

# Role of NADPH oxidase-2 and oxidative stress in children exposed to passive smoking

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Received 14 November 2017  
Revised 26 December 2017  
Accepted 22 January 2018  
Published Online First 15 February 2018

## ABSTRACT

This study explored oxidative stress, nicotinamide adenine dinucleotide phosphate oxidase-2 (Nox2) activity and endothelial function in children exposed or not to passive smoking. Compared with controls (n=57), Nox2 activity and isoprostanes were higher in children exposed to passive smoking (n=57); conversely, nitric oxide (NO) bioavailability and flow-mediated dilation were lower in children exposed to passive smoking. A bivariate analysis showed that Nox2 activity correlated with flow-mediated dilation, NO bioavailability and isoprostanes. A multivariate analysis showed that Nox2 activity was significantly associated with serum isoprostanes and cotinine levels; flow-mediated dilation was associated with isoprostanes and carotid intima-media thickness. In children exposed to passive smoking, Nox2-derived oxidative stress is upregulated and inversely associated with impaired artery dilation.

## INTRODUCTION

Children exposed to passive smoking have a higher risk to develop cardiovascular events in adulthood<sup>1</sup>; oxidative stress generated by cigarette smoke increases cardiovascular events through endothelial dysfunction, increased intimal-medial thickening (IMT) and platelet activation.<sup>1</sup>

Nicotinamide adenine dinucleotide phosphate oxidase (Nox) is the most important source of superoxide anion in humans.<sup>2</sup> There are various isoforms of this enzyme (Nox1, Nox2, Nox4 and Nox5) that contribute to determine oxidative stress and atherosclerosis.<sup>2</sup> Smokers have a greater activation of Nox2 with endothelial dysfunction, assessed by flow-mediated dilation (FMD) and lower bioavailability of nitric oxide (NO).<sup>3,4</sup> However, the mechanism responsible for oxidative stress generation in subjects exposed to passive smoking is still unclear. We hypothesised that Nox activity may be involved in the generation of oxidative stress by passive smoking and, thus, investigated the relationship between Nox2-derived oxidative stress and endothelial function in children exposed to passive smoking.

## MATERIALS AND METHODS

Fifty-seven consecutive children exposed to passive smoking (second-hand smoke) and 57 controls matched for age and sex were enrolled at the Pediatric Department of Sapienza University. A questionnaire was filled in by both parents of the children to assess the level and the type of passive smoking

exposition. Serum cotinine levels were assessed in children to measure exposition to passive smoking. Non-smoker parents reported in the questionnaire: no current cigarette, cigar or pipe smoking or any smoking in the past 5 years; exposure to passive smoking was based on self-reported smoking of parent and serum cotinine levels  $\geq 15$  ng/mL. Exclusion criteria were the presence of acute or chronic diseases, smoking and vitamin assumption.

FMD and carotid IMT were measured as previously described.<sup>5</sup>

Soluble Nox2 (sNox2-dp), a marker of Nox2 activation, was assessed in serum by ELISA method as previously described.<sup>5</sup> NO bioavailability and 8-iso-PGF2 $\alpha$  levels were measured in serum by using a colorimetric assay kit (Abcam and DRG International, USA). Serum cotinine was assessed by human Cotinine ELISA kit (Tema Ricerca, Italy).

## Statistical methods

Continuous variables are reported as mean $\pm$ SD when normally distributed or as median and IQR when non-normally distributed unless otherwise indicated. Normal distribution of parameters was assessed by the Kolmogorov-Smirnov test. Student's unpaired t-test and analysis of variance were used for normally distributed continuous variables. Appropriate non-parametric tests (Mann-Whitney and Kruskal-Wallis tests) were used for all the other variables (isoprostanes and FMD). Differences between percentages were assessed by the  $\chi^2$  test. Bivariate analysis was performed by Spearman's correlation; the variables with evidence of an association  $P < 0.10$  (sNox2-dp, serum cotinine, NO bioavailability, isoprostanes levels, IMT and FMD) were included in a multivariable linear regression using an automated procedure with forward selection; standardised beta coefficients were calculated by using an automated procedure.  $P < 0.05$  was considered as statistically significant. All analyses were carried out with SPSS V.18.0 (IBM SPSS Statistics V.25.0).

## Sample size determination

Cross-sectional study: sample size calculation was computed with respect to a two-tailed Student's t-test for independent groups, considering: 2.5% ( $\delta$ ) as difference for FMD between children exposed or not to passive smoking, 3.5% as SD, 0.05 ( $\alpha$ ) as type I error probability and 0.95 as power  $1 - \beta$ . The sample size was n=51 patients which was increased to n=57/group.

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**To cite:** Loffredo L, Zicari AM, Occasi F, et al. *Thorax* 2018;**73**:986–988.

