Histol Histopathol (2019) 34: 1141-1150

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Immunohistochemical expression and localization of MMP-9, MMP-13, E-Cadherin and Ki-67 in road pavers' skin chronically exposed to bitumen products

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Summary. To investigate the matrix metalloproteinase (MMP)-9, (MMP)-13, E-Cadherin and Ki-67 expressions in road pavers' skin chronically exposed to bitumen products in order to contribute to a better understanding of the earlier tissue alteration. Skin punch biopsies from 16 daily exposed workers and a control group were studied by immunohistochemistry. Morphometric and densitometric analyses were also conducted. Morphological specimen evaluation of skin of road pavers showed epidermal thinning, flattening and loss of intercellular junction with a decreased expression of E-cadherin confined to the basal skin layer, together with MMP-9 and MMP-13 overexpressions in all epidermis layers, vascular structures and adnexa. No immunohistochemical alteration was reported for Ki-67 vs normal skin. Results from this study show that overexpression of MMP-9 and MMP-13 may represent an early response of the first human barrier to exposure to bitumen products. Regulation of MMPs could be one of the strategies to prevent primary skin disease.

Key words; Cancer, Occupational exposure, PAHs, Matrix metalloproteinase, E-Cadherin, Ki-67, Workers

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DOI: 10.14670/HH-18-116

Introduction

The first interaction of the human body with the environment is represented by the skin that is regularly exposed to several external agents (ultraviolet radiation, temperature, chemical substances, etc.) which may cause epidermis and dermis alteration and secondary DNA injury, until skin cancer (IARC, 1987; Pittayapruek et al., 2016; Ledda et al., 2018a).

Several categories of workers are exposed to agents present in the outdoor environment (Rapisarda et al., 2015; Ledda et al., 2018b,c), among which there are road pavers, who are chronically exposed to numerous substances including asphalt fumes (Loreto et al., 2007; Rapisarda et al., 2009).

Asphalt, also known as *bitumen*, can generally be described as complex mixtures of hydrocarbons containing a large number of different chemical compounds of relatively high molecular weight and it contains polycyclic aromatic hydrocarbons (PAHs) that are part of the fumes emitted during handling of hot bitumen-containing products (Binet et al., 2002; IARC, 2013). Routes of exposure may occur via inhalation and/or skin absorption as PAHs may alter the skin hydrophilic film altering the barrier integrity by denaturation of keratin and other epidermal production, removal of superficial lipids surfactant transport to the upper epidermal layers, cell membrane damage and direct citotoxicity (Rougier et al., 1986; Froebe et al., 1990; Riala et al., 1998). Literature data reports an increased risk of skin cancers, genotoxic effects and development of skin tumors in mice after experimental

exposure to asphalt fumes (Machado et al., 1993; Sivak et al., 1997; Brandt et al., 2000). Apart from their carcinogenic potential, bitumen products may induce airway irritation (Raulf-Heimsoth et al., 2007), irritant and allergic contact dermatites in human (Riala et al., 1998), moderate hyperkeratosis and epidermal hyperplasia in rats (Poon et al., 1994). Workers chronically exposed to bitumen products may show thinning of the epidermal layer and flattening of the dermal papilla with loss of intercellular junctions and cell layers decrease (Loreto et al., 2007).

The International Agency for Research on Cancer (IARC) has demonstrated that bitumen contains mainly hydrocarbons, i.e. carbon, 79-88%; hydrogen, 7-13%; sulfur, traces up to 8%; oxygen, 2-8%; nitrogen, 3%; metals (such as vanadium and nickel) in parts per million (Speight, 2000). PAHs are a large group of chemicals with 2 to 7 fused aromatic rings (Kim et al., 2013). In particular, bitumen emissions tend to contain proportionally more two-ring PAHs, such as naphthalene and fewer five-ring PAHs, such as benzo[a]pyrene (B[a]P), than solid bitumen. B[a]P is one of the best-known PAH markers, categorized by IARC as carcinogenic to humans (group 1) (IARC 2010). Besides, B[a]P is commonly used as an indicator of PAH global concentrations in environmental monitoring; in fact, it has been found in many bitumen fume samples (IARC, 2010).

