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Short communication

Polymorphisms rs2745557 in *PTGS2* and rs2075797 in *PTGER2* are associated with the risk of chronic obstructive pulmonary disease development in a Tunisian cohort

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ABSTRACT

We hypothesized that polymorphisms of genes involved in the prostaglandin pathway could be associated with COPD. In this study we explored the involvement of genetic polymorphisms in *PTGS2, PTGER2* and *PTGER4* genes in the development and severity of COPD and their effects on plasma concentrations of inflammatory/oxidative stress markers. We identified genotypes of *PTGS2, PTGER2* and *PTGER4* SNPs in a Tunisian cohort including COPD patients ($n = 138$) and control subjects ($n = 216$) using PCR-RFLP and PCR TaqMan. Pulmonary function (FEV1 and FVC) were assessed by plethsmography. PGE_2, PGD_2 and cytokine plasma (IL-6, IL-18, TNF-α, TGF-β) concentrations were measured using ELISA and colorimetric standard methods were used to determine oxidative stress concentrations. Genotype frequencies of rs2745557 in *PTGS2* and rs2075797 in *PTGER2* were different between COPD cases and controls. There was no correlation between these polymorphisms and lung function parameters. For rs2745557, the A allele frequency was higher in COPD cases than in controls. For rs2075797, carriers of the GG genotype were more frequent in the COPD group than in controls. Only rs2745557 in *PTGS2* had an effect on PGD_2 and cytokine plasma concentrations. PGD_2 was significantly decreased in COPD patients with the GA or AA genotypes. In contrast, IL-18 and NO plasma concentrations were increased in COPD rs2745557 A allele carriers as compared to homozygous GG subjects. Our findings suggest that rs2745557 in *PTGS2* and rs2075797 in *PTGER2* are associated with COPD development but not with its severity.

1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a leading cause of death worldwide. It is a multifactorial disease characterized by airflow limitation which is not fully reversible. Airflow obstruction is usually progressive and is associated with dysregulated inflammation [1, 2]. Tobacco smoking is the main risk factor associated with approximately 80% of COPD cases [3, 4]. Due to genome wide associations studies (GWAS), many candidate genes for COPD were identified [5]. Numerous reviews have explored the relationship between gene polymorphisms and COPD susceptibility, severity and treatment effects [6]. But very few researches have focused on the effects of *PTGS2* (Prostaglandin-endoperoxide synthase) gene polymorphisms on COPD susceptibility. Lipid mediators, particularly those derived from the cyclooxygenase (COX) pathway play a critical role in inflammation attributed to airflow limitation and altered lung function in COPD patients. Cyclooxygenases also named prostaglandin endoperoxyde G/H synthases are key enzymes in PGE_2 synthesis and exist as both constitutive (COX-1) and inducible (COX-2) isoforms. COX-2 is predominantly expressed under

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Abbreviations

Chronic Obstructive Pulmonary Disease COPD
Single Nucleotide Polymorphism SNP
Forced Expiratory Volume FEV
Forced Vital Capacity FVC
Cycloxygenase COX
Prostaglandin D_2 PGD_2
Prostaglandin E_2 PGE_2
Interleukin IL
Tumor Necrosis Factor TNF
Transforming Growth Factor TGF

inflammatory conditions in response to stimulatory agents such as cytokines, hypoxia, growth factors and pro-mitogenic factors [7]. Several single nucleotide polymorphisms (SNPs) in the *PTGS2* gene have been identified. They may lead to different gene expression levels or different enzyme activities [8]. Some of these functional variants have been detected in the *PTGS2* promoter region; the others have been localized in coding regions of exons and 3′untranslated regions (UTR) [9].

Prostaglandin E_2 (PGE_2), a member of the prostaglandin family is involved in the pathogenesis of chronic pulmonary diseases such as asthma and COPD [10-12]. It is produced at sites of inflammation and can mediate many of the pathologic features of inflammation [13]. Previous studies reported an anti-inflammatory effect of PGE_2 in lung but more recent reports described its pro-inflammatory action [14]. In addition, a significant increase of its production was reported in COPD patients compared to healthy controls [15]. This lipid mediator derived from arachidonic acid has four distinct receptors (EP1–4) which differ widely in expression, tissue distribution, downstream intracellular signaling pathways and inflammatory effects. Prostaglandin receptor genes have also been investigated as candidate genes related to susceptibilities to many diseases such as asthma [11, 16], arterial hypertension [17] and Crohn disease [18, 19]. However, there have been few studies evaluating the effects of polymorphisms in EP receptors genes on COPD susceptibility/severity. In the current report, we hypothesized that *PTGS2, PTGER2* and *PTGER4* genetic variants could be risk factors for COPD susceptibility and/or may alter lung function. We therefore investigated the association between eight single nucleotide polymorphisms (SNPs) in the *PTGS2, PTGER2, PTGER4* genes and COPD susceptibility, lung function, inflammatory and oxidative stress markers.

2. Materials and methods

2.1. Cohort and data collection

Our study included a cohort of 138 COPD patients and 216 matched healthy controls, selected from the Tunisian population. Patients with symptomatic COPD were recruited from the Service of pneumology and allergology in the Farhat Hached Hospital (Sousse, Tunisia). Control subjects were selected from healthy blood donors in the local blood donation facility. Each patient with COPD answered a survey which included basic demographic (age, gender and origin), anthropometric data (height, weight, BMI) and tobacco smoking history. The study protocol was authorized by the ethics committee of Farhat Hached Hospital (acceptance number 06–2016) and written consent for research was also obtained from each participant.

We divided subjects with COPD into 4 groups according to GOLD (The Global Initiative for Obstructive Lung Disease) classification of severity: GOLD I (Mild): Forced expiratory volume FEV1≥80% predicted; GOLD II (Moderate): 50%≤FEV1<80%predicted; GOLD III (Severe): 30%≤FEV1<50% predicted and GOLD IV (Very severe): FEV1<30% predicted.

2.2. Pulmonary function evaluation

The diagnosis of COPD was essentially based on symptoms (cough, mucus secretions and dyspnea). Lung function tests in subjects with COPD were performed by measuring the forced vital capacity (FVC) and the FEV1 using a body plethysmograph (ZAN® 500, Body II, Messgeräte, Germany) by atrained pulmonary function technician/physician. To evaluate the reversibility of the airway obstruction in patients with a FEV1/FVC ratio below 0.7, measurements were repeated 15 min after given two sprays of bronchodilator (200 μg of salbutamol).

