Joint Bone Spine 86 (2019) 373–380

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Original article

Gene-environment interactions in Paget's disease of bone

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ARTICLE INFO

Article history:
Accepted 19 December 2018
Available online 27 December 2018

Keywords:
Paget's disease of bone
SQSTM1 gene
Heavy metals
Cadmium
Osteoclast
Oxidative stress

ABSTRACT

Objectives: This study explored the role of outdoor and indoor air pollutants in Paget's disease of bone (PDB).
Methods: We performed a survey in 140 French-Canadian patients with PDB, including 39 carriers of *p.Pro392Leu* mutation (*SQSTM1* gene) and 113 healthy not mutated controls. The survey covered outdoor air pollution near the residence and indoor air pollutants by focusing on heating fuels and exposure to tobacco smoke. In a subgroup of patients, urinary concentrations of 17 heavy metals and 11 polycyclic aromatic hydrocarbons were measured by mass spectrometry. In light of what we learned from the survey and urinary assays, we explored the in vitro effects of certain toxics on osteoclasts in PDB. We conducted in vitro monocytes differentiation from peripheral blood of more than 40 participants, whose osteoclasts were treated with or without the toxic. The morphology of osteoclasts, their bone resorption abilities, gene and protein expression levels, and cellular oxidative stress levels were assayed.
Results: An inhibitory effect of cigarette smoke condensate and heavy metals was observed on morphology and bone resorption activity of patients' osteoclasts. *SQSTM1* gene expression was upregulated in osteoclasts from patients with PDB versus healthy controls in presence of cadmium, and SQSTM1 protein expression was upregulated in presence of bismuth and tobacco smoke condensates, in particular in osteoclasts from carriers of the *SQSTM1* mutation. Furthermore, high levels of oxidative stress in patients' osteoclasts were observed.
Conclusions: Our in vitro experiments suggest an interaction between *SQSTM1* gene and exposure to cadmium and tobacco smoke condensates.

1. Introduction

Paget's disease of bone (PDB) is a common metabolic bone disease characterized by increased bone turnover in focal areas [1]. This disorder affects about 1% of European descents over the age of 55 and up to 5% in people over the age of 84 years old [2]. In familial forms of PDB, the disease is inherited in an autosomal dominant pattern with incomplete penetrance [3,4]. Up to 30 mutations within *SQSTM1* gene were linked to PDB [5]. The *p.Pro392Leu* mutation of *SQSTM1* gene is the most frequent mutation in PDB [6]. It is the only *SQSTM1* mutation found in the French-Canadian population in 46% of familial forms of PDB and 16% of unrelated affected patients [6]. *SQSTM1* gene encodes p62 protein that plays an important role in macro-autophagy [7]. Several common genetic variants were also found to be associated with PDB in GWAS [8,9].

Significant decrease in the prevalence and severity of PDB in UK, New Zealand and some European countries has been reported [1,2,10]. Lancashire, UK, one of the regions having the highest PDB prevalence, reported 60% of decline in PDB prevalence [1]. PDB was reported to be more frequent and more severe in rural areas than urban ones, in particular in Italy and Spain, where zoonosis in possible interaction with a genetic founder effect has been evocated [11,13–15]. Indeed, the disease outbreak has been attributed to possible viral infection such as Measles virus, or other Paramyxovirus, including Respiratory Syncytial virus, animal contact with dogs and cattle in rural areas [16], the incidence of PDB having decreased in countries using Measles vaccination in the 1960s [17]. An association between tobacco use and PDB was previously reported [18]. A survey in the French-Canadian cohort has suggested a potential association of PDB with wood heating during childhood and/or adolescence [19]. Furthermore, lead exposure was associated with an

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https://doi.org/10.1016/j.jbspin.2018.12.007

excessive bone turnover [20–22], in possible accordance with the high prevalence in air polluted cities [23].

Taken together, exposure to environmental factors may contribute to epidemiological changes in PDB. Herein, we performed an exploratory study focusing on indoor and outdoor air pollutants, in our French-Canadian cohort, and its effects on cellular phenotype of osteoclasts differentiated in vitro.

2. Methods

2.1. Recruitment of participants and survey

This study was approved by the CHU de Québec-Université Laval Ethics Committee and all participants signed a consent form before inclusion in the study. We studied 140 patients with PDB (familial and non-familial forms), including 39 carriers of the *p.Pro392Leu* mutation within *SQSTM1* gene and 113 healthy controls from the French-Canadian cohort, who previously answered a general survey on environmental factors and who agreed to participate to this new study [19]. Both questionnaires did not overlap, except for sex and education level which were reused from the previous survey. An affected participant was diagnosed with PDB in accordance with previously published criteria [19]. Controls were unrelated healthy adults without personal or familial history of PDB, with normal total serum alkaline phosphatase levels at inclusion, and not carrier of any *SQSTM1* mutation. Controls were not matched for age and sex with PDB patients. All participants lived in the same geographic area within a 120 km radius of Quebec City. In this survey, we focused on the history of residence in proximity to sources of indoor and outdoor air pollutants during childhood (age $\leq$ 20 years) and adulthood. Outdoor pollution sources were represented by residence close to ($\leq$ 1 km) a highway, an airport, a train, a bus, or a gas station, while indoor pollution sources were represented by frequent exposure to heating combustibles (coal, wood, and oil) and cigarette smoke exposure at home.