Experimental studies on the genotoxic activity of bitumen fumes showed contrasting results due to the different compositions of the bitumen samples, the temperature at which the fumes were generated and the experimental conditions (IARC, 2013).

Bitumen chronic exposure can cause several adverse health effects (Lutes et al., 1994), as a potential carcinogenic (Ledda et al., 2017, 2018a; Mundt et al., 2018). The main absorption pathways of PAHs are inhalation and dermal contact (Riala et al., 1998).

Epidemiological data show an increased risk of oral, stomach, lung and non-melanoma skin cancers (Brandt et al., 2000; Ledda et al., 2018a). In particular, IARC put bitumen and its emissions during road paving in Group 2B: possibly carcinogenic to humans (IARC, 2013).

Literature data showed the genotoxic and oxidative effects of PAH-related exposure to bitumen fumes (Machado et al., 1993; Binet et al., 2002; Mundt et al., 2018). Our working group already focused attention on the possible alterations of road pavers' skin exposed to these agents. Previously, we demonstrated an activation of programmed cell death and an overexpression of a cytokeratin pattern in workers' clinically healthy skin (Loreto et al., 2007; Rapisarda et al., 2009).

These molecular mechanisms are responsible for the activation of pathological processes that may at least determine the onset of pathology such as cancer (Loreto et al., 2007; Rapisarda et al., 2009).

A recent study has investigated the role of metalloproteinases (MMPs) in the onset of skin cancer (Pittayapruek et al., 2016). In particular, seems that they can regulate some processes related to tumor progression including tumor institution, growth, angiogenesis and metastasis (Pittayapruek et al., 2016).

MMPs are zinc-containing endopeptidases with an extensive range of substrate specificity (Leonardi et al., 2008, 2010; Quan et al., 2009). These enzymes are able to degrade various components of the extracellular matrix (ECM) proteins. To date, MMPs family includes at least 28 types of enzymes involved in various pathophysiological processes including tissue remodeling, inflammation, angiogenesis and cancer (García et al., 2006; Paur et al., 2011; Rundhaug et al., 2007). MMPs are enzymes capable of degrading all kinds of extracellular matrix proteins and also process a number of bioactive molecules (Leonardi et al., 2010). They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as FAS ligand) and chemokine/cytokine inactivation (Leonardi et al., 2007). MMPs are secreted by keratinocytes and dermal fibroblasts caused by various inductions (Quan et al., 2009; Sbardella et al., 2012). Depending on their structure and substrate specificity, they are classified in five groups: collagenases, gelatinases, stromelysins, matrilysins and membrane-type (MT) MMPs (Pittayapruek et al., 2016). Their activation produces alteration of ECM collagen fibers, fibronectin, elastin and proteoglycans (Pittayapruek et al., 2016).

MMP-13 is a collagenase that recognizes the substrate through a hemopenix-like domain and is able to degrade ECM fibrillary content (Bae et al., 2008). This proteinase shows higher cleavage specificity for type I, II and III collagen and is normally secreted by fibroblasts (Ciurea et al., 2013). MMP-13 may promote tumor skin angiogenesis, invasion and metastasis (Chu et al., 2007; Lederle et al., 2010a,b; Ciurea et al., 2013; Meides et al., 2014).

Furthermore, adherens junctions are a main of cell-cell adhesions system in keratinocytes. E-Cadherin, a 120-kDa transmembrane glycoprotein, is the constitutional component of adherens junctions (Papadavid et al., 2001). Its expression has been found abnormal in squamous cell carcinoma, thus the previously unreported analysis of this protein can be of interest in skin alteration induced by bitumen products (Lyakhovitsky et al., 2004; Vered et al., 2012).

Ki-67 is a well-known marker of cell proliferation, normally expressed in stratus basale of the epidermidis, which can exactly and rapidly determine the growth fraction of human cell population, regardless of whether it is normal or malignant (Papadavid et al., 2001).