2.3. DNA extraction

Five ml of EDTA (anticoagulant) blood samples were collected by vein puncture. Genomic DNA was obtained from peripheral whole blood leukocytes of patients and healthy controls using a salting out method. A lysis buffer (Sucrose 0.32 M, 1%Triton X-100, MgCl2 5 mM, TrisHCl 10 mM, pH 7.5) was mixed with peripheral blood for red blood cells lysis. Leukocytes were spun down, washed with H_2O and the pellet was

Table 1
Primer sequences, melting temperatures, restriction enzymes and fragment sizes after PCR-RFLP analysis of *PTGS2* gene polymorphisms.

Polymorphism(Location and SNP reference)	Primers	T_m	Restrictionenzyme	Restriction Fragment sizes (bp)
PTGS2 −765 (G/C) *rs20417*	F: 5′-ATTCTGGCCATCGCCGCTTC-3′ R: 5′-CTCCTTGTTTCTTGGAAAGAGACG-3′	60 °C	*BstFNI*	157+134+23
PTGS2 +202 (G/A) *rs2745557*	F: 5'-TCAGCCATACAGGTGAGTACC-3' R: 5'-CTGGGAGCAGGAAAGAACTG-3'	58 °C	*TaqI*	400+238+162
PTGS2 −1290 (A/G) *rs689465*	F: 5′-CAGGTTTTATGCTGTCATTTTCC-3′ R: 5′-TAGTGCTCAGGGAGGAGCAT-3′	62 °C	*RsaI*	173+121+53
PTGS2+306 (C/G) (Val 102Val) *rs5277*	F: 5′- CACTACATACTTACCCACTTC-3′ R: 5′-CCATCTGCTCACTATCCAAG-3′	58 °C	*HincII*	511+424+87
PTGS2+8473 (T/C) *rs5275*	F : 5′-GTTTGAAATTTTAAAGTACTTTTGAT-3′ R : 5′-TTTCAAATTATTGTTTCATTGC-3′	52 °C	*BclI*	147+124
PTGS2+9850 (A/G) *rs4648298*	F : 5′-CGTTCCCATTCTAATTAATGCCCTT-3′ R : 5′-TGTGTCAAGCACTGTGGGTTTTAAT-3′	59 °C	*AluI*	545+349+196

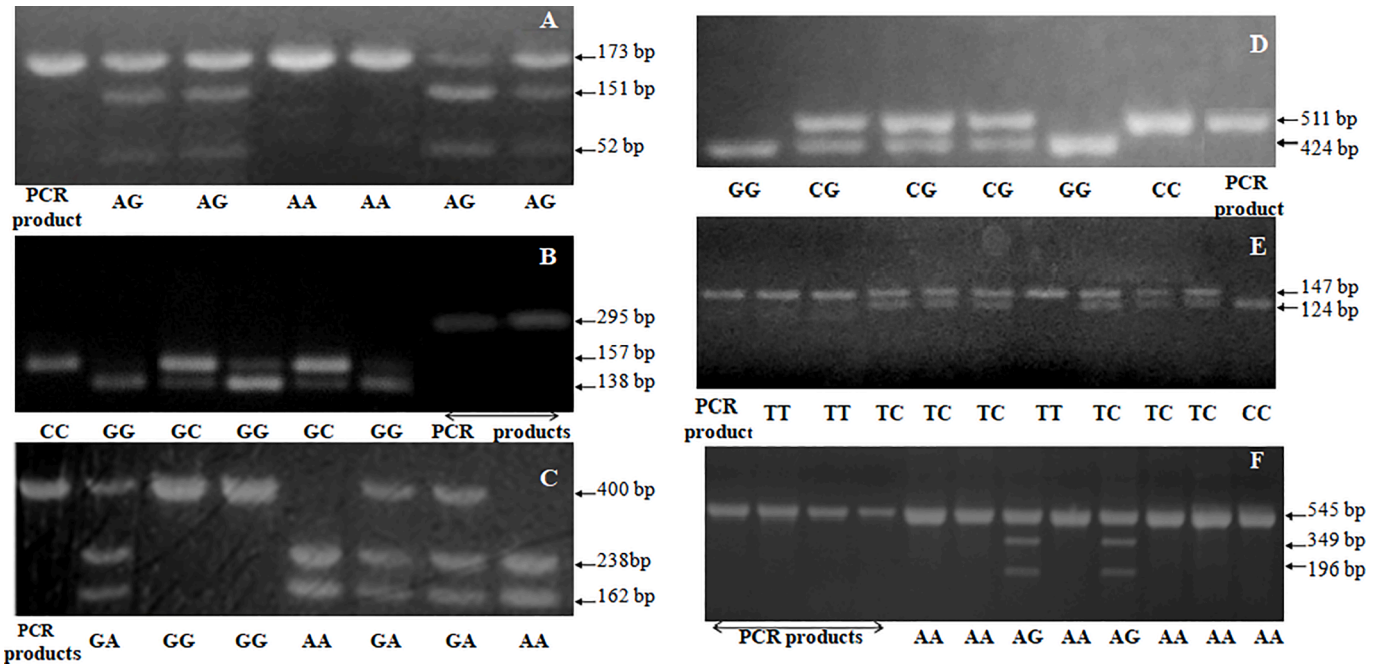

Fig. 1. Genotyping of *PTGS2* polymorphisms using PCR-RFLP. **A.** Genotyping of the rs689465 (A/G). The PCR product (173 bp) was digested with RsaI which cleaved the G allele into two fragments (121 bp and 52 bp) but did not cleave the A allele (173 bp). **B.** Genotyping of the rs20417 (G/C).The PCR product (295 bp) was digested with BstFNI. The G allele corresponds to the 138 bp fragment, the C allele corresponds to the 157 bp. **C.** Genotyping of the rs2745557 (G/A). The PCR product (400 bp) was digested with TaqI which cleaved the A allele and generated two fragments (238 bp and 162 bp) but did not cleave the G allele (400 bp). **D.** Genotyping of the rs5277 (C/G).The PCR product (511 bp) was digested with HincII, the C allele corresponds to the 511 bp and the G allele corresponds to the 424 bp. **E.** Genotyping of the rs5275 (T/C). The PCR product (147 bp) was digested with BclI, the T allele corresponds to the 147 bp and the C allele corresponds to the 124 bp. **F.** Genotyping of the rs4648298 (A/G). The PCR product (545 bp) was digested with AluI which cleaved the G allele to generate two fragments (349 bp and 196 bp) but did not cleave the A allele (545 bp).

