloperoxidase (MPO), $H_2O_2$ and chloride ($Cl^-$) form hypochorus acid (HOCl). Both $\cdot$OH and HOCl are potent oxidants. $H_2O_2$ accumulation is prevented by catalase (CAT) and glutathione peroxidase (GPx), the latter uses reduced glutathione (GSH) as the reducing factor. Oxidised glutathione (GSSG) is either exported from the cell or reduced to GSH by the action of glutathione reductase (GRd) using NADPH as the electron donor.

**Right:** High- and intermediate-risk AATD patients show diminished CAT activity, which leads to $H_2O_2$ accumulation. GPx activity is increased in these patients to compensate for the accumulation of $H_2O_2$. However, low levels of GSH would prevent its removal. Since $H_2O_2$ is a potent oxidant and a precursor of $\cdot$OH and HOCl, its accumulation would explain the increased levels of oxidative stress biomarkers observed in these patients.
Multiple hypothesis testing

Following the Kruskal-Wallis test, multiple hypothesis testing was performed using the Dunn’s multiple comparisons test to identify the significant pairwise differences among groups. When AATD patients were compared among themselves, a gradation was observed, so that high-risk (ZZ) patients showed a significantly higher GSSG/GSH ratio (p=0.01, p<0.0001; respectively), MDA (p=0.02, p=0.01), 8-OHdG (p=0.003, p<0.0001) and PC (p=0.04, p<0.001) and lower TG levels (p=0.02, p<0.0001) than intermediate (MZ; SZ) and low-risk patients (MS; SS). Regarding GSH levels, high-risk patients showed significantly higher levels than low-risk patients (p=0.004) and also higher levels than intermediate-risk patients, although these differences were not significant (p=0.56). Similarly, intermediate-risk patients showed a significantly higher GSSG/GSH ratio (p=0.01), MDA (p=0.02), 8-OHdG (p=0.02) and PC (p=0.04) and lower TG (p=0.02) and GSH (p=0.04) levels than low-risk patients.

Regarding antioxidant enzymatic activities, high-risk patients showed significantly lower CAT activity than intermediate- and low-risk patients (p=0.02, p=0.005; respectively), whereas no significant differences were observed between low and intermediate-risk patients (p=0.09). A significant increase in GPx activity was observed in high-risk compared to intermediate- (p=0.02) and low-risk patients (p=0.002) and no significant differences were observed between low- and intermediate-risk patients (p=0.35). No significant differences were observed in SOD and GRd activities. High-risk patients showed significantly higher H$_2$O$_2$ levels than either intermediate- (p=0.002) or low-risk patients (p=0.0004). In addition, intermediate-risk patients showed significantly higher H$_2$O$_2$ levels than low-risk patients (p=0.03).

Compared to controls and low-risk patients, diminished catalase activity and higher H$_2$O$_2$ levels were observed in high- and intermediate-risk patients. GPx activity (an enzyme that catalyses the reduction of H$_2$O$_2$ to H$_2$O using GSH as the reducing factor) is increased in these patients, probably to compensate for the accumulation of H$_2$O$_2$. However the significant low levels of GSH would prevent the removal of the H$_2$O$_2$, which accumulates in these patients. Moreover, no differences in the activity of GRd (an enzyme that catalyses the reduction of GSSG to GSH) were observed, as would be expected to compensate for low levels of GSH observed in these patients to maintain the reducing environment of the cell. Since H$_2$O$_2$ is itself a powerful oxidant and central precursor to both ·OH and HOCl (two extremely potent oxidants), its accumulation
would explain the significantly increased levels of oxidative stress biomarkers (MDA, 8-OHdG and PC) observed in high- and intermediate-risk patients (see Figure E5 online data supplement).

Overall, these results show a gradation in oxidative stress parameters suggesting that the expression of the Z allele produces higher oxidative stress status in homozygous (ZZ) than in heterozygous (MZ; SZ) patients.
Figure E1

A

Total Glutathione (µM)

Control Low risk Intermediate risk High risk

Kruskal-Wallis
p<0.001

B

GSH (µM)

Control Low risk Intermediate risk High risk

Kruskal-Wallis
p<0.001

C

GSSG (mM)

Control Low risk Intermediate risk High risk

Kruskal-Wallis
p=0.25

D

GSSG/GSH (%)

Control Low risk Intermediate risk High risk

Kruskal-Wallis
p<0.001

*** **
Figure E2
Figure E3
Table S1. Demographic and clinical characteristics in AATD and control children

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 38)</th>
<th>Low risk (n = 15)</th>
<th>Intermediate risk (n = 28)</th>
<th>High risk (n = 8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median; range)</td>
<td>9 (4-12)</td>
<td>11 (4-14)</td>
<td>9 (4-12)</td>
<td>9 (6-14)</td>
<td>0.182</td>
</tr>
<tr>
<td>Gender M/F (n, %)</td>
<td>25/17, 60/40</td>
<td>11/4, 73/27</td>
<td>17/11, 61/39</td>
<td>6/3, 67/33</td>
<td>0.691</td>
</tr>
<tr>
<td>AAT (mg/dl)</td>
<td>139.4 (161.0-119.1)</td>
<td>119.5 (127.5-99.0)</td>
<td>82.0 (95.0-67.8)</td>
<td>25.4 (34.2-22.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>18.22 ± 3.77</td>
<td>19.21 ± 2.55</td>
<td>19.17 ± 3.45</td>
<td>19.50 ± 3.31</td>
<td>0.536</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>91.90 (88.0-105.4)</td>
<td>100.2 (91.45-109.3)</td>
<td>103.9 (96.20-120.4)</td>
<td>101.0 (100.8-116.0)</td>
<td>0.620</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>100.0 (89.8-108.1)</td>
<td>100.0 (89.90-114.5)</td>
<td>108.4 (87.6-119.0)</td>
<td>107.0 (106.9-108.0)</td>
<td>0.951</td>
</tr>
<tr>
<td>FEV₁/FVC (%)</td>
<td>92.0 (78.0-102.0)</td>
<td>85.0 (76.4-107.7)</td>
<td>99.40 (88.8-109.7)</td>
<td>97.2 (83.0-111)</td>
<td>0.432</td>
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<tr>
<td>FEF₂₅₋₇₅ (%)</td>
<td>71.8 (56.7-90.2)</td>
<td>80.5 (59.1-97.3)</td>
<td>104.7 (98.6-108.3)</td>
<td>96.5 (84.1-97.0)</td>
<td>0.095</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>28.00 (24.50-37.50)</td>
<td>26.00 (22.75-30.75)</td>
<td>28.00 (25.00-37.00)</td>
<td>26.00 (25.00-29.00)</td>
<td>0.389</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>19.00 (18.00-23.50)</td>
<td>18.00 (16.00-20.00)</td>
<td>20.50 (17.25-25.00)</td>
<td>24.00 (19.00-32.00)</td>
<td>0.113</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>14.00 (12.00-17.25)</td>
<td>15.00 (11.75-16.25)</td>
<td>16.00 (14.00-18.00)</td>
<td>18.00 (17.00-20.00)</td>
<td>0.055</td>
</tr>
</tbody>
</table>

AAT, Alpha-1 antitrypsin; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gammaglutamyl-transferase; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; FEF₂₅₋₇₅, forced expiratory flow 25–75%. Data are expressed as median and interquartile range (IQR). Statistic significance after application of Kruskal-Wallis test. Comparison of proportions was performed by chi-square test. P-values lower than 0.05 were statistically significant (labelled in bold).