In order to describe the process of alteration of the skin induced by bitumen fumes exposure we investigated on sixteen samples road pavers' skin chronically exposed to bitumen products vs unexposed workers, the immunohistochemical expression and localization of MMP-9 and MMP-13, E-Cadherin and Ki-67.

Material and methods

Subjects

Sixteen male road pavers directly exposed to bitumen were recruited. The control group was composed of 16 road workers, who had no previous history of occupational exposure to bitumen. Exposed and non exposed workers were matched for: gender, ethnicity, age, working age and Body Mass Index (BMI).

Inclusion criterion for exposed workers was working as road pavers for at least seven years, usually these employees are exposed to bitumen for 5-7h/day for five working days.

Exclusion criteria for both groups were: skin diseases, diabetes, coronary heart disease, cerebrovascular and peripheral vascular disorder, renal disease and use of drugs.

The study was performed in accordance with the guidelines of the Declaration of Helsinki, and the procedures were approved on the 29/04/2014 by the Ethical Committee of the University Hospital of Catania (Italy). Written informed consent to participate in the study was obtained from all subjects and a copy was given to all partecipants. The study was conducted in the framework of regular occupational medical visits both exposed and for non exposed workers performed at the University Hospital of Catania (Italy).

All subjects underwent medical check-up with dermatological examination and several haematological parameters were analysed according to standard methods (including haemoglobin, haematocrit, platelets, white blood-cell count, lymphocytes and neutrophils).

Determination of urinary 1-hydroxypyrene (1-OHP)

One spot urine sample was collected from each subject at the end of an 8-h shift, on day 6 of the working week (Rapisarda et al., 2009). Furthermore, a diet was prescribed in order to not affect our study results over the whole sampling period.

The well-validated PAHs exposure biomarker 1-hydroxypyrene (1-OHP) was measured to serve as an indirect exposure indicator (Ledda et al., 2018a). Urinary 1-OHP was determined by HPLC (Agilent Technologies, Santa Clara, California, USA) with the fluorescence detection method, using commercial laboratory kit (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany).

The 1-OHP levels were adjusted by urinary creatinine excretion and expressed as μg/L. The method limit of quantification was 0.1 μg/L.

Morphological observations

All sixteen subjects, under local anesthesia (Pliaglis cream Galderma, Italy), underwent a single punch skin biopsy of 3 mm diameter and 6 mm thickness from the forearm which was fixed in 10% buffered formalin for 24 hrs for morphological and immunohistochemical analysis. The site of the biopsy was selected by a dermatologist in the volar forearm of the right arm. They were processed as described previously (Loreto et al., 2007; Rapisarda et al., 2009). Briefly, tissues were dehydrated using the following sequence: 50% EtOH × 1 h, 70% EtOH × 1 h, 80% EtOH × 1.5 h, 95% EtOH × 12 h, 100% EtOH × 1 h-repeat twice, xylene × 1 h, placed in a metal mold and embedded in paraffin at 60°C overnight. Two serial sections were selected: one for morphological evaluation with hematoxylin and eosin staining, the other for immunohistochemical staining.

Immunohistochemistry

For immunohistochemistry, sections were processed as previously described (Musumeci et al., 2014, 2015). Briefly, sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Italy). High-temperature antigen unmasking was conducted in a microwave oven. A mouse monoclonal anti-MMP-13 (anti-collagenase 3) (NeoMarkers, Lab Vision, Fremont, CA, USA), a rabbit polyclonal anti-MMP-9 (Novus Biologicals, Littleton, CO, USA) were used at 1:100 working dilution, a mouse monoclonal anti-E-Cadherin (Dako Corporation, Glostrup, Denmark) was used at 1:75 working dilution, and a ready to use MIB-1, a mouse monoclonal antibody directed against the Ki-67 antigen (Dako Corporation, Glostrup, Denmark). After overnight incubation in a humidified chamber (4°C), sections were incubated with the secondary antibody; detection was performed with the Streptavidin-biotin method using 3,3'-diaminobenzidine (DAB) as chromogen (LSAB 2 System-HRP, Dako, Denmark). Sections were counterstained with haematoxylin and observed under an Axioplan (Zeiss, Germany) light microscope.

