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Blood global DNA methylation is decreased in non-severe chronic obstructive pulmonary disease (COPD) patients

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Abbreviations
COPD, chronic obstructive pulmonary disease; Cyt: cytosine; FEV1, forced expiratory volume in 1 second; mCyt, methylcytosine; OS, Oxidative stress; PSH, Proteins–SH; TBARS, Thiobarbituric acid reactive substances.
Abstract

Background
Alterations in global DNA methylation have been associated with oxidative stress (OS). Since chronic obstructive pulmonary disease (COPD) is characterized by increased oxidative stress we aimed to evaluate the levels of global DNA methylation in this patient group.

Methods
We assessed methylcytosine (mCyt) levels in DNA from blood collected in 43 COPD patients (29 with mild and 14 with moderate disease) and 43 age- and sex-matched healthy controls.

Results
DNA methylation was significantly lower in COPD patients vs. controls (4.20±0.18 % mCyt vs. 4.29±0.18 % mCyt, p=0.02). Furthermore, DNA methylation in COPD patients with moderate disease was significantly lower than that in patients with mild disease (4.14 ± 0.15 % mCyt vs. 4.23±0.19 % mCyt, p<0.05). Univariate logistic regression analysis showed that lower DNA methylation levels were associated with presence of COPD (crude OR=0.06, 95% CI 0.00 to 0.67, p=0.023). This relationship remained significant after adjusting for several confounders (OR 0.03, 95% CI 0.00 to 0.67; p=0.028). Receiver operating characteristics (ROC) curve analysis demonstrated the area under the curve of mCyt was 0.646, with 46.6% sensitivity and 79.1% specificity for presence of COPD.

Conclusions
There were no significant correlations between methylation and OS indices. The presence and severity of COPD is associated with progressively lower DNA methylation in blood. However, this epigenetic alteration seems independent of oxidative stress.

Keywords: Chronic obstructive pulmonary disease; DNA methylation; Methylcytosine; Oxidative stress.
Introduction

Epigenetics refers to various processes that modify the functionality of genome under exogenous stimuli, and also provide a molecular mechanism that allows a stable transmission of gene expression states to the next generation of cells [3]. Methylation of DNA cytosines of dinucleotide sequence CpG is an epigenetic mechanism involved in the regulation of gene expression and cellular differentiation [1-2]. The methyl donor for this reaction is S-adenosylmethionine (SAM) whereas the addition of the methyl group is catalyzed by one of the three active DNA methyltransferases; DNMT1 [4], DNMT3a, or DNMT3b [5]. DNMT1 is generally responsible for copying the methylation pattern from the parent strand to the daughter strand during DNA replication [4], while DNMT3A/B exhibit de novo activity during cellular transitions [5]. The inclusion of a methylated cytosine within a regulatory element can prevent transcription factors from binding the DNA in some occurrences, which in turn limits transcription at that locus [6]. Thus, normally DNA hypomethylation is associated with gene transcriptional activity while hypermethylation is related to gene silencing. Previous studies support a role for DNA methylation in some diseases such as cancer and cardiovascular disease [7-8]. Furthermore, there is evidence that, in these disease states, global DNA methylation is strictly related to oxidative stress [7-10]. Oxidative stress occurs when reactive oxygen species (ROS) production in the body exceeds the defense mechanisms conferred by protective anti-oxidants, leading to oxidative attack on cellular structures such as DNA. Transient increases in oxidative bout lead to the generation of DNA base adducts, such as 8-hydroxyl-2′-deoxyguanosine (8-OH-dG) and O6-methylguanine, that strongly inhibit methylation of adjacent cytosine residues, ultimately leading to global DNA hypomethylation [11]. Moreover, it has been reported that a more oxidized cellular redox state may lead to a decrease in genomic DNA methylation through redox regulation of related enzymes. In particular, the presence of cysteine residues has led to the identification of the SAM-dependent methyltransferases as potentially redox-sensitive enzymes [12]. Furthermore, the activity of methionine adenosyltransferase, which catalyzes the enzymatic addition of methionine to adenosine
for the synthesis of SAM, is decreased in a more oxidized environment [13]. In consideration of the metabolic link between alterations in DNA methylation and oxidative stress we assessed global DNA methylation in chronic obstructive pulmonary disease (COPD), a chronic condition characterized by increased local and systemic oxidative stress. In particular, we tested the hypothesis that a) DNA methylation is associated with COPD presence and severity and b) DNA methylation is associated with OS markers.