2.2. Urinary dosages

We measured by mass spectrometry concentrations of 17 heavy metals related to air pollution and 11 polycyclic aromatic hydrocarbons (PAHs) in urinary samples of a subgroup of patients (n = 15 to 46) and healthy controls (n = 12 to 48).

2.3. Toxics selection

We performed a literature review on toxics that can accumulate in the human body [Appendix A, Table S1; See the supplementary material associated with this article online]. Subsequently, we performed in vitro experiments using monocytes from peripheral blood differentiated in vitro into mature osteoclasts to examine the impact of these toxics on osteoclast phenotype in patients with PDB and healthy controls. Four heavy metals (lead, cadmium, bismuth, and mercury) and cigarette smoke condensate were selected for in vitro experiments in accordance with the results of urinary dosages and feasibility to obtain toxic for our experiments. All heavy metal powders (lead nitrate, cadmium chloride, mercury chloride, and bismuth trinitrate pentahydrate) were purchased from Sigma Aldrich co. The Cigarette Smoke Condensate (CSC) was purchased from Murty Pharmaceuticals, Lexington, KY, which was prepared using Federal Trade Commission's smoke machines and University of Kentucky's 3R4F Standard Research Cigarettes [24]. In order to determine the optimal dose of toxic for our in vitro experiments, we prepared a gradient of diluted heavy metals from 20 μM to 500 μM and CSC solutions from 5 μg/mL to 100 μg/mL applied on lymphoblasts immortalized by Epstein-Barr virus cultures. Next, we selected the highest dose of toxic that has no effect on lymphoblasts' viability and proliferation (Appendix A, table S2).

2.4. Cell cultures

We collected 50 mL of peripheral blood from participants in order to harvest monocytes from healthy controls, patients with non-familial form of PDB, and *p.Pro392Leu SQSTM1* mutation healthy carriers. Mononuclear cells from human peripheral blood (PBMCs) were isolated by density gradient centrifugation using a Ficoll-Paque, and cells' concentration was adjusted up to 3×10^6 cells/mL. Cell cultures were exposed to one of the heavy metals or CSC and differentiated in vitro by the use of RANKL (Peprotech, Rocky Hill) and hMCSF (eBioscience, San Diego) during 21 days. Sterilized, demineralized, and distilled water was added to 2 wells and toxic was added to 2 other wells of 8 chamber slide system Lab-Tek: 400 μL of suspended cells/well (1.2×10^6 cells). Additionally, in order to examine the bone resorption abilities of mature osteoclasts, cells were seeded in 4 wells of Osteoassay. Sterilized, demineralized, and distilled water was added to 2 wells; one contained 40 ng/mL RANKL called ctrl 40; toxics (using concentrations cited in Appendix A, table S2) were added to 2 other wells; one contained 40 ng/mL RANKL. All wells were using a 36-well Osteoassay plate (Fisher Scientific, Ottawa): 300 μL of suspended cells/well (1×10^6 cells). Cells were left overnight at 37 °C with 5% CO2 in order to facilitate monocytes to attach to the plastic surface of the culture plate. Mediums were changed every 3–4 days: Alpha-MEM medium, which contains 10% FBS + 1% Penicillin-Streptomycin (Wisent, St-Bruno, Canada). Fluorescence-based staining for TRAP relying on ELF97 phosphatase substrate (Thermo Fisher Scientific) was performed as well as DAPI Fluorescent stain (Thermo Fisher Scientific) for nuclei and phalloidin (Thermo Fisher Scientific) for cell membrane.

2.5. Quantitative real-time PCR

Total RNA extraction from cell lysates was performed as published [25]. We used the PCR reaction protocol as reported [25]. Designation of the Oligoprimer pairs of genes involved in heavy metals binding, detoxification and macro-autophagy was performed using GeneTool 2.0 software (Biotools Inc, Edmonton) (Appendix A, Table S3). LightCycler 480 SYBRGreen I Master Reagent (Roche Diagnostics, Indianapolis) with 2% DMSO and LightCycler 480 (Roche Diagnostics, Mannheim, DE) were used in the reaction. Following Luu-The et al. methods [26], the mRNA copies numbers were calculated. In addition, Glucose-6-phosphate dehydrogenase (G6PD), peptidylprolyl isomerase B (cyclophilin B) (PPIB), and 18S ribosomal RNA (18S) were used as reference genes [27]. Quantitative Real-Time PCR reactions used previously published conditions [25]. Experiments were performed by the CHU de Québec Research Center (CHUL) Gene Expression Platform, and were compliant with MIQE guidelines, as reported [25].

2.6. Oxidative stress levels measurement

Quantitative measurement of 8-OHG (8-hydroxyguanosine), which indicates the oxidative damage on RNA, was performed using a competitive ELISA (OxiSelect™ Oxidative RNA Damage ELISA Kit, CELL BIOLABS) on RNA extracted from osteoclast lysates. 8-OHG/BSA conjugate preabsorbed EIA plate was added to 8-OHG samples and controls followed by incubation. An anti-8-OHG monoclonal antibody and an HRP-conjugated secondary antibody were added.

2.7. Protein expression analyses by Western Blot

We separated cell lysates by SDS-PAGE and transferred them to a PVDF membrane, which was incubated with primary antibod-

Table 1
Environmental factors associated with familial forms of Paget's disease of bone (PDB) and non-familial forms of PDB, results of multivariate analysis. OR = odds ratio.