Positive controls consisted of tissue specimens with known antigenic positivity. Negative control sections were processed like the experimental slides except that they were incubated with preimmune rabbit serum instead of the primary antibody.

Evaluation of immunohistochemistry (IHC)

The antibodies-staining (MMP-9, MMP-13 and E-Cadherin) status was identified as either negative or positive. Immunohistochemical positive staining was defined by the presence of brown chromogen on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation with light microscope as previously described (Loreto et al., 2007; Rapisarda et al., 2009). Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at a protein level. Positive controls consisted of tissue specimens with known antigenic positivity. Sections treated with PBS without the primary antibodies served as negative

controls. Seven fields of the 16 samples, randomly selected from each section, were analyzed for morphometric and densitometric analysis. The percentage areas (morphometric analysis) stained with antibodies (MMP-9 and MMP-13 and E-Cadherin), expressed as % positive, dark brown pixels of the analyzed fields, and the level (high/low) of staining intensity of positive areas (densitometric analysis), expressed as densitometric count (pixel^2) of positive, dark brown pixels of the analyzed fields, were calculated using an image acquisition software (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

MIB-1, a monoclonal protein that identify Ki-67 protein in paraffine included tissue, represents a labeling index and was evaluated in the highest immunoreactivity fields. It was expressed as percentage and was determined by dividing the number of positive staining nuclei by 1000 tumor cells.

Statistical analysis

Data analysis was performed using SPSS software (Ver. 23.0, Chicago, USA). The data were summarized with the mean as a measure of central tendency and standard deviation as a measure of dispersion. All variables were normally distributed. The difference between two means was tested with unpaired Student's t-test. A p value of 0.05 was chosen as the limit of significance.

The graphs were made using Graph Pad Prism (Ver. 7, La Jolla, USA).

Results

All subjects enrolled in the study were current non-smokers for at least 6 months, being employed at least during the same period, had no history of chronic or recent illnesses and had not been taking any medication that could interfere with the study results.

None of the workers had a history of dermatological diseases. None used skin cream, oil or ointment or was taking any medications. Dermatological evaluation showed neither atopia nor skin infections. On visual examination the skin was intact.

Blood routine parameters (haemoglobin, haematocrit, platelets, white blood-cell count, lymphocytes and neutrophils) were in the normal range both for exposed subjects and control group (data not show). Table 1 shows the main demographics features of the exposed subjects and control group.

Urinary 1-hydroxypyrene

PAH exposure measured through urinary 1-OHP showed significantly greater ($P<0.01$) mean concentrations in road pavers than in controls (Table 1).

Histology

Hematoxylin & Eosin staining demonstrated an epidermal thinning and flattened derma papilla of the exposed specimens. Basal layer exhibited heterogeneous morphology of its constitutive cells. Suprabasal layers keratinocytes showed loosening of intercellular junctions and thus of normal intercellular relationships that could be translated in an alteration of the physiological cell flattening from the spinous to the granular layer (Fig. 1a). Non exposed skin samples demonstrated absence of histomorphological changes (Fig. 2b).

Immunohistochemistry (IHC)

The immunohistochemical analysis performed in bitumen exposed skin samples demonstrated a strong immunoexpression of both MMP-9 (Fig. 1b) and MMP-13 (Fig. 1c) in all the layers (from the basal to the corneum layer) of the epidermis including follicular adnexa, acrosyringium and vascular structures.

No immunoreaction was observed in the negative controls treated with PBS without the primary antibodies that exhibited only a melanic pigmentation of the stratum basale (Fig. 2b,c).

Conversely, bitumen exposed skin samples showed a low immunoexpression of E-Cadherin confined to the basal layer (Fig. 1d) respect to the physiological immunoexpression, found in all layers, of non exposed skin (Fig. 2d). As in normal skin (data not shown), Ki-67 immunoexpression was limited to the basal layer (Fig. 1e).