**Methods**

**Subjects**

This was a case–control study of 43 patients with COPD (mean age 74.8±5.9 years, range 52-85 years), with mild or moderate form of disease recently diagnosed according to pre-defined clinical criteria, and 43 sex- and age matched healthy controls. All subjects were recruited from the Respiratory Unit of the University of Sassari. COPD patients with significant symptom deterioration, indicative of disease exacerbation, within the last three months were not enrolled. None of the selected patients had a previous diagnosis of COPD, took inhaled corticosteroids within four weeks prior to the study, or was on long- or short-acting \( \beta \)-agonists or long-acting muscarinic antagonists at the time of the assessments. Each patient underwent physical examination, chest radiographs, routine blood tests, and respiratory function tests. The latter included forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV1) and FEV1/FVC. A structured questionnaire was administered to collect clinical and demographic data, including age, body mass index (BMI), occupation and smoking status.

COPD diagnosis and severity was assessed according to a patient’s smoking history, physical examination, respiratory symptoms, and spirometric results based on the Global Initiative for Chronic Obstructive Lung Disease criteria [14]. Diagnosis of COPD required confirmation of airflow limitation that is not fully reversible (post-bronchodilator FEV1/FVC ratio <0.7). Classification of COPD severity was based on spirometric values.
Controls were selected from general population. Exclusion criteria for controls included the presence of concomitant inflammatory disease such as autoimmune disorders and infections, cancer, liver, kidney and heart disease. Also controls completed a structured questionnaire and underwent physical examination, routine blood tests and respiratory function tests.

This study was approved by the Institutional Local Ethics Committee (Azienda Sanitaria Locale n°1 di Sassari (Italy) (prot. 2175/CE del 21/04/2015), and was in accordance with the principles of Declaration of Helsinki. All subjects provided written informed consent.

**Biochemical analysis**

Whole blood DNA methylation was determined as follows. Genomic DNA extraction was performed by using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the instructions supplied by the manufacturer. The purified DNA was then hydrolyzed by 90% formic acid. After hydrolysis, samples were evaporated and the dry residue containing free bases was dissolved in ultrapure water and immediately analyzed by capillary electrophoresis as described previously [15]. The percentage of methylated to total cytosine (mCyt/tCyt) was calculated using the formula: 
\[
\frac{[mC]}{[mC] + [C]} \times 100
\]
The inter-assay CV for mCyt/tCyt measurements was 3.3%.

Plasma PSH determination was performed by spectrophotometry with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as titrating agent by measuring the absorbance of conjugate at 405 nm [16]. Concentration in samples was determined from a GSH standard curve. PSH refers to total protein sulfhydryl groups in plasma. The most abundant reduced -SH group in plasma is that of albumin Cys34 that accounts for about 80% of all reduced thiols in human plasma. It is an important scavenger of reactive oxygen and nitrogen species in the vascular compartment and then a significant redox buffer system of blood [17]. In presence of oxidative stress the –SH groups of proteins become oxidized to disulfide or sulfenic acid, leading to a reduction in –SH groups.

TBARS were determined according to the method described by Esterbauer and Cheeseman [18]. TBARS assay measures malonyledialdehyde (MDA) and other aldehydes produced as a result of lipid peroxidation induced by hydroxyl free radicals. Aldehydes react in acidic condition with
thiobarbituric acid yielding a product that can be detected spectrophotometrically by measuring the absorbance at 535 nm. A calibration curve was obtained using standard MDA. The measurement of TBARS is a well-established method for screening and monitoring lipid peroxidation [19].

**Statistical analysis**

All results are expressed as mean values (mean ± SD) or median values (median and range). Variables distribution was assessed by the Kolmogorov-Smirnov test. Statistical differences between groups were compared using unpaired Student’s t-test or Mann-Whitney rank sum test, as appropriate. Correlation analysis between variables was performed by Pearson's correlation or Spearman’s correlation as appropriate. Multiple comparisons were performed by one-way ANOVA. Levene’s test for equality of error variances was employed, while student-Newman-Keuls test for all pairwise comparisons was used. Non-normally distributed variables were log10-transformed prior to being used with parametric tests. Normal distribution of the residuals was checked to assess the goodness of fit of the transformations.

Logistic regression analysis with COPD absence vs. presence as dependent variable was performed to determine independent associations between variables potentially involved in disease development.

A further logistic regression analysis with mild or moderate condition as dependent variable was conducted to determine associations between COPD severity and variables potentially involved in disease progression.