Characteristics	Category	Without correlation, variable conserved in the final multivariate model if *P*-value < 0.05		
		OR Familial PDB (95% CI)	OR Non-familial PDB (95% CI)	*P*-value
Sex				
	Men	4.126 (1.823–9.342)	6.734 (3.180–14.260)	*P* < 0.0001
	Women	REF	REF	
Education level				
	Primary or secondary	1.231 (0.561–2.699)	2.891 (1.350–6.189)	*P* = 0.0210
	Collegial or university	REF	REF	
Residence close to (≤ 1 km) a highway during childhood (age ≤ 20 years)				
	Yes	0.053 (0.023–0.122)	0.172 (0.081–0.365)	*P* < 0.0001
	No	REF	REF	
Coal heating exposure during childhood (age ≤ 20 years)				
	Yes	0.193 (0.068–0.546)	0.923 (0.432–1.972)	*P* = 0.0045
	No	REF	REF	

ies against SQSTM1 (Cell signaling technology), GSTM3 (Abcam), GSTM4 (Proteintech), LC3B (Cell signaling technology), MT3 (Abcam), and anti-α-tubulin antibody (Cell signaling technology) as control. We used HRP-conjugated secondary anti-rabbit or anti-mouse antibodies (Cell signaling technology) to perform the detection with a chemiluminescent system. We quantified the protein expression by the use of the Molecular Imager Gel Doc XR and Imaging System.

2.8. Statistical analyses

Using multivariate logistic mixed model, exploration of potential associations between exposures to indoor or outdoor air pollutants were performed by comparing 140 affected participants (both familial and non-familial from), and 113 healthy unrelated controls. Only factors with a *P*-value < 0.10 in univariate analysis were included in the multivariate analysis and a backward algorithm was then performed. Factors with a *P*-value < 0.05 were retained in the final model. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated. Moreover, multinomial analyses to explore the associations between exposure to indoor or outdoor air pollutants and familial or non-familial PDB versus healthy controls were also performed, using the same selection algorithm as described above. Subsequently, we compared distribution of urinary heavy metal concentrations in patients versus controls by the use of Wilcoxon tests, because of the small sample size and urinary concentrations not being normally distributed. For the assessment of osteoclast phenotypes, ratio of multinucleated cells, consisting of actin positive cells with at least three nuclei per cell over all actin positive cells, mean nuclei number, and bone resorption abilities were compared between patients and healthy controls using Friedmann test and one-way ANOVA followed by Tukey's post hoc test. For gene expression analysis and oxidative stress measurement, the results were presented as ratios of values measured when toxic was added in the culture medium over the value measured without any exposure to the toxic. Statistical analysis relied on *t*-tests. Statistical analyses of protein expression analyses, performed by the use of GraphPadPrism, relied on ANOVA followed by Tukey post-tests. A *P*-value less than 0.05 was considered statistically significant.

2.9. Role of the funding source

The funding source has supported the salary of the interviewer and the master student who has worked on this project, as well as office expenses, all laboratory consumables to perform in vitro experiments, urinary toxic measurements and fees for biostatical analyses.

3. Results

We studied 140 patients with PDB (familial and non-familial forms) and 113 healthy controls from the French-Canadian cohort. The mean age of participants with PDB was 72.2 ± 8.1 years old and 70.1 ± 9.6 in the healthy control group (*P* = 0.0662). 50.7% of participants with PDB were male versus 24.8% in the healthy controls (*P* < 0.0001). 72.1% of participants with PDB had a low education level versus 53.1% of healthy controls (*P* = 0.0017). The main clinical characteristics of participants with PDB are summarized in the Appendix A, table S4.

3.1. Association of indoor and outdoor air pollutants with PDB

In univariate analyses, ninety percent (90%) of PDB patients were exposed to wood heating during childhood in comparison to 80.5% of healthy controls; OR = 2.396 (0.968–5.931), *P* = 0.0583 (Appendix A, table S5). In multivariate analysis, with correlation for intrafamilial relativeness and including sex as adjustment variables to avoid some bias, healthy individuals were more likely to have lived close to highway during childhood than patients with PDB; OR = 0.092, (0.044–0.191); *P* < 0.0001. Healthy controls were also more likely to have lived close to a bus, train or airport station during childhood (Appendix A, table S6). In univariate analyses considering familial and non-familial forms of PDB, 20.9% of patients with familial form and 42.3% of patients with non-familial form lived close to a highway during childhood in comparison to 78.8% of healthy controls, *P* < 0.0001 (Appendix A, table S7). In multivariate analysis without intrafamilial correlation and including sex and educational level as adjustment variables, coal heating at home during childhood was found to be more frequent in healthy controls than in patients with a familial form of PDB, but not significantly different from what was found in the non-familial form of PDB (Table 1).

3.2. Urinary measurements of heavy metals and polycyclic aromatic hydrocarbons

Lower levels of some heavy metals were detected in patients' urinary samples when compared to healthy controls: total arsenic (*P* = 0.0009), cesium (*P* = 0.0055), lead (*P* = 0.0031) (Appendix A, table S8). Total arsenic was lower in patients with familial form of PDB than in healthy controls and lowest levels were found in patients with non-familial form of PDB (*P* = 0.0026). Cesium uri-

Table 2
Comparison of urinary dosages of heavy metals and polycyclic aromatic hydrocarbons between patients with familial form of Paget's disease of bone (PDB), non-familial PDB, and healthy controls, non-parametric test (Wilcoxon) was applied (subgroup analysis). The extreme values have been excluded from the table.