Densiometric analysis

Densitometric count (pixel^2) of stained areas by MMP-9, MMP-13 and E-Cadherin expressed by dark brown pixels of the analyzed fields demonstrated insignificant traces of immunoreactions in unexposed skin samples while in road pavers' skin sections high (red color) expressions were detected. All layers of the epithelium showed the same high immunoreactions with no different staining between MMP-9 and MMP-13. Statistical analysis showed that the difference between

Table 1. Demographics and urinary levels of 1-OHP of the control group and exposed subjects.

	Road pavers	Control	p - Value
Gender (Male)	16 (100%)	16 (100%)	n.s.
Age (yrs)	42.4±5.7	43.1±4.2	n.s.
BMI (kg/m^2)	22.7±2.1	23.1±3.3	n.s.
Working age (yrs)	14.3±3.4	14.6±4.1	n.s.
1-OHP (μg/L)	0.93±0.36	0.38±0.27	<0.01

MMP-9, MMP-13 and E-Cadherin expression in exposed skin tissue vs. non-exposed skin tissue was statistically significant ($p<0.05$) (Fig. 3).

Morphometric analysis

The percentages of MMP-9 and MMP-13 stained areas, expressed by dark brown pixels of the analyzed fields, were considered. From analysis of immunolabeling extension, the percentage of immunostained areas by both these MMPs was much higher in exposed skin compared with non-exposed ones ($p<0.05$). No differences in % of stained areas were observed between the basal, spinous, granulosus and corneum layers. Unexposed skin samples showed no immunostaining in any epidermal layers.

Discussion

The purpose of the present study was to investigate the process of alteration of the skin induced by bitumen products by analyzing the immunohistochemical expression and localization of MMP-9 and MMP-13, E-Cadherin and Ki-67.

Bitumen products may contain high levels of PAHs that for their lipophilic properties could alter the skin hydrophilic film and cause diseases (Riala et al., 1998). It has already been demonstrated that asphalt workers are subject to high PAH exposure/absorption through the skin despite wearing ad hoc protective clothing (Cirla et al., 2005; Cavallo et al., 2006).

1-OHP levels relevated in exposed workers are considered analogous to workers who perform similar

