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 alpha-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 AATD at early stages, opening up a new rationale for the use of antioxidant therapies in the treatment of the disease.

α-1 Antitrypsin deficiency (AATD) is a rare genetic and hereditary condition characterised by low circulating levels of the α-1 antitrypsin (AAT) protein, a serine protease inhibitor that protects lung tissues from damage caused by proteolytic enzymes, such as neutrophil elastase and proteinase-3. Deficiency of circulating AAT is associated with an increased risk of developing emphysema, hepatic cirrhosis, panniculitis, bronchiectasis and vasculitis.[1]

Clinical data indicate that there is a great variability in the severity of the symptoms found in patients with AATD, and neither plasma AAT levels nor phenotype are sufficient to identify those patients who will develop severe lung or liver disease, indicating that there may be other mechanisms, in addition to the protease-antiprotease imbalance, that contribute to the development of emphysema or liver disease.[2] Recent studies in animal models have shown an association between oxidative stress (OS) and the pathophysiology of AATD.[3 4]

We hypothesised that OS is increased in patients with AATD at early stages before the development of severe clinical manifestations. Thus, the present study was undertaken to investigate the role of OS in children with AATD. The OS status was evaluated in plasma by monitoring the total glutathione, the oxidised versus reduced glutathione ratio (GSSG/GSH), the oxidation products 8-hydroxydeoxyguanosine (8-OHdG), malonyldialdehyde (MDA) and protein carbonyl (PC), and the activity of the main antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRd).

Fifty-one children diagnosed with AATD, and 38 control individuals, were prospectively included in the study. Demographic and clinical characteristics of patients included in the study are presented in online supplementary table S1. There were no differences in age, sex, Body Mass Index and lung or liver damage markers, and all the children were asymptomatic according to their physical status and the pulmonary and liver tests. Intermediate- (MZ; SZ) and high-risk (ZZ) patients showed significantly higher GSSG/GSH ratio ($p=0.001$; $p<0.001$, respectively), MDA ($p=0.004$; $p<0.001$), 8-OHdG ($p<0.001$; $p<0.001$) and PC ($p=0.010$; $p=0.002$) than the control group (MM). When compared with the control group, intermediate and high-risk patients showed significantly lower total glutathione ($p<0.001$; $p<0.001$), GSH levels ($p<0.001$; $p<0.001$), decreased CAT activity ($p=0.003$; $p<0.001$) and increased GPx activity ($p=0.040$; $p<0.001$) leading to an accumulation of hydrogen peroxide ($p=0.040$; $p=0.001$) (table 1 and see online supplementary figures E1–E4) that would explain the significantly increased levels of OS biomarkers observed in these patients (see online supplementary figure E5). No differences were observed between the control and the low-risk (MS; SS) groups. A gradation in OS parameters was observed when patients were compared among themselves, in that the expression of the Z allele produces a higher OS status in homozygous (ZZ) than in heterozygous (MZ;SZ) patients (see online supplement