Urinary dosage	Familial form of PDB			Healthy controls			*P*-value Wilcoxon test
	n	Mean (SD)	Median (Q1–Q3)	*n*	Mean (SD)	Median (Q1–Q3)	
Antimony	15	0.75 (0.85)	0.53 (0.20–0.95)	12	0.59 (0.49)	0.35 (0.20–0.89)	0.8805
Total Arsenic	45	0.18 (0.4)	0.09 (0.06–0.15)	48	0.24 (0.2)	0.15 (0.10–0.33)	0.0026
Beryllium		NA	NA		NA	NA	
Bismuth	15	0.80 (0.8)	0.63 (0.45–1.00)	12	0.56 (0.3)	0.49 (0.31–0.84)	0.4639
Cadmium	45	7.66 (16.6)	3.30 (2.30–6.60)	48	29.87 (157.9)	4.15 (2.95–8.70)	0.2375
Cesium	15	31.0 (16.3)	3.25 (2.20–6.55)	12	62.8 (28.6)	4.0 (2.9–8.5)	0.0025
Chrome	15	3.62 (2.07)	34.0 (13.0–49.0)	12	7.37 (9.8)	59.5 (45.0–81.0)	0.1912
Cobalt	15	6.1 (2.3)	3.0 (3.0–3.0)	12	6.06 (2.6)	3.0 (3.0–4.7)	0.9416
Copper	30	0.38 (0.34)	6.0 (4.6–8.0)	36	0.29 (0.4)	6.3 (4.5–7.4)	0.2552
Urinary density	45	1.015 (0.007)	0.25 (0.16–0.52)	48	1.017 (0.006)	0.20 (0.15–0.27)	0.1960
Tin	15	9.03 (6.3)	1.015 (1.010–1.020)	12	28.1 (39.6)	1.017 (1.014–1.021)	0.3929
Manganese	45	18.5 (21.2)	9.20 (3.9–14.0)	48	17.7 (26.4)	11.8 (3.7–30.5)	0.6895
Mercury	45	5.1 (6.5)	12.0 (6.6–20.0)	48	3.42 (3.0)	11.0 (6.85–16.0)	0.3218
Molybdenum	15	419.3 (279.2)	3.30 (1.90–5.60)	12	548.5 (381.5)	2.50 (1.75–4.55)	0.5258
Nickel	15	25.2 (18.9)	370 (210–570)	12	26.8 (21.4)	470 (305–625)	0.8450
Lead	45	0.0130 (0.0105)	21.0 (9.0–32.0)	48	0.0136 (0.0468)	25.5 (13.0–30.0)	0.0097
Selenium	15	0.654 (0.390)	0.010 (0.006–0.017)	12	0.6833 (0.2883)	0.006 (0.003–0.010)	0.6079
Zinc	30	9.19 (5.5)	0.56 (0.35–1.10)	36	7.68 (8.3)	0.72 (0.50–0.87)	0.1370
1- hydroxyphenanthrene	15	0.31 (0.2)	8.95 (4.50–12.0)	12	0.35 (0.4)	4.70 (1.85–9.05)	0.8262
1- hydroxypyrene	15	0.16 (0.2)	0.31 (0.13–0.49)	12	0.09 (0.1)	0.22 (0.10–0.46)	0.2319
1-Naphtol	15	11.2 (23.1)	0.10 (0.05–0.22)	12	3.9 (5.9)	0.06 (0.04–0.09)	0.1641
2-Hydroxyfluorene	15	0.71 (0.97)	3.80 (1.20–15.0)	12	0.49 (0.75)	1.30 (0.96–3.05)	0.7323
2-Hydroxyphenanthrene	15	0.13 (0.11)	0.36 (0.12–0.61)	12	0.12 (0.11)	0.30 (0.17–0.39)	0.6961
2-Naphtol	15	7.7 (11.9)	0.11 (0.05–0.23)	12	4.5 (7.3)	0.08 (0.05–0.15)	0.9222
3-Hydroxyfluorene	15	0.36 (0.60)	2.20 (0.58–11.0)	12	0.27 (0.64)	2.35 (1.80–2.85)	0.8451
3-Hydroxyphenanthrene	15	0.22 (0.20)	0.07 (0.04–0.18)	12	0.17 (0.20)	0.08 (0.05–0.13)	0.3929
4-Hydroxyphenanthrene	15	0.05 (0.04)	0.18 (0.07–0.31)	12	0.04 (0.05)	0.09 (0.05–0.23)	0.6603
9-Hydroxyfluorene	15	0.24 (0.19)	0.04 (0.01–0.08)	12	0.27 (0.19)	0.02 (0.01–0.06)	0.7144
9-Hydroxyphenanthrene	15	0.10 (0.09)	0.20 (0.09–0.36)	12	0.09 (0.12)	0.24 (0.10–0.38)	0.6253

nary concentration was lower in patients with familial form of PDB than in healthy controls ($P = 0.0025$). For lead, levels were the lowest in patients with non-familial form of PDB and the highest in healthy controls ($P = 0.0097$). A trend for a slight increase in bismuth, mercury and cadmium urinary concentrations was found in patients with PDB (Table 2). We did not find any statistically significant differences in polycyclic aromatic hydrocarbons urinary concentrations.