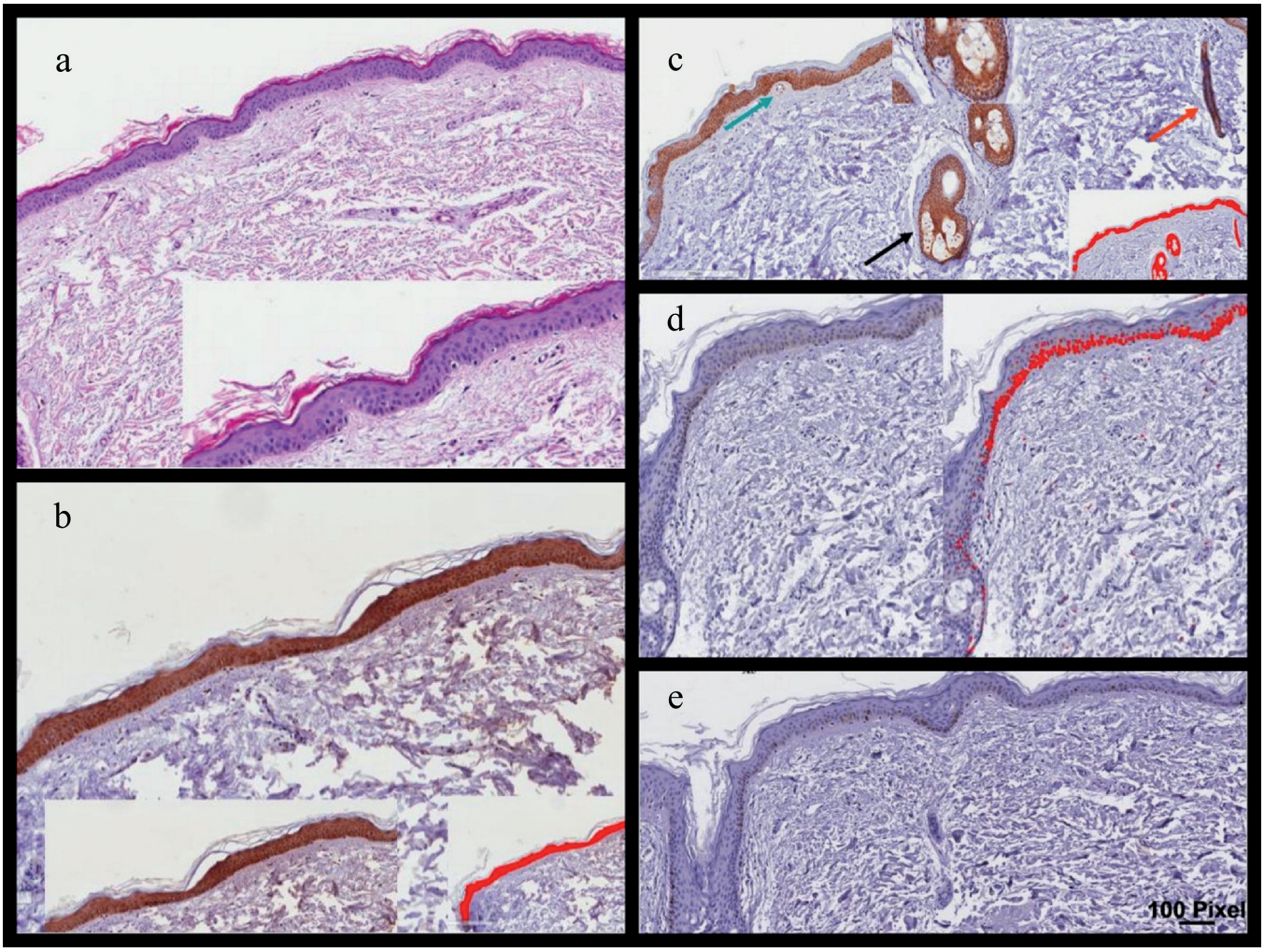

Fig. 1. a. H&E - Skin of bitumen exposed workers with high magnification insert. **b.** MMP-9 overexpression in skin of bitumen exposed workers with densitometric and high magnification inserts. **c.** MMP-13 overexpression in skin of bitumen exposed workers with densitometric and high magnification inserts. Green arrow showing vascular channel, red arrow showing acrosyringium and black arrow showing adnexa. **d.** E-cadherin underexpression in skin of bitumen exposed workers with densitometric insert and high. **e.** Ki-67 expression in skin of bitumen exposed workers. a-c, e, x 30; d, x 40.

job (Ledda et al., 2018a,b).

The results of the present study showed an overexpression of MMP-9 and MMP-13 in the basal and suprabasal epidermic layers, suggesting an activation of these enzymes towards bitumen exposure. Because of the histology evidence we decided to evaluate also E-Cadherin and analyze Ki-67 proliferative molecular expressions.

Immunohistochemical results confirmed alteration of cell-cell adhesion in epidermal skin of road pavers demonstrated by a decreased immunoexpression of E-Cadherin, while Ki-67 staining was confined to few basal keratinocytes as in normal squamous epithelial (Onoue et al., 2003).

Our results demonstrate that bitumen exposure may induce alteration of keratinocyte adhesion mediated by low expression of E-Cadherin. Literature data show that E-cadherin in keratinocytes leads to a loss of adherens junctions and altered epidermal differentiation without accompanying signs of inflammation (Young et al., 2003). At the same time they did not modify epidermal proliferation as demonstrated by basal immuno-localization of Ki67.

MMPs are also thought to play a major role in cell behavior such as cell proliferation, migration, (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defense (Joo and Seomun, 2008).