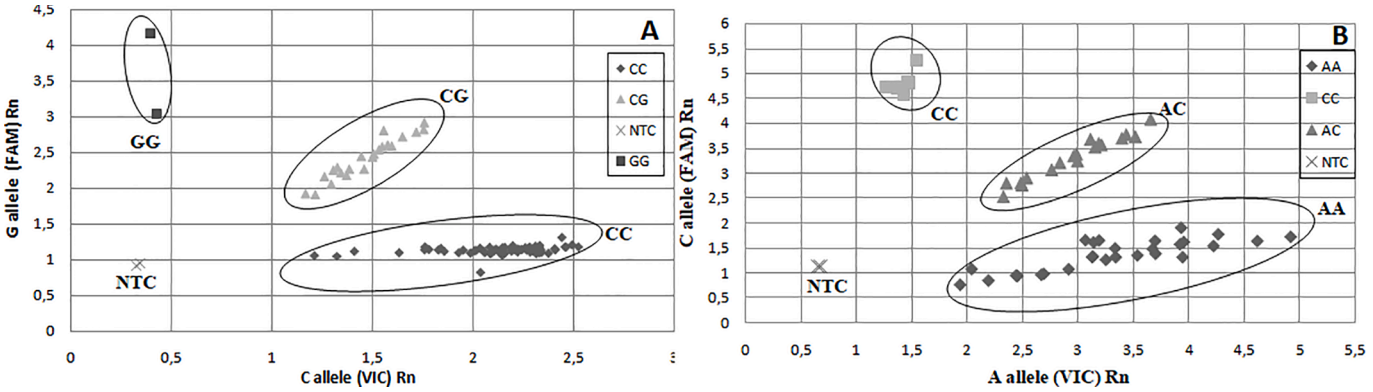

Fig. 2. Schematic representation of quantitative metrics measured using the VIC and FAM Rn values from the Applied Biosystems 7300 real-time fast system software. Graph A: allele discrimination graph of *PTGER2* rs2075797 (C/G). Graph B: allele discrimination of *PTGER4* rs4495224 (A/C). On an entire plate the data are resolved into three discrete clusters representing three different genotypes CC, CG and GG for rs2075797; AA, AC and CC for rs4495224 of each SNP. NTC: no template control; Rn: normalized reporter.

incubated with proteinase K at 37 °C then salted out at 4 °C by a saturated NaCl solution. Proteins were eliminated by centrifugation and DNA precipitated with ethanol.

2.4. *PTGS2* polymorphism genotyping

PTGS2 polymorphisms were detected by the PCR-Restriction Fragment Length method (RFLP).Genomic DNA was amplified using specific primers and specific restriction enzymes for each polymorphism as summarized in Table 1.The PCR was performed in a volume of 25 μl reaction mixture containing 2 ng/μl of deoxynucleotide triphosphates (dNTPs), 5 μl of 5X PCR Buffer, 50 pmol of each primer, 1.5 mM of $MgCl_2$ and 0.5 units of Taq DNA polymerase (Promega, Madison, WI, USA).

The PCR reaction for each polymorphism was performed as follows: 95 °C for 1 min of DNA double stranded denaturation, 35 cycles of 95 °C for 45 s, 60 °C for 1 min, 72 °C for 45 s and final extension at 72 °C for 7 min. Then, amplified products were digested by specific restriction endonucleases (Table 1).The digestion products were separated by electrophoresis on a 2% agarose gel marked with ethidium bromide and visualized under ultraviolet light. Analysis of *PTGS2* polymorphisms by PCR-RFLP revealed bands whose number and size depended on the presence of the restriction site (Fig. 1).

Table 2
Demographic and clinical characteristics in chronic obstructive pulmonary disease cases and control subjects.

Characteristics	COPD Cases(n = 138)	controls(n = 216)	P-value
Age (years)	61.8 ± 13.2	58.15 ± 10.7	0.1
Sex			
Male	122 (88.4%)	156 (72.2%)	**0.000**
Female	16 (11.6%)	60 (27.8%)	**0.000**
BMI (Kg/m^2)	25.1 ± 5.61	26.65 ± 4.25	0.4
Smoking status			
Never-smoked	21(15.2%)	106 (49.1%)	**0.000**
Smokers	63(45.7%)	95 (44%)	0.35
Ex-Smokers	54(39.1%)	15 (6.9%)	**0.000**
Pack years	46 ± 23	18 ± 16	**0.02**
FEV1 (mL)	1577± 653	3006± 748	**0.000**
FEV1(% predicted)	55.5 ± 18.5	93.8 ± 8.5	**0.000**
FVC (mL)	2785± 912	3660 ± 900	**0.000**
FVC (%predicted)	77.9 ± 9.5	96.3 ± 19.6	**0.000**
FEV1/FVC (%)	56±11	82 ± 5	**0.000**
GOLD stage			
GOLD I	13 (9.4%)	0	
GOLD II	63(45.7%)	0	
GOLD III	54 (39.1%)	0	
GOLD IV	8 (5.8%)	0	
PGE_2 (pg/ml)	2478.9 (1064.5–2698.8)	1497.6 (1000.3–1801.2)	**0.01**
PGD_2 (pg/ml)	4786.8 (3958.9–4980.2)	9000.1 (7947.9–9800.3)	**0.002**
Interleukin 6 (pg/ml)	16 (13.1–16.7)	6.8 (5.2–7.1)	**0.02**
Interleukin 18 (pg/ml)	1480 (960–1520)	1600(760–1610)	0.42
TNF α (pg/ml)	1 (0.5–1.2)	1.3 (1–1.5)	0.66
TGF β (pg/ml)	1820 (870–1855)	1780(875–1800)	0.79
AOPP (μmol/l)	13.9 ± 1.8	12.8 ± 2.5	0.35
NO (pmol/mg)	203.6 ± 112	318 ± 141	**0.03**
Total thiols (nmol/ml)	620 ± 256	699 ± 305	**0.01**
GSH (nmol/ml)	49.7 ± 31.0	96.6 ± 57.7	**0.001**
PCO (μM)	0.08 ± 0.03	0.07 ± 0.02	0.8
MDA (μM)	1.15 (0.86–1.24)	1 (0.75–1.15)	0.62
Peroxynitrite (μmol/ml)	1.05 (0.75–1.1)	1.12 (0.7–1.24)	0.5
TAS (mmoltroloxequivalent/l)	0.62(0.24–0.7)	0.8 (0.25–0.93)	**0.008**
Iron (μM)	11.4 ± 3.8	11 ± 3.7	0.7
Ascorbicacid (μg/ml)	12.1 ± 6.7	12.2 ± 8.1	0.91