3.3. Osteoclast morphology and bone resorption abilities

In *p.Pro392Leu* mutation carriers, mean number of nuclei in mature osteoclasts without lead was higher than with lead; *P*-value = 0.0010 (Appendix A, Fig. S1). In three categories of cell cultures, there was a marked inhibitory effect of CSC on osteoclast formation and bone resorption activity. Osteoclast formation was higher in patients with non-familial PDB in the absence of tobacco than in its presence, *P*-value < 0.0001. A similar observation was made for the *p.Pro392Leu* mutation carrier. Mean number of nuclei per mature osteoclast was greater in the absence of tobacco among patients with non-familial PDB, $P < 0.0001$. Similarly, the mean number of nuclei was higher in *p.Pro392Leu* mutation carriers in the absence of tobacco, $P < 0.0001$ (Fig. 1). Cadmium was also found to have a marked inhibitory effect on osteoclastogenesis and bone resorption activity in all cell cultures. In the absence of cadmium, the ratio of multinucleated cells was higher in patients with non-familial PDB ($P \leq 0.001$). Mean number of nuclei was higher in patients with non-familial forms of PDB in the absence of cadmium, $P \leq 0.0001$ (Appendix A, Fig. S2). There was an inhibitory effect of mercury on osteoclast formation, on mean number of nuclei, and on bone resorption activities in both patients with PDB and healthy controls. A significant inhibitory effect of mercury was found on mean number of nuclei in mature osteoclasts from patients with non-familial PDB, $P < 0.0001$ (Appendix A, Fig. S3). Bismuth was found to have an inhibitory effect on osteoclast formation and bone resorption abilities both in patients with PDB and healthy controls. Ratio of mature osteoclasts was higher in the absence of bismuth in cells from patients with non-familial forms of PDB, $P \leq 0.0001$. Mean number of nuclei in mature osteoclasts was higher in the absence of bismuth in patients with non-familial PDB, $P \leq 0.05$. Bone resorption was also higher in the absence of bismuth; $P \leq 0.001$ (Fig. 2).

3.4. Gene expression analyses in osteoclasts

Gene expression of *MAP1LC3B (LC3)* was significantly downregulated in patients with PDB versus healthy controls after lead exposure. However, after cadmium exposure, *MAP1LC3B (LC3)* was significantly upregulated in patients with PDB versus healthy controls. In addition, *SQSTM1* expression was significantly upregulated in patients with PDB after cadmium exposure. *GSTM3* was significantly downregulated in PDB patients versus healthy controls after exposure to CSC (Fig. 3).

3.5. Impact of toxic on oxidative stress in osteoclasts

Ratio of oxidative stress was increased in patients' osteoclasts after exposure to all studied toxics, except for cadmium. The highest ratio of 8-Hydroxyguanosine (8-OHG) was found in healthy *p.Pro392Leu* mutated individuals, after exposure to CSC, which suggests a high level of induced oxidative stress in mutation carrier cells (Fig. 4).

3.6. Protein expression analyses by Western Blot

Statistically significant differences were found for SQSTM1 expression after exposure to bismuth ($P = 0.04$) and after exposure to CSC ($P = 0.03$), in particular when comparing osteoclasts from patients with PDB in the presence of tobacco versus healthy carriers