Physiologically, these enzymes act on ECM by regulating collagen degradation, together with their natural inhibitors (Tissue Inhibitor of Metallo-proteinases-TIMPs). An increase of MMPs activity is an important factor inducing alteration of the composition and distribution of all skin layers (Cavallo et al., 2006; Tewari et al., 2014). Chronic exposure to bitumen fumes, like ultraviolet (UV) radiation, may disrupt the normal skin structure leading to a host of skin issues

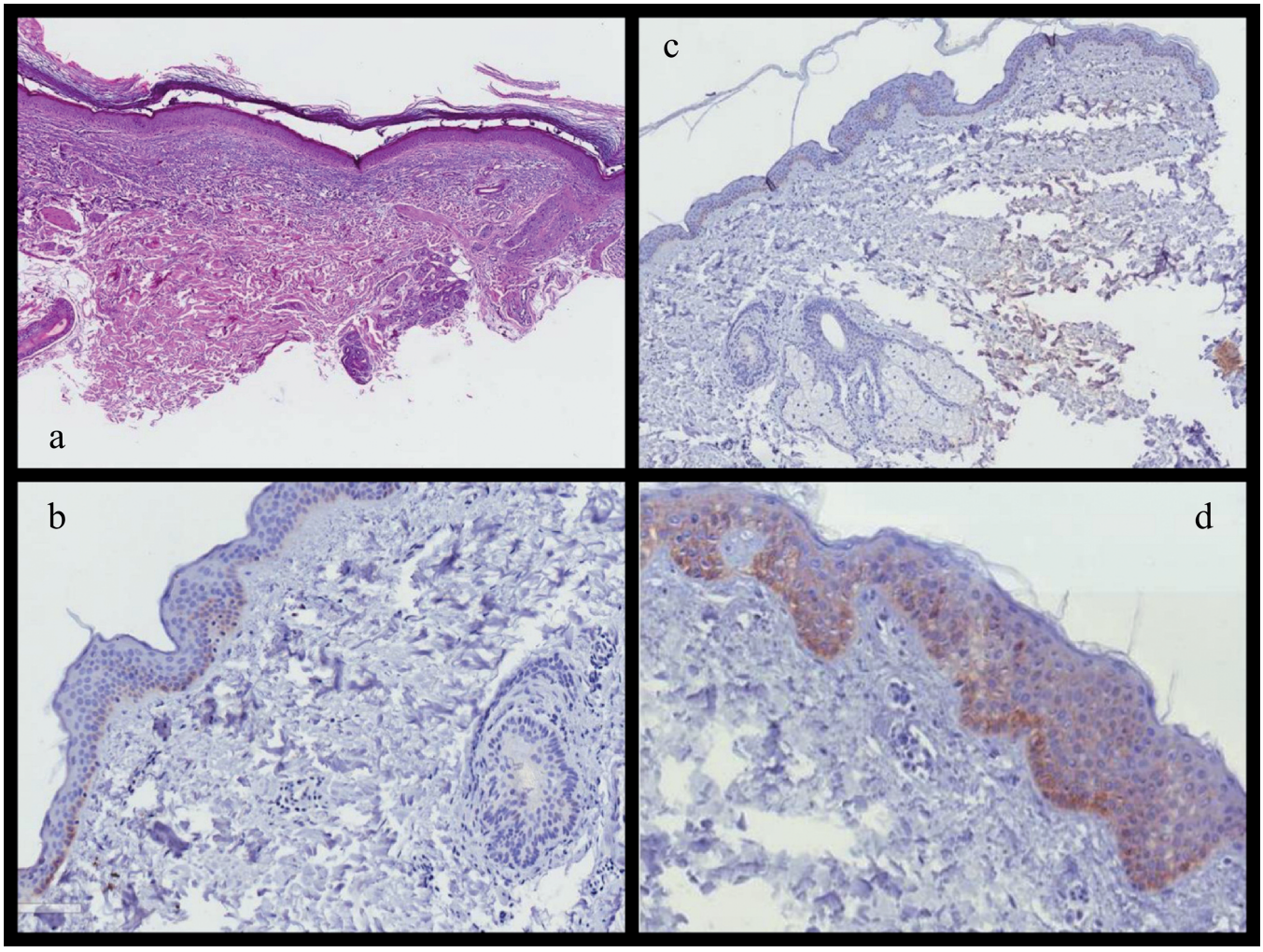

Fig. 2. a. H&E - Skin of bitumen non-exposed workers. **b.** MMP-9 overexpression in skin of bitumen non-exposed workers. **c.** MMP-13 overexpression in skin of bitumen non-exposed workers. **d.** E-cadherin expression in skin of bitumen non-exposed workers. a-c, x 30; d, x 40.