Continuous data are expressed as mean ± Standard Deviation if normally distributed, otherwise as median (IQR); Difference was considered significant at $P<0.05$.Prostaglandins, cytokines and oxidative stress markers concentrations were measured in plasma.
BMI : Body Mass Index; FEV1 : Forced Expiratory Volume in 1second; FVC : Forced Vital Capacity; COPD : chronic obstructive pulmonary Disease;% predicted : percentage of predicted value; GOLD : Global initiative for Chronic Obstructive Lung Disease;AOPP: advanced oxidation protein products; NO: nitric oxide; GSH: reduced glutathione; PCO: Protein carbonyl oxide; TAS: total antioxidant status; IQR: interquartile range.

2.5. PTGER2 and PTGER4 polymorphisms genotyping

The *PTGER2* rs2075797 (NG_013082.1:g.4636C>*G*) and *PTGER4* rs4495224 genotypes were determined by the PCR-TaqMan allelic discrimination using assays-on-demand Kits (Thermo Fisher Scientific Life Technologies, CA 92,008 USA). The PCR was carried out in a final volume of 25 μl reaction mixture. Allelic discrimination was performed with ABI 7300 Software (7300Real Time PCR system) and data are shown on Fig. 2).

2.6. Measurement of inflammatory mediators and oxidative stress markers in plasma

The concentrations of PGE_2, PGD_2, IL-6, IL-18, TNF-α and TGF-β in plasma were measured using commercially available sandwich ELISA kits (PGE_2: Invitrogen Corporation, Canada; PGD_2-methoxime: Cayman Chemical, Ann Arbor MI, USA; Human IL-6, IL-8, TNF-α and TGF-β: 4A Biotech, Beijing101111 China). Since PGD_2 is a relatively unstable metabolite, we measured PGD2-MOX, the stable derivate of PGD_2. For analysis, we averaged duplicate measurements of each sample. Plasma concentrations of oxidative stress markers (AOPP, NO, total thiol, GSH, PCO, MDA, peroxynitrite, TAS, H_2O_2, Iron, Ascorbic acid) were measured using standard colorimetric methods [20].

2.7. Statistical analyses

Statistical analyses were performed using SPSS 13.0 (INC., Chicago, IL, USA) and EpiInfo-7. We used the Chi-square (X^2) test for the determination of genotype distributions between groups. Odds ratios (ORs) and 95% confidence intervals (CI) were estimated using unconditional logistic regression analysis based on the comparison of genotype and allele frequencies between patients with COPD and controls. The logistic regression was performed with the adjustment for age and tobacco smoking history using categorical variables representing each combination of age of COPD patients (groups of 10 years-interval) and tobacco smoking (Current smokers, ex-smokers and non-smokers). Comparisons between groups of means were performed using student *t*-test or with the Mann-Whitney test as adequate. Results are presented as mean ± SD or median (IQR) as adequate. The data were considered as significant when the statistical P-value was < 0.05.

3. Results

3.1. Patients' characteristics

The demographic and clinical characteristics of the study subjects are indicated in Table 2. There was no significant difference in mean age between COPD cases and controls. The frequency of tobacco ex-smokers was significantly higher in COPD cases compared to controls (39.1% *vs.* 6.9%; $P<0.001$) and mean smoking pack year was significantly higher in COPD patients than in the control group (46±23 *vs.* 18±16, $P = 0.02$). As expected, FEV1 (ml) and FEV1 (%) post-bronchodilator as well as FEV1/FVC values were significantly reduced in COPD as compared to controls (1577±653 *vs.* 3006±748; 55.5 ± 18.5% *vs.* 93.8 ± 8.5%; 56±11% *vs.* 82±5%, $P<0.001$ respectively; results are expressed as percentages of predicted values).

3.2. Inflammatory and oxidative stress markers of the study subjects

The concentrations of prostaglandins PGE_2 and PGD_2, of pro-inflammatory cytokines and oxidative stress markers measured in plasma from COPD cases and controls and presented in Table 2. There was a significant difference in PGE_2 and PGD_2 plasma concentrations between COPD subjects and controls (2478.9 (1064.5–2698.9) *vs.*1497.6 (1000.3–1801.2) pg/ml; $P = 0.01$ and 4786.8 (3958.9–4980.2) *vs.* 9000 (7947.9–9800.3) pg/ml; $P = 0.002$ respectively). A significant difference in Interleukin-6 concentrations between COPD and controls (16(13.1–16.7) *vs.*6.8 (5.2–7.1); $P = 0.02$) was also observed. Regarding oxidative stress markers, NO, total thiols, reduced glutathione (GSH) and total antioxidant status (TAS) plasma concentrations were significant decreased in COPD cases compared to controls (NO: 203.6 ± 112 *vs.* 318±141 pmol/mg, $P = 0.03$; total thiols 620±256 *vs.* 699±305 nmol/ml, $P = 0.01$; reduced glutathione: 49.7 ± 31 *vs.* 96.6 ± 57.7 nmol/ml, $P = 0.001$; TAS: 0.62(0.24±0.7) *vs.* 0.8 (0.25–0.93) mmol trolox equivalent/l; $P = 0.008$ respectively).

3.3. Genotype distribution of PTGS2, PTGER2 and PTGER4 polymorphisms

Genotype distribution of the eight polymorphisms was in accordance with the Hardy Weinberg equilibrium in the control and COPD groups. Genotype and allele frequencies of the polymorphisms in the *PTGS2,*

Table 3
Comparisons of genotype and allele frequencies of *PTGS2* polymorphisms between chronic obstructive pulmonary disease cases and controls.