**Table 1** Clinical characteristics of children exposed or not exposed to passive smoking

	Children exposed to passive smoking	Children not exposed to passive smoking	P
N	57	57	–
Age (years)	8.7 (7.8 to 9.5)	8.7 (8.0 to 9.4)	0.949
Females/males	26/31	26/31	1.0
Systolic blood pressure, mm Hg	115 (112 to 118)	112 (109 to 115)	0.198
Diastolic blood pressure, mm Hg	75 (71 to 78)	70 (68 to 73)	0.60
Parents cigarettes/day, No.	12 (11 to 14)	–	<0.001
Serum cotinine (ng/mL)	35.7 (33 to 38)	1.5 (1.2 to 1.8)	<0.001
Body mass index	18 (17 to 19)	17 (16 to 18)	0.157
Total cholesterol level, mg/dL	169 (162 to 175)	160 (155 to 166)	0.052
Fasting glucose level, mg/dL	88 (84 to 91)	87 (83 to 89)	0.617
sNOX-2dp (pg/mL)	26 (24 to 29)	18 (16 to 20)	<0.001
8-iso-PGF2 $\alpha$ (pmol/L)	169 (142 to 185)	150 (130 to 159)	<0.001
NO bioavailability ( $\mu$ M)	51 (48 to 54)	59 (55 to 62)	<0.001
FMD (%)	6.1 (4 to 9.1)	7.7 (5.1 to 10)	0.006
IMT (mm)	0.50 (0.48 to 0.52)	0.49 (0.46 to 0.51)	0.502

Data are reported as mean and 95% CI. Italicised values show a P value statistically significant.

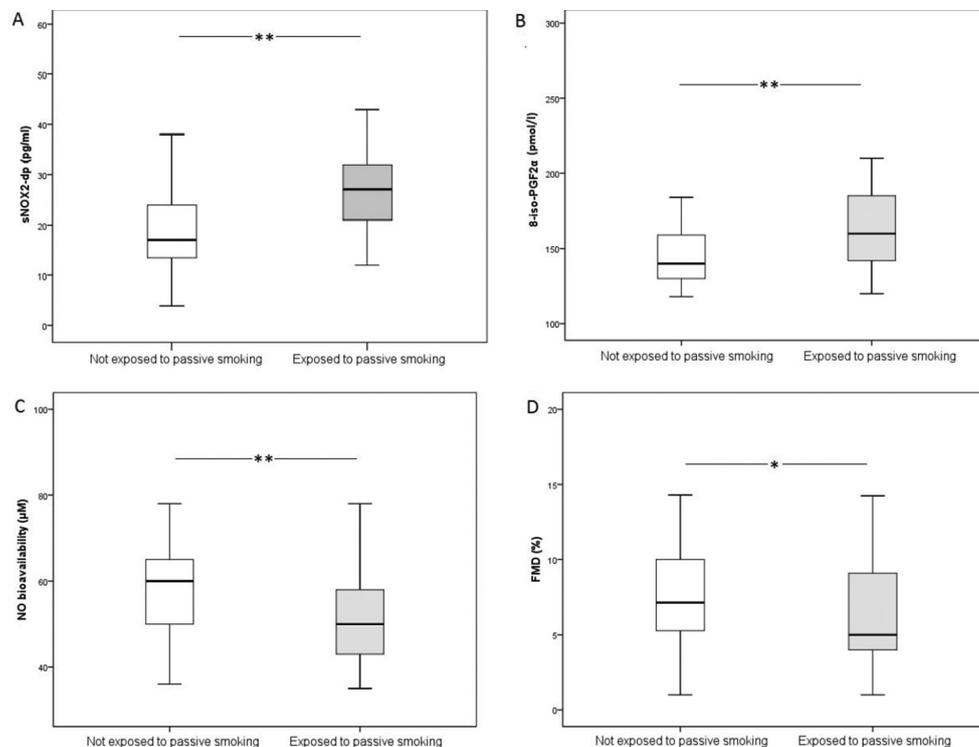
FMD, flow-mediated dilation; IMT, intimal-medial thickening; NO, nitric oxide; sNOX-2dp, soluble Nox2.

## RESULTS

Clinical characteristics of children exposed ( $n=57$ , age:  $9\pm 3$  years, 26 females and 31 males) or not exposed ( $n=57$ , age:  $9\pm 3$  years, 26 females and 31 males) to passive smoking are reported in [table 1](#). No significant differences in age, gender, arterial blood pressure, body mass index (BMI), total cholesterol, fasting glucose levels were found between the groups ([table 1](#)). As expected, children exposed to passive smoking had higher plasma levels of cotinine ( $35.7\pm 8.8$  vs  $1.5\pm 1.0$  ng/

mL;  $P<0.001$ ) ([table 1](#)). All the parents of children exposed to passive smoking declared to currently smoke cigarettes; they smoke prevalently at home or in car.