Table 1 Biomarkers of oxidative stress in AATD and control children

Oxidative stress biomarkers	Control (MM)	Low risk (MS; SS)	Intermediate risk (MZ; SZ)	High risk (ZZ)	p Value
GSSG/GSH (%)	2.42 (1.97–3.57)	2.62 (1.07–3.77)	4.14 (2.60–6.32)	6.47 (4.56–9.50)	**<0.001**
TG (μM)	2930 (2592–3687)	2684 (2261–2831)	1818 (1474–2535)	1630 (1292–1800)	**<0.001**
GSH (μM)	2864 (2532–3620)	2183 (1947–2622)	1741 (1464–2326)	1713 (1376–1937)	**<0.001**
GSSG (μM)	72.98 (63.96–91.81)	85.86 (75.32–108.5)	64.0 (32.11–102.2)	75.60 (68.05–101.6)	0.254
MDA (pmol/mg)	0.85 (0.75–1.07)	0.72 (0.52–1.85)	1.15 (0.85–1.68)	1.94 (1.44–3.76)	**<0.001**
PC (nmol/mg)	0.15 (0.12–0.20)	0.16 (0.13–0.23)	0.17 (0.16–0.18)	0.25 (0.19–0.26)	**0.010**
8-OHdG (ng/mL)	0.61 (0.54–0.71)	0.68 (0.60–0.82)	0.97 (0.68–1.11)	2.00 (1.16–2.54)	**<0.001**
SOD (U/mL)	5.38 (4.56–5.61)	5.90 (4.38–6.30)	6.16 (5.41–7.53)	5.76 (3.78–6.17)	0.080
CAT (nmol/min/mL)	37.66 (32.05–40.57)	36.95 (31.39–43.01)	26.92 (16.47–38.89)	17.67 (14.05–23.26)	**<0.001**
GPx (nmol/min/mL)	21.52 (18.32–23.18)	22.16 (20.79–24.48)	24.52 (19.37–26.79)	32.35 (29.16–37.05)	**<0.001**
GRd (nmol/min/mL)	3.69 (3.41–4.38)	3.04 (2.79–4.97)	4.46 (2.67–5.22)	4.20 (2.86–4.87)	0.610
H_2O_2 (fluorescence units)	198.50 (138.3–333–3)	251.0 (212.0–335.0)	323.0 (258.5–350.0)	584.0 (369.0–601.0)	**0.001**

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, malonyldialdehyde; PC, protein carbonyls; 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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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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ONLINE DATA SUPPLEMENT

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

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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 (·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 ·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 ·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_2O_2 levels than either intermediate- ($p=0.002$) or low-risk patients ($p=0.0004$). In addition, intermediate-risk patients showed significantly higher H_2O_2 levels than low-risk patients ($p=0.03$).

Compared to controls and low-risk patients, diminished catalase activity and higher H_2O_2 levels were observed in high- and intermediate-risk patients. GPx activity (an enzyme that catalyses the reduction of H_2O_2 to H_2O using GSH as the reducing factor) is increased in these patients, probably to compensate for the accumulation of H_2O_2. However the significant low levels of GSH would prevent the removal of the H_2O_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_2O_2 is itself a powerful oxidant and central precursor to both $^{\cdot}OH$ 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.

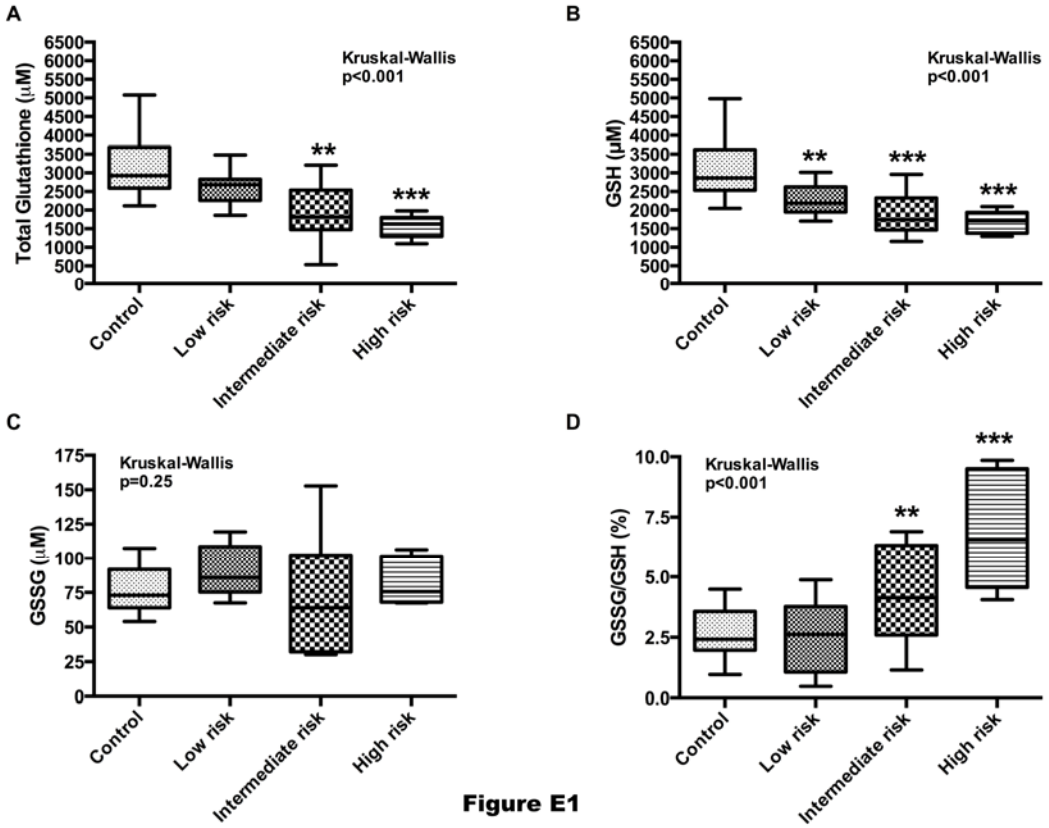
A
Total Glutathione (µM)
Kruskal-Wallis
p<0.001
**
***
Control
Low risk
Intermediate risk
High risk
B
GSH (µM)
Kruskal-Wallis
p<0.001
**
***
***
C
GSSG (µM)
Kruskal-Wallis
p=0.25
D
GSSG/GSH (%)
Kruskal-Wallis
p<0.001
**
***