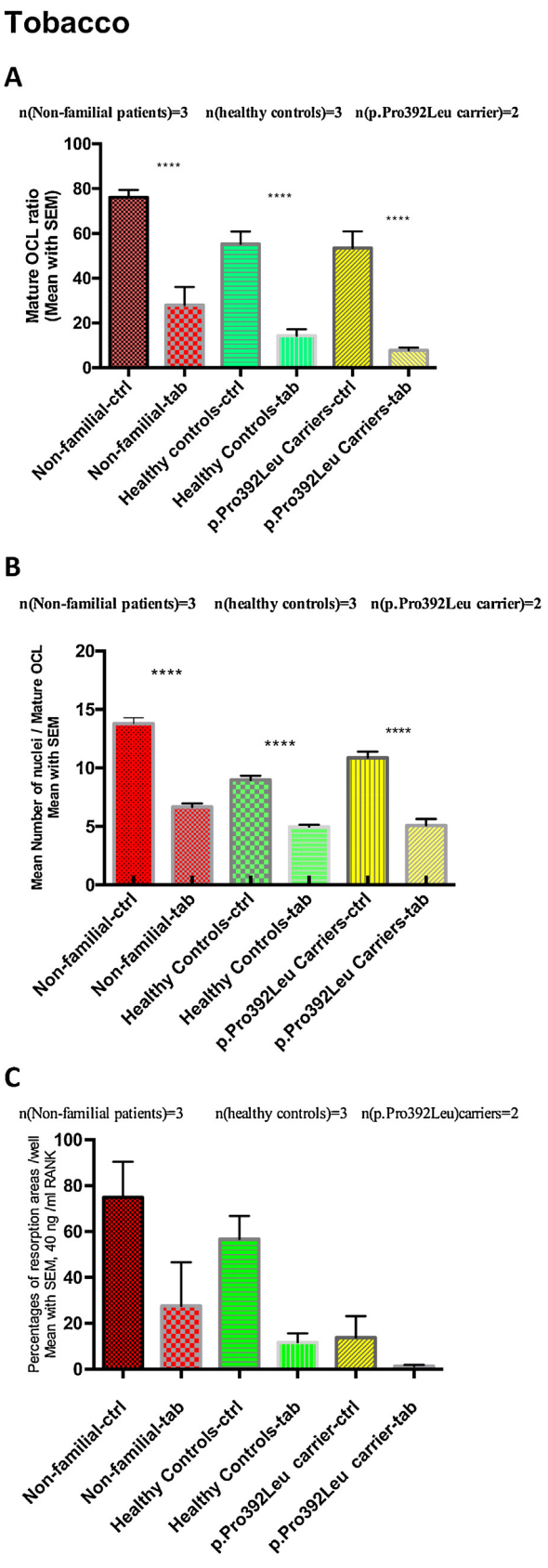

Fig. 1. Osteoclast formation and bone resorption activities in vitro in presence (tab) or absence (ctrl) of administration of tobacco smoke condensate. A. Ratio of multinucleated cells. B. Mean number of nuclei in 20 randomly chosen mature osteoclasts. C. Percentage of bone resorbed area. (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$).

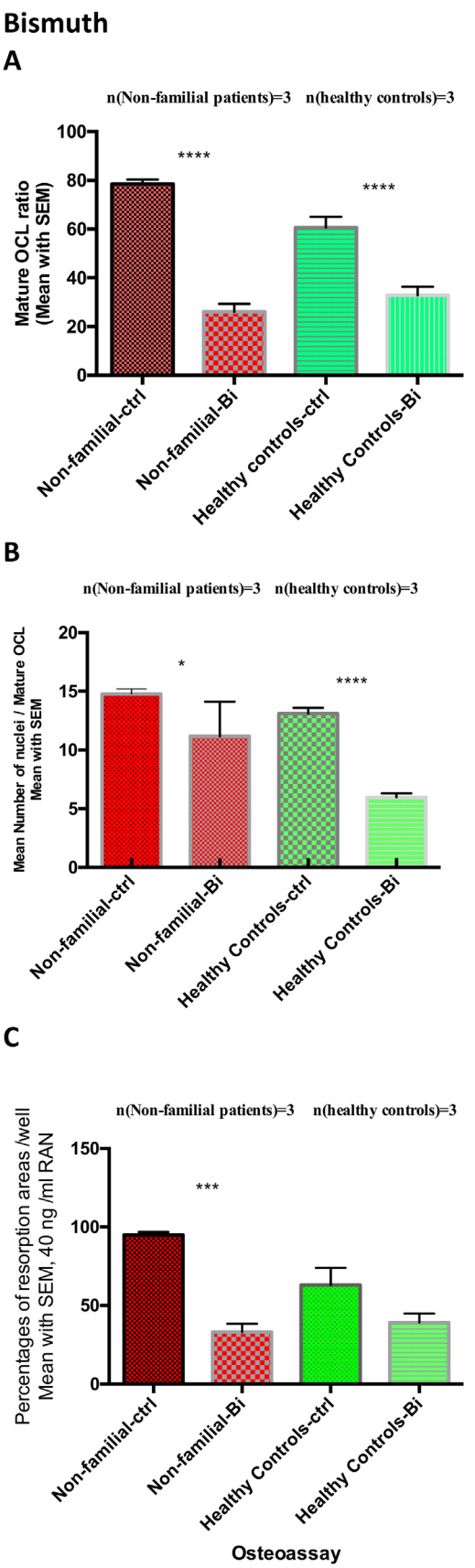

Fig. 2. Osteoclast formation and bone resorption activities in vitro in presence (Bi) or absence (ctrl) of bismuth. A. Ratio of multinucleated cells. B. Mean number of nuclei in 20 mature osteoclasts randomly chosen. C. Percentage of bone resorbed area (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$).