SNP	Genotypes and alleles	COPD Cases (n = 138) [n(%)]	Controls (n = 216) [n(%)]	Unadjusted		Adjusted for age and tobacco smoking	
				OR (95%CI)	P-value	OR (95%CI)	P-value
PTGS2 rs689465	AA	114 (82.6)	166 (78.7)	1(Reference)	0.41	1(Reference)	0.72
	AG	24 (17.4)	44 (20.9)	0.79 (0.46–1.38)	1	1.15 (0.51 – 2.62)	0.79
	GG	0	1 (0.4)	0	0.36	1.76 (0.61 - 5.05)	
	AG+GG	24 (17.4)	45(21.3)	0.77 (0.45–1.34)	0.37		
	A allele	252 (91.3)	376 (89)	1(Reference)			
	G allele	24 (8.7)	46 (11)	1.28 (0.76–2.16)			
PTGS2 rs20417	GG	93 (67.4)	132 (62.6)	1(Reference)		1(Reference)	0.91
	GC	38 (27.5)	72 (34.1)	0.75 (0.46–1.20)	0.23	0.37 (0.41–2.68)	0.94
	CC	7 (5.1)	7 (3.30)	1.42 (0.48–4.18)	0.52	0.96 (0.32 – 2.86)	0.37
	GC+CC	45 (32.6)	79 (37.44)	0.8 (0.51–1.27)	0.36	1.49 (0.62 - 3.58)	
	G allele	224 (81.2)	336 (79.6)	1(Reference)	0.63		
	C allele	52 (18.8)	86 (20.4)	1.1 (0.75–1.62)			
PTGS2 rs2745557	GG	53 (38.4)	110 (50.9)	1(Reference)	0.07	1(Reference)	0.55
	GA	65 (47.1)	88 (40.7)	1.53 (0.97–2.42)	**0.02**	0.75 (0.29 −1.93)	**0.03**
	AA	20 (14.5)	18(8.3)	2.3 (1.13–4.72)	**0.02**	3.23 (1.2–11.6)	0.37
	GA+AA	85 (61.6)	106 (49)	1.66 (0.54–2.41)	**0.009**	1.34 (0.7 - 2.59)	
	G allele	171 (61.96)	308 (71.3)	1(Reference)			
	A allele	105 (38.04)	124 (28.7)	1.52 (1.1–2.1)			
PTGS2 rs5277	CC	10 (7.2)	11 (5.3)	1(Reference)	0.85	1(Reference)	0.7
	CG	43 (31.2)	70 (33.8)	0.82 (0.10–6.61)	0.96	0.74 (0.17- 3.33)	0.9
	GG	85 (61.6)	126 (60.9)	1.05 (0.14–7.86)	0.72	0.99 (0.23 – 4.23)	0.97
	CG+GG	128(92.7)	196 (94.7)	0.46 (0.30–1.74)	0.85	0.88 (0.21 – 3.71)	
	C allele	63(22.8)	92 (22.2)	1(Reference)			
	G allele	213 (77.2)	322 (77.8)	1.03 (0.71 −1.49)			
PTGS2 rs5275	TT	76 (55.1)	113 (53.8)	1(Reference)	0.44	1(Reference)	0.68
	TC	49 (35.5)	87 (41.4)	0.83 (0.53–1.32)	0.14	1.15 (0.48–2.75)	0.12
	CC	13 (9.4)	10 (4.8)	1.93 (0.80–4.63)	0.82	3.18 (0.73–5.54)	0.53
	TC+CC	62 (44.9)	97 (46.2)	0.95 (0.62–1.64)	0.10	1.3 (0.57–3)	
	T allele	201 (72.8)	313 (74.5)	1(Reference)			
	C allele	75(27.2)	107 (25.5)	1.34 (0.94–1.92)			
PTGS2 rs4648298	AA	128 (92.8)	200 (93.9)	1(Reference)		1(Reference)	
	AG	10 (7.2)	13 (6.1)	1.2 (0.51–2.82)	0.67	0.92 (0.92–3.27)	0.15
	A allele	266(96.38)	413 (96.95)	1(Reference)	0.67		
	G allele	10 (3.62)	13 (3.05)	0.83 (0.36–1.93)			

The Chi-square test was used to compare genotype distributions between the cases and controls. Adjustments for age and tobacco smoking were performed by logistic regression analysis. OR: Odds Ratio; CI: Confidence Interval.
Difference was considered significant at $P<0.05$.OR = Odds Ratio, CI = Confidence Interval.

PTGER4 and *PTGER2* genes are indicated in Table 3 and Table 4. We used logistic regression as statistical test, to analyze the differences between cases and controls in the distribution of *PTGS2* SNPs genotypes and their risk of developing COPD. The genotype distribution of rs2745557 in *PTGS2* and rs2075797 in *PTGER2* were significantly different between COPD cases and controls. While those for rs689465, rs20417, rs5277, rs5275, rs4648298 in *PTGS2* and rs4495224 in *PTGER4* were not ($P > 0.05$) (Table 3, Table 4). For rs2745557 in *PTGS2*, the frequencies of the GG, GA and AA genotypes were respectively 38.4%, 47.1%, 14.5% in COPD cases and 50.9%, 40.7%, 8.3% in the control group. The GA heterozygotes were markedly over-represented in COPD subjects compared with the control group (47.1% *vs*. 40.1% respectively, $P = 0.07$). Considering the GG genotype as a reference, a significant association was observed between the homozygous AA genotype and COPD risk (OR= 2.3; 95%CI=1.13–4.72; $P = 0.02$). Similarly, an association was observed also with GA and AA genotypes combined together (OR=1.66; 95%CI=0.54–2.41; $P = 0.02$). Analysis after adjustments made for age and smoking status showed that COPD subjects carrying the AA genotype were at increased risk to develop COPD than GG wild type carrying (OR= 3.23; 95%CI=1.2–11.6; $P = 0.03$). The A allele frequency was significantly higher in COPD cases compared to controls (38.04% *vs*. 28.7%). Thus the A allele was associated to COPD risk (OR=1.52; 95%CI=1.1–2.1; $P = 0.009$) (Table 3). Similar results were obtained with adjustment including sex with age and smoking status as covariates (data not shown).