Figure E1

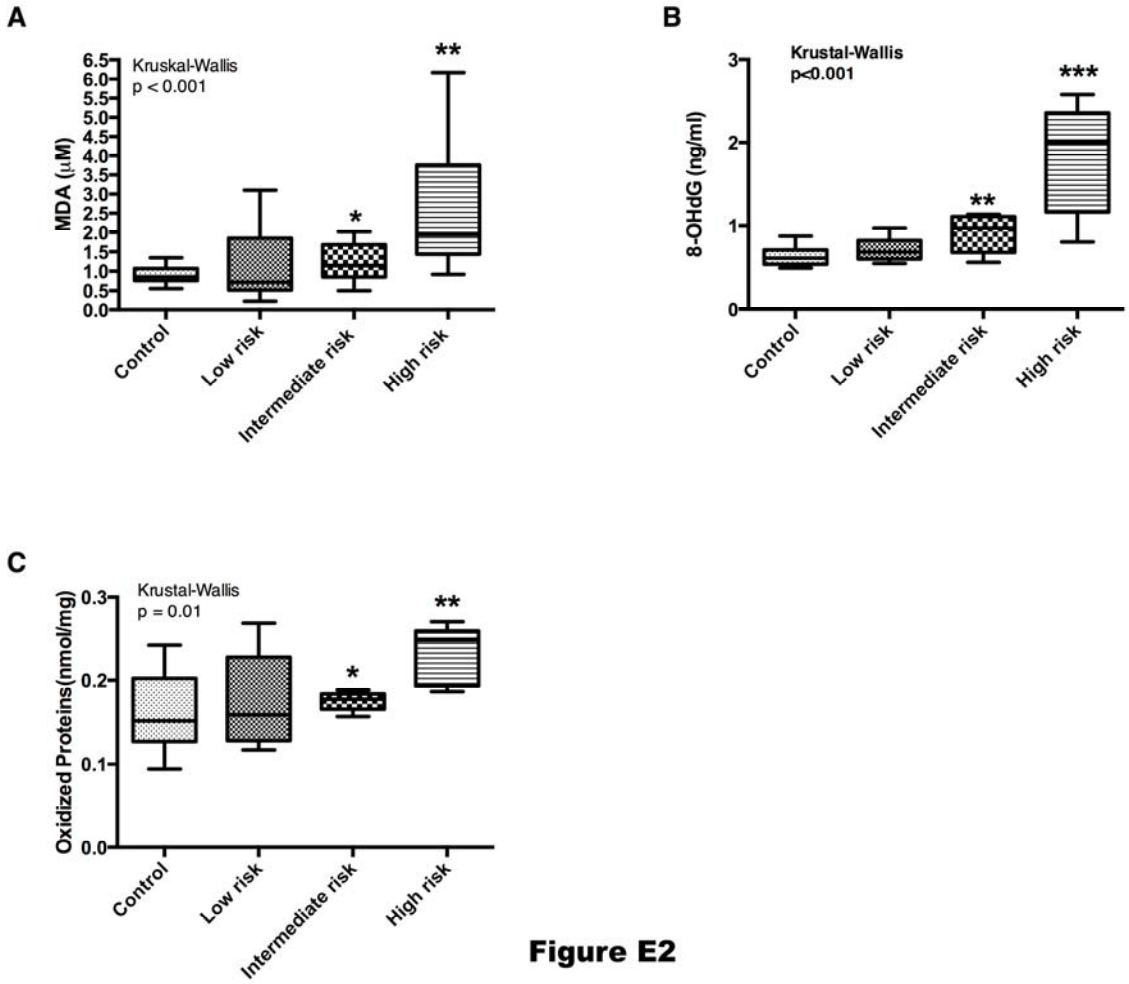
A
Kruskal-Wallis
p < 0.001
MDA (μM)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
Low risk
Intermediate risk
High risk
B
Krustal-Wallis
p<0.001
8-OHdG (ng/ml)
3
2
1
0
**
***
Control
Low risk
Intermediate risk
High risk
C
Krustal-Wallis
p = 0.01
Oxidized Proteins(nmol/mg)
0.3
0.2
0.1
0.0
*
**
Control
Low risk
Intermediate risk
High risk