The ability of global DNA methylation to discriminate COPD versus controls was analyzed using receiver operating characteristics (ROC) curve analysis. Optimal cutoff maximizing sensitivity and specificity was selected. Sensitivity and specificity were reported using the optimal ROC curve value according to the Youden Index.

Statistical analyses were performed using MedCalc for Windows, version 15.4 64 bit (MedCalc Software, Ostend, Belgium) and SPSS for Windows, version 14.0 32 bit (IBM Corporation; Armonk, NY, USA).
Results

Table 1 reports demographic and clinical characteristics of controls and COPD patients. As expected, COPD patients showed a reduced FEV1 (2.02±0.59 L vs. 2.75±0.59 L, p<0.0001), FVC (2.94 ± 0.78 L vs. 3.40 ± 0.73 L, p<0.01) and FEV1/FVC ratio (68.4±5.7 vs. 80.8 ± 4.9, p<0.001) when compared with controls. There were no between-group differences in smoking status or BMI.

As expected, COPD patients with moderate disease severity had a lower FEV1 (1.56±0.32 L vs. 2.24±0.56 L, p<0.0001), FVC (2.44 ± 0.54 L vs. 3.18 ± 0.77, p<0.01) and FEV1/FVC ratio (64.8±5.7 vs. 70.2±4.9, p<0.01) when compared with patients with mild disease (Table 2). The distribution plots of global DNA methylation, reported in figure 1, show a lower degree of methylation in COPD patients (range 3.88-4.67 % mCyt; IQR 4.04-4.48 % mCyt) when compared with controls (range 3.98-4.69 % mCyt; IQR 4.18-4.40 % mCyt). Furthermore, as reported in figure 2, DNA methylation was significantly lower in COPD patients than in controls (4.20±0.18 % mCyt vs. 4.29±0.18 % mCyt, p=0.02). A significant trend towards lower DNA methylation was observed according to COPD presence and severity (p=0.018, Figure 3). Furthermore, patients with moderate disease had significantly lower DNA methylation than patients with mild disease (4.14±0.15 % mCyt vs. 4.23±0.19 % mCyt, p<0.05). As previously reported [20], TBARS concentrations significantly increased, and PSH concentrations significantly decreased, according to COPD presence and severity. However, no significant correlations were observed between global DNA methylation and oxidative stress indices. In univariate logistic regression, lower DNA methylation levels were independently associated with presence of COPD (crude OR=0.06, 95% CI 0.00 to 0.67, p=0.023). This association remained significant also after adjusting for age, sex, BMI, smoking status, and oxidative stress indices (OR 0.03, 95% CI 0.00–0.67; p=0.028).

In ROC curve analysis, evaluating the sensitivity, specificity, and diagnostic accuracy of DNA methylation degree in distinguishing COPD from healthy subjects, % mCyt with a threshold of 4.15 discriminated COPD from controls with 46.6% sensitivity and 79.1% specificity (AUC=0.646, 95% CI 0.535-0.746, p=0.014; Figure 4).
A multiple logistic regression analysis on 43 COPD patients according to disease severity (mild vs. moderate), after adjusting for the abovementioned parameters, showed that lower global DNA methylation was independently associated to more advanced disease (OR 0.00, 95% CI 0.00-0.78, p=0.043). Multiple logistic regression analysis of mild COPD patients vs. controls showed a trend towards significant associations between global DNA methylation and mild COPD (OR 0.06, 95% CI 0.00-1.67, p=0.098).