As shown in Table 4, subjects carrying the CG genotype of rs2075797 in *PTGER2* had an unadjusted OR of 6.62 (95%CI=3.9–11.1) of COPD risk compared to those having the CC genotype. Additionally, COPD patients with GG genotype had an increased risk (OR=9.22; 95% CI=3.35–25.4; $P < 0.001$) as compared to those with CC genotype. Considering CG and GG genotypes together, a significant association was observed between the combined genotypes CG+GG and COPD risk (OR=7.05; 95% CI =3.5–19.5; $P< 0.001$). The G allele of rs2075797 was significantly more frequent in COPD cases compared to controls (38.3% *vs*. 12%; $P< 0.001$). This variant was associated to the risk of COPD with an unadjusted OR=4.55 and 95% CI=3.5–8.9; $P< 0.001$. Significant differences in genotype frequencies between COPD cases and controls and persisted after adjustments made for age and smoking status (Table 4).

3.4. Relationship between lung-function tests values and PTGS2, PTGER2, PTGER4, polymorphisms in COPD cases

Overall, there was no statistically significant relationship between

Table 4
Comparison of genotype and allele frequencies of *PTGER2* and *PTGER4* polymorphisms between chronic obstructive pulmonary disease cases and controls.

SNP	Genotypes and alleles	BPCO Cases ($n = 138$) [n(%)]	Controls ($n = 216$) [n(%)]	Unadjusted		Adjusted for age and tobacco smoking	
				OR (95%CI)	P-value	OR (95%CI)	P-value
PTGER2 rs2075797	CC	43 (34.7)	170 (78.8)	1(Reference)	**<0.001**	1(Reference)	**0.02**
	CG	67 (54)	40 (18.5)	6.62 (3.9–11.1)	**<0.001**	3.2 (1.5- 2.7)	**0.003**
	GG	14 (11.3)	6(2.7)	9.22 (3.35–25.4)	**<0.001**	6(3.8- 12.5)	**0.03**
	CG+GG	111(89.5)	46(21.3)	7.05 (3.5–19.5)	**<0.001**	5.4 (3.2 −8.1)	
	C allele	153(61.7)	380 (88)	1(Reference)			
	G allele	95(38.3)	52 (12)	4.55 (3.5–8.9)			
PTGER4 rs4495224	AA	48 (38.1)	76 (38.6)	1(Reference)	0.54	1(Reference)	0.94
	AC	58 (46)	81 (41.1)	1.13 (0.69–1.86)	0.62	1.21 (0.46–3.2)	0.29
	CC	20 (15.9)	40 (20.3)	0.79 (0.41–1.51)	0.48	1.89 (0.58–6.2)	0.46
	AC+CC	121(61.4)	78 (61.9)	1.02 (0.64–1.62)	0 .93	1.4 (0.57–3.43)	
	A allele	154 (61.1)	233 (59.1)	1(Reference)	0.61		
	C allele	98 (38.9)	161 (40.9)	1.08 (0.78–1.5)			

The Chi-square test was used for genotype distributions between the cases and controls. Adjustments for age and tobacco smoking were performed by logistic regression analysis.
Difference was considered significant at $P<0.05$. OR : Odds Ratio ; CI : Confidence Interval.

pulmonary function parameters tested and the different genotype groups of *PTGS2, PTGER2* and *PTGER4* SNPs in COPD patients (Supplementary Tables 1 and 2). A significant association was observed, however between the CC genotype of rs20417 in *PTGS2* and the decline of FEV1 and FVC values ($P = 0.02$).

3.5. Relationship between rs274557 in PTGS2 and inflammatory markers concentrations

We evaluated whether the *PTGS2, PTGER2* and *PTGER4* polymorphisms were related to prostaglandins plasma concentrations. As shown in Table 5, for rs274557, the PGD_2 concentration was decreased in AA as compared to GG carriers: 4050.1(2019.7–4133) *vs*. 4859.8 (2049.9–4918) pg/ml; $P = 0.03$ among COPD patients. It was significantly decreased in COPD patients with GA and AA combined together: 3959.8 (2130–4022) pg/ml; $P = 0.02$. There was a significant increase of IL-18 plasma concentration associated to the GA and AA genotypes combined together compared to the GG genotype: 1630(1050–1655) pg/ml *vs*. 975 (927–1280) pg/ml; $P = 0.03$ among COPD subjects.

3.6. Relationship between rs274557 in PTGS2 and oxidative stress markers

Only the rs274557 GA and AA genotypes were associated with significantly increased NO concentrations among COPD subjects compared to those with GG genotype (234.1 ± 14.9 pmol/mg *vs*.170.4 ± 13.1 pmol/mg; $P = 0.004$), while no differences were observed for the other oxidative stress markers.

4. Discussion

In the present study, we screened eight genetic polymorphisms for proteins involved in the prostaglandin pathway including the COX-2 enzyme and two EP receptors (EP2 and EP4). Our aims were to explore their association with COPD susceptibility and to evaluate their effects on inflammatory and oxidative stress markers and selected lung function parameters.

In previous studies, COX-2 expression has been shown to be an important risk factor for COPD development and has been related to the inflammatory status of multiple diseases [21, 22]. This has made this enzyme an important target in several genetic studies [23, 24]. In the present report we genotyped six variants of the *PTGS2* gene including rs689465, rs20417, rs5277, rs5275 and rs4648298 but no significant relationships with COPD development were found. We also investigated the role of another genetic variant, rs2745557, located in the intron1 of *PTGS2* gene. Multiple studies examined the impact of this SNP in cancer susceptibility. As an example, Shalaby et al. reported that rs2745557 was not associated to colorectal cancer (CRC) susceptibility in the Saudi population [25]. In another report, CRC patients carrying GA or AA genotypes and having a smoking history had an increased risk of CRC compared to healthy controls [9]. Few studies have reported a correlation between concentration of lipid mediators including (PGD_2, LTE4 and EPA) and lung function (FEV1) in COPD [26, 27].Besides, other reports found that *PTGS2* gene polymorphisms have no effect on lung function (FEV1 and FVC) in bronchial asthma, lung fibrosis [28, 29].