Figure E2

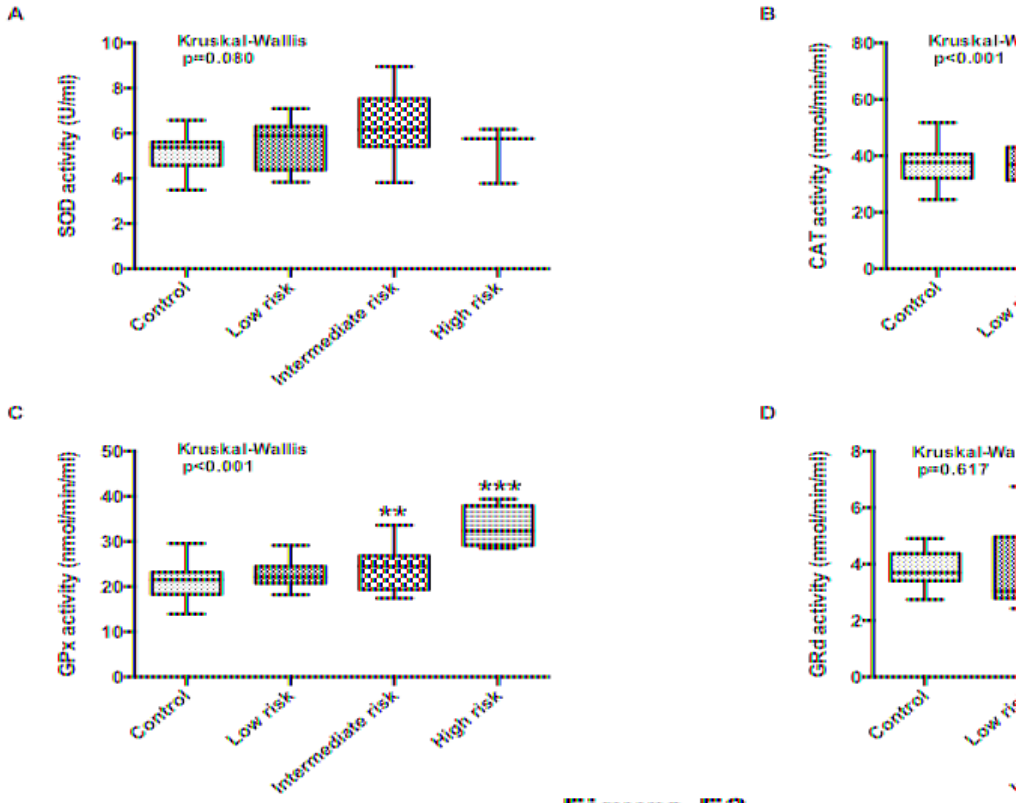
A
Kruskal-Wallis
p=0.080
SOD activity (U/ml)
10
8
6
4
2
0
Control
Low risk
Intermediate risk
High risk
B
Kruskal-W
p<0.001
CAT activity (nmol/min/ml)
80
60
40
20
0
Control
Low
C
Kruskal-Wallis
p<0.001
GPx activity (nmol/min/ml)
50
40
30
20
10
0
**
***
Control
Low risk
Intermediate risk
High risk
D
Kruskal-Wa
p=0.617
GRd activity (nmol/min/ml)
8
6
4
2
0
Control
Low ri