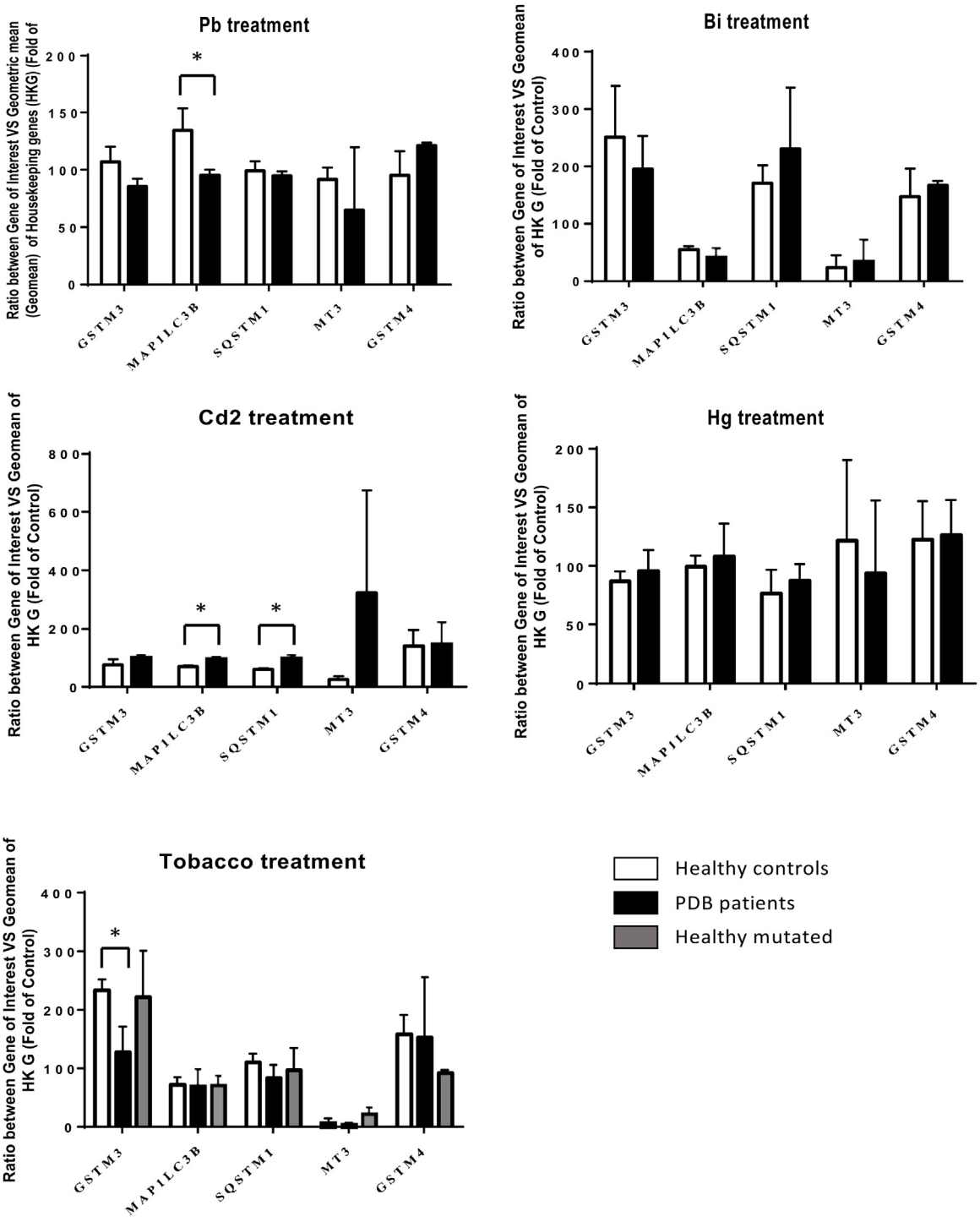

Fig. 3. Osteoclast gene expression analyses of candidate genes over housekeeping genes (HKG) standardized for the exposure to different toxics: lead (Pb), bismuth (Bi), cadmium (Cd2), mercury (Hg) and cigarette smoke condensates (tobacco). $^{*}P \leq 0.05$.

of *SQSTM1* mutation in the presence of tobacco ($P = 0.03$) (Appendix A, Fig. S4).

4. Discussion

In this exploratory study, outdoor air pollutants were more associated with healthy controls than with patients with PDB. Patients who were less exposed to urban outdoor air pollution displayed lower urinary levels of some heavy metals than healthy controls, and were more likely to develop both forms of PDB, familial and non-familial. These results are in accordance with the known association of PDB with rural residence, mainly reported in Italy [12] and Spain [11,23,28–30].

In vitro, some heavy metals and CSC were found to have an inhibitory effect on osteoclastogenesis and bone resorption in osteoclasts from both healthy controls and patients with PDB. Interestingly, *SQSTM1* expression was upregulated by cadmium in osteoclasts from patients with PDB versus healthy controls, and SQSTM1 expression was upregulated by bismuth and tobacco smoke condensates, in particular in the presence of *SQSTM1* mutation, suggesting a possible interaction between SQSTM1 and some

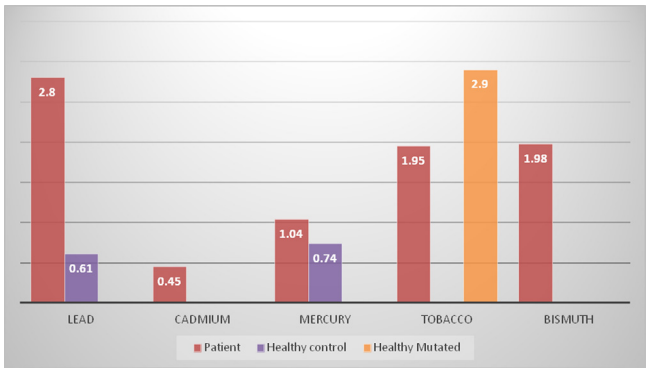

Fig. 4. Available data of the ratio of 8-Hydroxyguanosine (8-OHG), a marker of oxidative stress, in osteoclasts from patients with PDB, healthy controls and healthy carrier of *SQSTM1* mutation participants, after exposure to heavy metals or tobacco smoke condensate.

heavy metals and CSC. In addition, cadmium has significantly upregulated *LC3* and *SQSTM1* genes expression in patients with PDB. Lead may have a suppressive effect on macro-autophagy machinery by downregulating *LC3* gene expression in patients with PDB. The inhibitory effects of lead on osteoclastogenesis may be more related to its role on generating oxidative stress than macro-autophagy dysfunction. Furthermore, *GSTM3, SQSTM1, MT3* genes expressions were downregulated in patients' osteoclasts after treatment with tobacco smoke condensate. This downregulation of *GSTM3* and *MT3* genes may be an indicator of the negative effect of tobacco on the detoxification process and may predispose cells to several injuries by free radicals. The levels of oxidative stress were higher in patient's osteoclasts than in healthy controls, and the healthy carriers of *p.Pro392Leu* mutation had the highest levels of oxidative stress after exposure to CSC.