Until now, the functional role of the rs2745557 on COX-2 expression and activity in COPD is still not clear. In the current report, we found that the frequency of the rs2745557 A allele was significantly different between the two groups; the rs2745557 (GA+AA) genotypes being more frequent in COPD patients. However, no significant relationships were found between this polymorphism and both lung function parameters and severity of disease. We also failed to find significant effects of this SNP on PGE_2 concentration, which is a mediator involved in airway inflammation and remodeling of airway smooth muscle cells and fibroblasts [30]. Interestingly, higher PGE_2 concentrations were observed in Tunisian COPD patients, which is consistent with previous data indicating that PGE_2 is induced in the sputum and lung fibroblasts from COPD patients and correlates with COX-2 expression and severity of airflow limitation [23, 31]. Overall, these results may indicate the involvement of other undetermined genetic factors that may affect PGE_2 plasma concentration in our studied cohort. Interestingly, rs2745557 genotypes significantly affected PGD_2 plasma concentrations in COPD patients.

Of note, a significant decrease in PGD_2 plasma concentration was found in COPD patients, as well as in those carrying the rs2745557 A allele. These findings support data from [32], indicating that decreased PGD_2 production in COPD patients may lead to an abnormal inflammatory response and an alteration of airway permeability. In contrast to our findings, Csansky and colleagues reported higher PGD_2 concentrations in the bronchoalveolar fluid from COPD subjects compared with controls. PGD_2 concentration was also negatively correlated with lung function decline in COPD patients, showing an eventual link between inflammation and lung function [26] . Overall, these results and ours may indicate that this SNP could have an impact on COX-2 activity since some intronic variants in COX-2 gene may affect the splicing phenotype even if they do not occur within transcripts splice junctions [33, 34].

Table 5
Plasma concentrations of prostaglandins, cytokines and oxidative markers according to the PTGS2 +202 (G/A) rs2745557polymorphism genotypes in COPD patients and healthy controls.

	rs2745557 in *PTGS2*													
	COPD							Controls						
	GG	GA	P^a	AA	P^b	GA+AA	P^c	GG	GA	P^a	AA	P^b	GA+AA	P^c
PGE_2 (pg/ml)	2184.9 (1200–2230)	2280.1 (1244–2300)	0.7	2519.8 (1250–2600)	0.54	2680.2 (1290–2750)	0.7	1699.8 (1450–1725)	1630.1 (1180–1695)	0.3	1649.9 (1460–1720)	0.5	1420 (1280–1500)	0.5
PGD_2 (pg/ml)	4859.8 (2049.9–4918)	4231.9 (2250–4178)	0.1	4050.1 (2019.7–4133)	**0.03**	3959.8 (2130–4022)	**0.02**	7732.2 (7400–8005)	8849.8 (8105–9155)	0.1	8123.7 (7555–8420)	0.8	8509.9 (7858–7250)	0.6
IL-6 (pg/ml)	16.4 (9.2–17)	17.5 (9.6–18.2)	0.9	15.7 (8.4–16.9)	0.9	16.5 (9.4–18.8)	0.99	8.7 (6.9–9.4)	9.2 (7.5–9.6)	0.6	6.8 (4.4–7.2)	0.4	8.3 (4.7–9.3)	0.7
IL-18 (pg/ml)	975 (927–1280)	1655 (1140–1690)	**0.02**	1342 (1110–1415)	**0.04**	1630 (1050–1655)	**0.03**	1675 (760–1740)	1446 (690–1580)	0.3	1410 (669–1405)	0.3	1432 (718–1488)	0.2
TNF-α(pg/ml)	1.5 (0.5–1.6)	1.1 (0.5–1.4)	0.7	0.9 (0.5–1.2)	0.6	1 (0.5–1.05)	0.8	1.1 (0.8–1.6)	1.5 (0.9–1.8)	0.79	1.2 (0.8–1.4)	0.9	1.4 (0.9–1.7)	0.8
TGF β(pg/ml)	1657 (1522–1744)	1760 (1654–1788)	0.6	1810 (1732–1825)	0.2	1832 (1784–1873)	0.3	1720 (1615–1850)	1836 (1655–1910)	0.64	1745 (1622–1895)	0.9	1799 (1705–1825)	0.9
AOPP (μM)	14.2 ± 0.18	14.5 ± 0.13	0.08	13.9 ± 0.37	0.6	14.4 ± 0.13	0.3	14±0.19	13.9 ± 0.2	0.81	13.2 ± 0.4	0.2	13.78±0.23	0.5
NO (pmol/mg)	170.4 ± 13.2	235.6 ± 16.7	**0.004**	229 ± 33.5	**0.04**	234.1 ± 14.9	**0.004**	254.6 ± 13.6	252.6 ± 15.4	0.74	290.6 ± 62.7	0.3	259.2 ± 16.7	0.5
Thiol (nmol/ml)	605.6 ± 32	601.3 ± 17.5	0.4	587.1 ± 75.8	0.8	629.3 ± 30.3	0.6	692.3 ± 28	719.1 ± 36.1	0.55	605.19±54.4	0.2	699.2 ± 31.4	0.8
GSH (nmol/ml)	51.2 ± 4.4	43.4 ± 3.3	0.1	71.52 ± 15.5	0.08	48.8 ± 4.2	0.7	100.5 ± 5.7	94.7 ± 6.1	0.53	86.8 ± 15.6	0.4	93.9 ± 5.7	0.4
PCO (μM)	1.3 ± 0.13	1.3 ± 0.1	0.9	1.03 ± 0.18	0.6	0.08 ± 0.003	0.9	0.07 ± 0.002	0.07 ± 0.002	0.38	0.07±0.007	0.6	0.07 ± 0.002	0.3
MDA (μM)	1.11± 0.08	1.07 ± 0.06	0.7	0.89 ± 0.12	0.2	1.22 ± 0.09	0.4	0.97±0.07	1.8 ± 0.09	0.09	0.8 ± 0.1	0.5	1 .5 ± 0.08	0.2
Peroxynitrite (μM)	1.12±0.08	1.05 ± 0.03	0.6	0.9 ± 0.02	0.1	1.03 ± 0.06	0.3	1.2 ± 0.07	1.2 ± 0.07	0.78	0.9 ± 0.09	0.1	1.2 ± 0.06	0.8
TAS (mmol/L)	0.66±0.03	0.68±0.02	0.5	0.61 ± 0.04	0.3	0.66 ± 0.02	0.9	0.8 ± 0.02	0.8 ± 0.02	0.46	0.8 ± 0.05	0.5	0.8 ± 0.2	0.4
H_2O_2 (μM)	350.2 ± 18.5	332.7 ± 18.5	0.92	353.6 ± 26.5	0.92	337.5 ± 15.4	0.61	334.6 ± 13.3	367.5 ± 15.9	0.11	310.9 ± 20.5	0.5	357.6 ± 13.7	0.2
Iron (μM)	12.9 ± 1.17	10.84 ± 0.77	0.53	11.86 ± 0.86	0.77	11.47±0.4	0.85	11.4 ± 0.34	10.49 ± 0.4	0.1	11.2 ± 0.9	0.9	10.6 ± 0.4	0.1
Ascorbic acid (μg/ml)	14.16±0.18	14.55 ± 0.13	0.43	14.86±0.92	0.4	11.69±0.7	0.37	12.38 ± 0.85	12.6 ± 0.88	0.86	10.6 ± 1.3	0.4	12.2 ± 0.76	0.9