Compared with controls, serum sNox2-dp and isoprostanes were higher in children exposed to passive smoking ( $26\pm 9$  vs  $18\pm 9$  pg/mL,  $P<0.001$  and  $160$  (95% CI 142 to 185) vs  $140$  (95% CI 130 to 159) pmol/L,  $P<0.001$ , respectively) ([figure 1A,B](#)); conversely, NO bioavailability and FMD were lower in children exposed to passive smoking ( $51\pm 10$   $\mu$ M vs



**Figure 1** The box plots depict the non-outlier ranges of sNOX2-dp (A), 8-iso-PGF2 $\alpha$  (B), NO bioavailability (C) and FMD (D). The median is identified by a line inside the box. The length of the box represents the IQR. The extreme lines of the box plot depict the maximum and the minimum of the non-outlier range. \* $P\leq 0.01$ ; \*\* $P<0.001$ . FMD, flow-mediated dilation; IMT, intimal-medial thickening; NO, nitric oxide; sNOX-2dp, soluble Nox2.

59±11,  $P<0.001$  and 5 (95% CI 4 to 9.1) vs 7.1 (95% CI 5.1 to 10),  $P=0.006$ , respectively) (figure 1C,D).

No significant difference for IMT was found between children exposed or not exposed to passive smoking ( $0.50\pm 0.08$  vs  $0.49\pm 0.09$  mm,  $P=0.502$ ).

Bivariate analysis showed that serum sNox2-dp correlated with FMD (Rs:  $-0.221$ ,  $P=0.01$ ), serum cotinine (Rs:  $0.316$ ,  $P<0.001$ ), NO bioavailability (Rs:  $-0.547$ ,  $P<0.001$ ) and isoprostanes (Rs:  $0.754$ ,  $P<0.001$ ) levels and that FMD correlated with NO bioavailability (Rs:  $0.228$ ,  $P=0.01$ ), isoprostanes (Rs:  $-0.339$ ,  $P<0.001$ ) levels and IMT (Rs:  $-0.334$ ,  $P<0.001$ ). Furthermore, cotinine levels correlated with NO bioavailability (Rs:  $-0.297$ ,  $P<0.001$ ) and isoprostanes (Rs:  $0.277$ ,  $P<0.001$ ) levels.

A multiple linear regression analysis, including the variables linearly associated with the dependent variable, was performed to define the independent predictors of sNox2-dp and FMD. Serum isoprostanes (SE: 0.02; standardised coefficient  $\beta$ : 0.547;  $P<0.001$ ) and serum cotinine levels (SE: 0.04; standardised coefficient  $\beta$ : 0.257;  $P<0.001$ ) were significantly associated to sNox2-dp ( $R^2$ : 0.44); FMD was independently correlated to serum isoprostanes (SE: 0.008; standardised coefficient  $\beta$ :  $-0.292$ ;  $P<0.001$ ) and IMT (SE: 3.3; standardised coefficient  $\beta$ :  $-0.280$ ;  $P=0.002$ ;  $R^2$ : 0.2).

## DISCUSSION

This study provides the first evidence that Nox2-derived oxidative stress is higher in children exposed to passive smoking and is closely related to the grade of smoking exposition. Coincidentally and in agreement with a previous report,<sup>6</sup> we found an increase of isoprostanes that are bioactive products of lipid peroxidation exerting vasoconstriction and eventually endothelial dysfunction.<sup>7</sup>

Previous study showed that oxidative stress plays a pivotal role in modulating endothelial function<sup>8</sup>; in particular, Nox2 influences FMD by impairing NO bioavailability and/or biosynthesis.<sup>2-9</sup> Consistently with this, we observed a reduced NO generation along with an inverse correlation between serum sNOX2-dp and FMD, which lead us to hypothesise that Nox2-generated oxidative stress reduces NO bioavailability thus impairing FMD in children exposed to second-hand smoke. This hypothesis is in accordance with Adams *et al*,<sup>10</sup> who found low brachial FMD coincidentally with reduced NO bioavailability in subjects exposed to passive smoking.

This study has the following limitations. We did not evaluate other NADPH isoforms, as NOX1 and NOX4, that could

contribute to generate oxidative stress and endothelial dysfunction. The low sample size limits the study, thereby future studies with larger sample size are needed to confirm and extend the data reported.

In children exposed to passive smoking, Nox2-derived oxidative stress is upregulated and inversely associated with impaired artery dilation.

**Acknowledgements** Thanks to Dr Francesco Baratta for his help in data acquisition.

**Collaborators** Francesco Baratta.

**Contributors** Study concept and design and guarantors: LL and AMZ. Acquisition of data: FO, LP, GDC, SB and FM. Analysis and interpretation of data: FO, RC, CN, VC and LP. Drafting of the manuscript: LL and AMZ. Critical revision of the manuscript for important intellectual content: FA, MDB, MD and FV. Statistical analysis: LL and FO. Study supervision: FV and MD.

**Funding** This study was supported by a grant from the University of Rome 'La Sapienza' (Università degli Studi di ROMA 'la Sapienza' RM11715C743A15B7, funds to LL).

**Competing interests** None declared.

**Ethics approval** Ethical Committee of 'Sapienza' University of Rome (ref. n.2339/26.01.2012).

**Provenance and peer review** Not commissioned; externally peer reviewed.

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