A previous study in our French-Canadian cohort showed a statistically significant association between wood fire heating during childhood and/or adolescence and PDB [19]. In the present study which investigated a subset of this cohort, this association was found only in univariate analysis, possibly due to the lower sample size. In addition to wood heating exposure, tobacco smoke was previously associated with PDB in a French cohort in which one third of families was linked to *SQSTM1* mutations [18]. Our observations of an upregulation of *SQSTM1* gene by cadmium and of SQSTM1 protein by tobacco in osteoclasts are very interesting, the interaction between tobacco and SQSTM1 being not restricted to bronchial cells [31].

The effect of tobacco smoke as a trigger to oxidative stress in normal human monocytes was also reported [32]. Here, we found a higher level of oxidative stress after exposure to CSC in osteoclasts in the presence of *SQSTM1* mutation. Deleterious effects of lead due to overproduction of free radicals and reactive oxygen species have been reported [33,34]. The cellular damage caused by lead may result of an increase in lipid peroxidation and a change in the composition of fatty acids, that negatively impacts the cell membrane functions, including signals transduction and transport mechanisms [34]. Lead can stimulate cellular oxidative stress [35]. Furthermore, the inflammatory response resulting from lead exposure was associated with polymorphisms in *GSTs* genes [35], but no evidence of association with *GSTM3* and *GSTM4* were found in our study. Cadmium stimulated the expression of genes related to the synthesis and regeneration of glutathione antioxidant, and was associated with *GSTM4* gene expression upregulation in mouse embryonic fibroblasts [36]. P62/SQSTM1 protein expression and mRNA expression were strongly upregulated by cadmium, sodium arsenite, and oxidants [37]. Here, we reported a significant upregulation of *SQSTM1* gene after cadmium exposure in osteoclasts from patients with non-familial PDB. Although we did not test cadmium exposure on familial PDB patients' osteoclasts due to the limited number of patients, further investigations should be performed to search for a possible association between cadmium exposure, particularly during childhood, and functional effect on *p.Pro392Leu* mutation. Nevertheless, cigarette smoke and one of its major components, cadmium, were associated with *GSTM3* gene expression and induced cellular oxidative stress in prostate cancer patients' cells [38]. Similarly, cadmium exposure was associated with the upregulation of *GSTM3*, *GSTM4* and *SQSTM1* genes. Association between oxidative stress induced by Nrf2 activation and *SQSTM1* gene expression was reported with exposure to some heavy metals such as nickel, chromium, and cadmium [39]. More efforts to investigate a potential association between *p.Pro392Leu* mutation and heavy metals and CSC are needed to better understand the potential gene-environment interactions in familial forms of PDB.

The number of participants, patients with PDB and healthy controls, in our survey was smaller than in our previous study in the French-Canadian cohort [19], which may represent a study limitation, although we still have more than 10 patients with PDB per studied variable in the model. The associations found in this study may be specific to the studied population, i.e. a founder effect population living in Nordic area conditions. In addition, since urinary samples were available only for a subset of participants from our biobank, the small number of participants may have limited our ability to detect statistically significant associations and did not allow us to determine when they were exposed. Other pollutants which have not been measured in the present study may have also contributed to the observed differences. Moreover, since we aimed at performing in vitro exploratory analyses with the exposure to four different heavy metals in addition to CSC, the number of patients and healthy controls for each exposure was limited. In addition, the low number of participant carrier of the *p.Pro392Leu* mutation also represents a study limitation. Although we performed an extensive review of the literature on the in vitro concentration of heavy metals that may mimic in vivo lifetime chronic exposure, we cannot ascertain whether the inhibitory effect on osteoclastogenesis and bone resorption activity of osteoclasts observed in our study results from an excess of heavy metal concentration.

Investigating in vitro the effect of different concentrations of heavy metals during osteoclastogenesis should be an interesting way to go forward, as well as measuring other markers of oxidative stress. Moreover, the effect of heavy metals and CSC in a more physiological bone microenvironment should be investigated in vitro by performing coculture experiments with osteoclast precursors and stromal cell lines. The interaction between wood heating, tobacco smoke, and *SQSTM1* gene, should be further investigated by in vitro and in vivo experiments.

Author's contributions

Study design: MSN, SJ, LM. Patient recruitment and acquisition of data: MSN, MD, EG, NA, JPB, LM. Analysis and interpretation of data: MSN, SJ, MD, EG, NA, JPB, LM. Revision of manuscript content (all authors). Approving final version of manuscript (all authors).

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

Mohamed Numan was supported by scholarships from the Réseau de santé bucco-dentaire et osseuse, from Louise-Côté

scholarship from Université Laval, and the Fondation du CHU de Québec. Dr. Michou was supported by a career award from the Fonds de recherche du Québec-santé (FRQ-S). This study was funded by Canadian Institutes for Health Research (catalyst grant 201304CEN), CHU de Québec Foundation, Canadian Foundation for Innovation, Fonds de recherche du Québec-santé, Université Laval and CHU de Québec-Université Laval Research Centre. The authors thank Mrs. Janette Dumont for performing telephone interviews with study participants and Mr Thomas Pornin for preparing the Appendix A, Fig. S4. The authors would also like to acknowledge the CHU de Québec Research Centre (CHUL) Gene Expression Platform, Quebec, Canada for the quantitative real-time PCR measurements, and Dr Alain LeBlanc from the toxicology centre at Institut national de santé publique du Québec for urinary measurements of toxics.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.jbspin.2018.12.007.

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