Figure E3

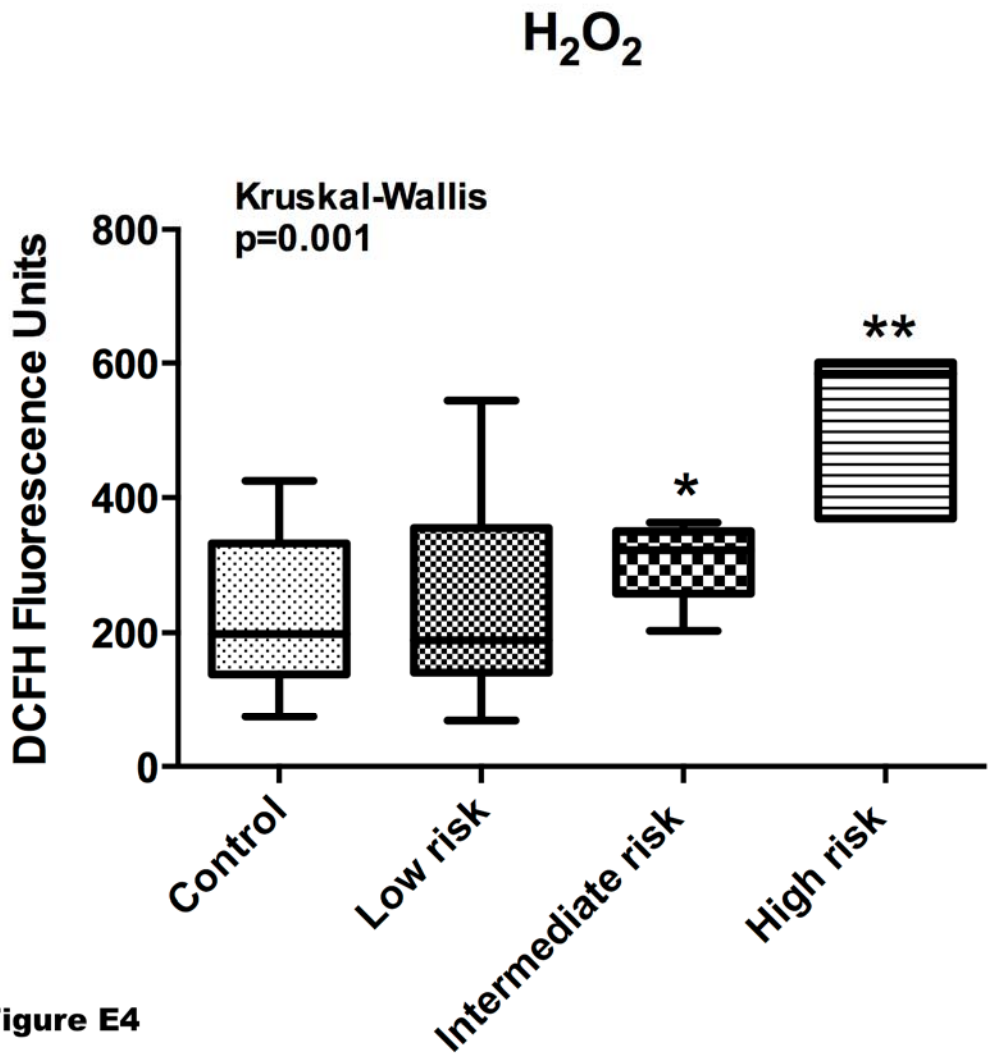
H_2O_2
Kruskal-Wallis
p=0.001
DCFH Fluorescence Units
800
600
400
200
0
*
**
Control
Low risk
Intermediate risk
High risk