Difference was considered significant at $P<0.05$: P^a: GG *vs.* GA ; P^b: GG *vs.* AA ; P^c: GG *vs.* GA+AA. Continuous data are expressed as mean ± Standard Deviation if normally distributed, otherwiseas median (IQR).
AOPP: advanced oxidation protein products; NO: nitric oxide; GSH: reduced glutathione; PCO: Protein carobonyl oxide; MDA: malondialdehyde; TAS: total antioxidant status; H_2O_2: hydrogen peroxide.

Based on this previous hypothesis and our current findings we can speculate that rs2745557 in *PTGS2* can affect the alternative splicing machinery as a consequence of the localization within an intron splicing enhancer or silencer or by activating a cryptic splice junction. A decreased PGD_2 production could also be due to downstream regulation mechanisms that affect PGD synthase expression or activity.

According to the literature, COX-2 derived metabolites exert their effects through binding to four different E-prostanoid receptors (EP1-EP4) [35]. Among these, the EP2 and EP4 receptors are expressed in human bronchi and mediate the broncho-protective effects of PGE_2 in airways by inhibiting chemo-attractant mediators production by alveolar macrophages and bronchial epithelium [36]. The role of the EP4 receptors in mediating the bronchodilator effects of PGE_2 in human bronchi has been confirmed by [37].To the best of our knowledge, this is the first report that investigates the role of *PTGER* polymorphisms in COPD susceptibility and severity. Among these, rs2075797 is located in the promoter region of *PTGER2* gene coding for the EP2 receptor [10]. Until now, three studies have examined the association between this SNP and the risk of asthma [10, 11, 38].

In this report, significant differences in the frequency of the rs2075797 alleles were found between COPD patients and healthy controls. The frequencies of the rs2075797 (CG+GG) genotypes were higher in the COPD than in control patients. Of note, the rs2075797 G allele was more frequent in COPD patients and significantly associates with disease risk. These results were different, from those reported in Japanese subjects with arterial hypertension showing no significant impact of this polymorphism on vascular tone [17]. In contrast, Kim et al. found that rs2075797 associates to an aspirin-intolerant phenotype ($P = 0.038$, OR= 0.64) in an asthmatic Korean cohort [10].

Our preliminary unpublished results indicate, however a reduced expression of EP2 and EP4 receptors in bronchial specimens from COPD patients resulting in a reduction of the PGE2 braking mechanisms of inflammation (data not shown). Although additional functional studies are required, one can speculate that rs2075797 may correlate with a lower EP2 receptors signaling or even deactivates constitutively the corresponding receptors. Further studies are required however, to decipher whether there is a correlation between EP2 receptors expression, rs2075797and PGE_2 levels in our studied population. Since prostanoid synthesis is extremely complex and highly gene-regulated, one can argue, that genetic polymorphisms in the *PTGS2* gene such as rs2745557 could affect prostaglandin synthesis. Otherwise, a downstream genetic regulation involving rs2075797 in *PTGER2* could alter EP2 receptor expression and disturb its signal transduction.

Several limitations of this study should be enumerated. First, the COPD group is relatively small and our data needs to be replicated in a larger cohort. Results with *PTGS2* and *PTGER4* variants should also be interpreted cautiously because of the limited sample size of our COPD cohort. Second, the genes explored in our current report were not previously identified in the genetic landscape of COPD [39]. Third, for *PTGER2* and *PTGER4* genes only one SNP per gene was screened, which is not sufficient to study their role in COPD pathophysiology. Further studies screening other polymorphisms in *PTGER2* and *PTGER4* genes should be considered. In our experiments, we chose to use ELISA for measurement of PGE_2 and PGD_2 plasma concentrations because of its availability and its simpler procedure (antigen-antibody reaction). Future work could consider using liquid chromatography/ mass spectrometry (LC/MS) as an alternative to ELISA to provide further specificity, speed and accuracy and to avoid the possibility of false positive or negative results.

In summary, this is the first study which focused on the role of SNPs in *PTGS2* and EP receptor genes in COPD pathogenesis in a Tunisian cohort. In this community-based case-control study, we provided evidence that subjects with the rs2745557 A allele in *PTGS2* gene and the rs2075797 G allele in *PTGER2*gene are particularly associated with higher risk to develop COPD. Moreover, the rs2745557 A allele in *PTGS2* contributes to increased inflammation and oxidative stress but does not associate to altered lung function. Further studies are required, however to explore the present findings for functionality and protein expression.

CRediT authorship contribution statement

Salma Mani: Conceptualization, Resources, Investigation, Formal analysis, Writing - original draft. **Xavier Norel:** Writing - original draft, Validation. **Mathilde Varret:** Formal analysis, Writing - original draft, Validation. **Sarra Bchir:** Investigation, Resources. **Amel ben Anes:** Investigation, Resources. **Abdelahamid Garrouch:** Resources. **Zouhair Tabka:** Resources. **Dan Longrois:** Formal analysis, Writing - original draft, Resources, Validation, Supervision. **Karim Chahed:** Formal analysis, Writing - original draft, Validation, Supervision.

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Supplementary materials

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