Discussion

A central role in the pathophysiology of COPD is played by the chronic inflammation of the small and distal airways. This is characterized by a significant increase in the number of activated neutrophils and macrophages, inflammatory mediators [21-22] and anion superoxide (O2·-), probably through an impairment of the nicotinamide adenine dinucleotide phosphate oxidase pathway. In addition, hypoxaemia, a common feature in COPD, may induce a reduction in haemoglobin oxygen saturation levels, resulting in local tissue hypoxia [23]. Experimental studies suggest that hypoxaemia enhances oxidative stress in COPD, probably through the increase of ROS production at the respiratory chain level of mitochondria [24-25]. It has been proposed that in COPD the increased oxidant burden may not be adequately counterbalanced by the lung antioxidant systems, resulting in oxidative stress. Although the pathogenesis of COPD remains unclear, the central role of oxidative stress is well established [20, 26-27]. Oxidative stress adversely affects cellular functions, since excessive quantity of ROS leads to protein, lipids, and DNA damage [28]. In addition, it has been reported that oxidative stress may be associated with a DNA hypomethylation pattern through several mechanisms, involving guanine hydroxylation in CpG dinucleotides (thus generating 8-OH-dG that inhibit proximal cytosine methylation) or reduced activity of the redox sensitive enzymes SAM-dependent methyltransferases [11-12]. Moreover, it has been postulated that GSH depletion during chronic oxidative stress may lead to decreased global DNA methylation through the depletion of SAM in the folate/homocysteine pathway [29]. In
support of this hypothesis, treatment with the GSH-depleting hepatotoxin bromobenzene has been reported to induce extensive depletion of methionine levels and DNA hypomethylation in the liver of Syrian hamsters [30]. Niedzwiecki et al. [9] recently reported that a more oxidized blood GSH redox status is associated with decreased global DNA methylation in peripheral blood mononuclear cell. However, blood SAM does not appear to mediate this association. In addition, a study of 45 infertile men showed that seminal ROS production was negatively correlated with sperm global DNA methylation. In this study, a three-month supplementation with antioxidants significantly reduced seminal ROS, increasing sperm global DNA methylation [31]. Therefore, we tested the hypothesis that the increased oxidative stress in COPD may lead to alterations in global DNA methylation. This hypothesis was confirmed by the observation of significantly lower DNA methylation indexes in patients with COPD vs. healthy controls. A further reduction in DNA methylation was associated with disease progression. Notably, logistic regression analysis showed that the degree of DNA methylation was independently associated with COPD after adjusting for key demographic and clinical confounders, and oxidative stress indices. A cut-off value of 4.15 for the % mCyt determined on the ROC curve yielded the best sensitivity (46.6%) and specificity (79.1%) towards COPD presence. Further logistic regression analyses showed significant associations between DNA methylation and disease severity in COPD patients and a trend towards significant associations between DNA methylation and early COPD disease in healthy controls and COPD patients with mild disease. This suggests that DNA methylation is primarily involved in disease progression, when oxidative stress is more marked. However, contrary to this hypothesis, we observed no significant correlations between global DNA methylation and oxidative stress indices. It is likely that this might be due to the relatively small study population and/or the absence of patients with severe or unstable form of the disease, likely to be characterized by a particularly high oxidative stress. Therefore, it would be interesting to evaluate associations between DNA methylation and oxidative stress indices in patients with more severe symptoms (COPD stages 3 and 4) and/or during symptom exacerbations. It should be stressed that the individual DNA
methylation values showed a relatively narrow distribution, with a ratio between the inter-quartile difference and the median of 0.05, suggesting a particularly low biological variation (inter-individual CV 4.2%). This might reduce the likelihood of identifying strong associations between methylation levels and other biochemical or clinical variables, particularly when the inter-assay CV was above the defined threshold values for the sample size analyzed [32].

**Conclusions**

The present study demonstrates for the first time that global DNA methylation indexes are independently associated with COPD in patients with stable disease. Further studies, with a larger sample size, also including those with more severe symptoms, are required to elucidate the relationship between DNA mCyt levels and oxidative stress, and to fully characterize the impact of methylation degree on disease worsening and clinical outcomes.

**Acknowledgements**

A Visiting Professorship granted to Professor Mangoni by the Department of Biomedical Sciences, University of Sassari (Italy), facilitated this work.

**Ethics approval**

This study was approved by the ethics committee of Azienda Sanitaria Locale n°1 di Sassari (Italy) (prot. 2175/CE del 21/04/2015).

**Declaration of interest**

The authors declare that they have no competing interests.

**Funding**

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References


LEGENDS

**Figure 1.** Distribution plots of % mCyt in control subjects and COPD patients. The biological variation expressed as inter-individual CV was respectively 4.2 and 4.5% and the ratio between the inter-quartile difference and the median was respectively 0.05 and 0.06.

**Figure 2.** % of mCyt in DNA extracted from blood in healthy subjects (n=43) and in all COPD patients (n=43). The central horizontal line on each box represents the median, the ends of the boxes are 25 and 75 percentiles and the error bars 5% and 95%. P-values derived from Student-Newman-Keuls test.

**Figure 3.** % of mCyt in DNA extracted from blood in healthy subjects (n=43) and in COPD patients after sorting in mild (n=29) and moderate disease (n=14). The central horizontal line on each box represents the median, the ends of the boxes are 25 and 75 percentiles and the error bars 5% and 95%. P-values derived from Student-Newman-Keuls test.