Figure E4

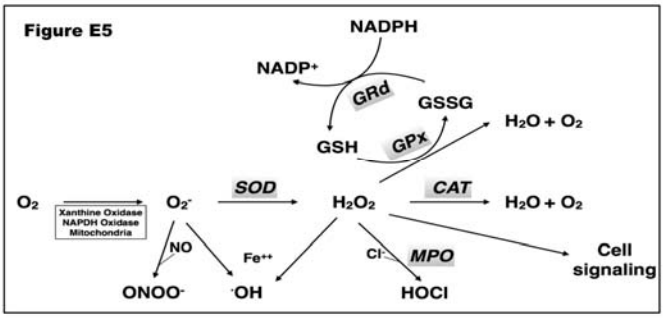
Figure E5
NADPH
NADP+
GRd
GSSG
GSH
GPx
H2O + O2
O2
Xanthine Oxidase
NAPDH Oxidase
Mitochondria
O2-
SOD
H2O2
CAT
H2O + O2
NO
Fe++
Cl-
MPO
Cell signaling
ONOO-
·OH
HOCl

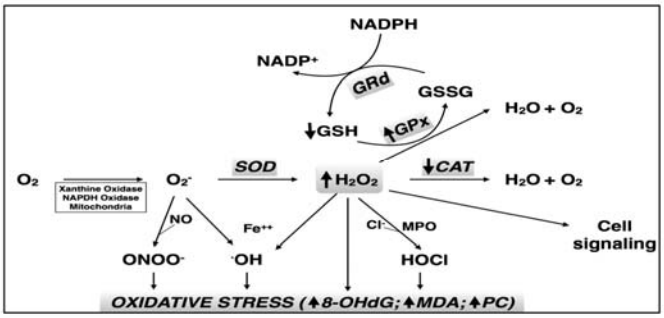
NADPH
NADP+
GRd
GSSG
↓GSH
↑GPx
H2O + O2
O2
Xanthine Oxidase
NAPDH Oxidase
Mitochondria
O2-
SOD
↑H2O2
↓CAT
H2O + O2
NO
Fe++
Cl-
MPO
Cell signaling
ONOO-
·OH
HOCl
OXIDATIVE STRESS (↑8-OHdG; ↑MDA; ↑PC)

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

Variable	Control (n = 38)	Low risk (n = 15)	Intermediate risk (n = 28)	High risk (n = 8)	p-value
Age (median; range)	9 (4-12)	11 (4-14)	9 (4-12)	9 (6-14)	0.182
Gender M/F (n, %)	25/17, 60/40	11/4, 73/27	17/11, 61/39	6/3, 67/33	0.691
AAT (mg/dl)	139.4 (161.0-119.1)	119.5 (127.5-99.0)	82.0 (95.0-67.8)	25.4 (34.2-22.0)	**< 0.001**
BMI (Kg/m^2)	18.22 ± 3.77	19.21 ± 2.55	19.17 ± 3.45	19.50 ± 3.31	0.536
FEV_1 (%)	91.90 (88.0-105.4)	100.2 (91.45-109.3)	103.9 (96.20-120.4)	101.0 (100.8-116.0)	0.620
FVC (%)	100.0 (89.8-108.1)	100.0 (89.90-114.5)	108.4 (87.6-119.0)	107.0 (106.9-108.0)	0.951
FEV_1/FVC (%)	92.0 (78.0-102.0)	85.0 (76.4-107.7)	99.40 (88.8-109.7)	97.2 (83.0-111)	0.432
FEF_{25-75} (%)	71.8 (56.7-90.2)	80.5 (59.1-97.3)	104.7 (98.6-108.3)	96.5 (84.1-97.0)	0.095
AST (U/L)	28.00 (24.50-37.50)	26.00 (22.75-30.75)	28.00 (25.00-37.00)	26.00 (25.00-29.00)	0.389
ALT (U/L)	19.00 (18.00-23.50)	18.00 (16.00-20.00)	20.50 (17.25-25.00)	24.00 (19.00-32.00)	0.113
GGT (U/L)	14.00 (12.00-17.25)	15.00 (11.75-16.25)	16.00 (14.00-18.00)	18.00 (17.00-20.00)	0.055

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; FEF_{25-75}, 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).