**Figure 4.** The area under receiver operating characteristic curves of % mCyt.
Table 1. Characteristic features of the study groups.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>COPD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>43</td>
<td>43</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>73.4±6.9</td>
<td>74.8±5.9</td>
<td>NS</td>
</tr>
<tr>
<td>Males, n</td>
<td>34</td>
<td>34</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.4±3.6</td>
<td>27.4±3.7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Lung function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>2.75±0.59</td>
<td>2.02±0.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>112.1±14.4</td>
<td>80.1±17.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.40±0.73</td>
<td>2.94±0.78</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>107.9±15.4</td>
<td>88.0±15.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>80.4±3.9</td>
<td>66.6±4.8</td>
<td>&lt;0.001</td>
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<tr>
<td>RV (L)</td>
<td>3.07±0.6</td>
<td>3.42±0.9</td>
<td>NS</td>
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<tr>
<td>RV (% predicted)</td>
<td>125.4±22.4</td>
<td>137.3±32.5</td>
<td>NS</td>
</tr>
<tr>
<td>TLC (L)</td>
<td>6.6±1.1</td>
<td>6.4±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>TLC (% predicted)</td>
<td>110.3±13.8</td>
<td>107.8±14.4</td>
<td>NS</td>
</tr>
<tr>
<td>RV/TLC (%)</td>
<td>46.8±5.6</td>
<td>53.4±8.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Smoking status</strong></td>
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<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>3 (7%)</td>
<td>3 (7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Never smoked</td>
<td>14 (32.6%)</td>
<td>10 (23.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>26 (60.4%)</td>
<td>30 (69.8%)</td>
<td>NS</td>
</tr>
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</table>

Table 2. Clinical and functional characteristics of mild versus moderate COPD patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mild COPD</th>
<th>Moderate COPD</th>
<th>p-values</th>
</tr>
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<tbody>
<tr>
<td>Subjects, n</td>
<td>29</td>
<td>14</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.4±4.8</td>
<td>73.4±7.7</td>
<td>NS</td>
</tr>
<tr>
<td>Males, n</td>
<td>22</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4±3.4</td>
<td>27.4±4.5</td>
<td>NS</td>
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</tbody>
</table>

**Lung function**

<table>
<thead>
<tr>
<th></th>
<th>Mild COPD</th>
<th>Moderate COPD</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 (L)</td>
<td>2.24±0.56</td>
<td>1.56±0.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>92.8±6.9</td>
<td>62.7±12.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.18±0.77</td>
<td>2.44±0.54</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>97.9±5.3</td>
<td>74.7±14.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>68.6±1.4</td>
<td>63.4±6.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV (L)</td>
<td>3.10±0.55</td>
<td>3.85±1.07</td>
<td>0.025</td>
</tr>
<tr>
<td>RV (% predicted)</td>
<td>127.3±26.0</td>
<td>150.8±36.4</td>
<td>0.02</td>
</tr>
<tr>
<td>TLC (L)</td>
<td>6.26±0.89</td>
<td>6.55±1.38</td>
<td>NS</td>
</tr>
<tr>
<td>TLC (% predicted)</td>
<td>108.9±10.6</td>
<td>106.3±18.4</td>
<td>NS</td>
</tr>
<tr>
<td>RV/TLC (%)</td>
<td>49.9±8.0</td>
<td>58.2±7.3</td>
<td>&lt;0.001</td>
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</table>

**Smoking status**

<table>
<thead>
<tr>
<th></th>
<th>Mild COPD</th>
<th>Moderate COPD</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smokers</td>
<td>2 (6.9 %)</td>
<td>1 (7.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Never smoked</td>
<td>8 (27.6%)</td>
<td>2 (14.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>19 (65.5%)</td>
<td>11 (78.6%)</td>
<td>NS</td>
</tr>
</tbody>
</table>
DNA methylation (% mCyt)

**Controls**
- n: 43
- Mean: 4.29
- SD: 0.18
- CV: 4.2%
- (P75-P25)/P50 = 0.05

**COPD**
- n: 43
- Mean: 4.20
- SD: 0.19
- CV: 4.5%
- (P75-P25)/P50 = 0.06
A

P<0.05

DNA methylation (% mCyt)

CTRL COPD
DNA methylation (% mCyt)

CTRL    Mild COPD    Moderate COPD

Linear Trend: p=0.018

P<0.05