(Steinbrenner et al., 2003); thus, the overexpressions of MMP-9 and MMP-13 in skin of road pavers may play a crucial role by regulating/affecting various processes related to tumor institution, growth, angiogenesis and metastasis. Based on our results we speculate that MMPs may act initially as a defense mechanism against the exogenous stimuli but prolonged chemical exposure, together with activation of tumorigenic mechanisms, may promote tumor onset.

Several relations exist between the various MMPs, in particular MMP-13 seems to be upperregulated by a multifunctional growth factor called TGF-β that is activated by gelatinolytic enzymes such as MMP-9 (Williamson and Carughi, 2010; Murbach et al., 2015). In particular, MMP-9 is produced by human keratinocytes and digests ECM components such as collagen type I and IV (Chiang et al., 2013; Poswar et al., 2013), important constituents of skin basement membrane. Thereby, it is fundamental for epidermal adhesion, hence for epidermal integrity (Kim et al., 2012). Indeed, our evidence highlights that MMP-9 and MMP-13 were overexpressed in response to bitumen exposure (Ma et al., 2003).

Physiologically MMP-9 controls epidermal differentiation (Jung et al., 2010; Kim et al., 2012; Sbardella et al., 2012) and it is mostly secreted by inflammatory cells such as macrophages, neutrophils and mast cells rather than by tumor cells (Boyd et al., 2008; Hartmann-Petersen et al., 2009).

Overexpression of MMP-9 also induces secretion of angiogenic factors, playing a fundamental role in tumor skin cancer growth, proliferation and metastasis (Hofmann et al., 2005; Foda and Zucker, 2001).

The results of our previous studies showed that chronic exposure to bitumen fumes induces skin morphological alterations and activation of the apoptotic cell death as a defense mechanism against bitumen exposure (Loreto et al., 2007; Rapisarda et al., 2009, 2016). Bitumen exposure, like other environmental agents, may induce excess intracellular reactive oxygene species (ROS) that play as secondary messengers, activating the mitogen-activated protein kinase (MAPK) family (Rapisarda et al., 2015; Parrado et al., 2016). The latter in turn, apart from being strictly related to the apoptotic cascade, determines through the nuclear factor-kappa B (NF-κB), an enhancement of several MMPs expression (Xu et al., 2006; Youn et al., 2011). Regulation of MMPs is one of the strategies to prevent primary skin disease.

Immunohistochemical and morphological data suggest that although road pavers' skin was directly exposed to bitumen vapors and appeared clinically healthy, an MMP-9 and MMP-13 strong immunoreaction were detected; the immunolocalization was demonstrated in all epidermis layers and in vascular structures.

The role of these MMPs is fundamental for the maintenance of the basal membrane, intercellular junction and skin thickness; therefore their overexpression may be responsible for the histological modifications (such as loosening of intercellular junctions, with loss of the normal intercellular relationships) observed in the subjects studied. These data could be interpreted as an early response of the first human barrier to the exposure to bitumen products.

Recently, it has been proposed, for the prevention of skin photodamage, the use of plant-based ointments which act as protective agents through activation of tissue inhibitors of metalloproteinases (TIMPs) (Park et al., 2014). This strategy may be suggested also to workers exposed to bitumen fumes.

Further studies will be carried out, *in vivo* to investigate TIMPs and *in vitro* on human keratinocytes in order to assess the response to chemical bitumen products stimulation at different doses and times by analyzing MMPs immunocytochemical expressions and western blot quantifications. Furthermore, recently, there has been considerable interest in regulation of MMPs via microRNAs (Da Silva Melo et al., 2015). In particular, microRNA-mediated MMP regulation may lead to the

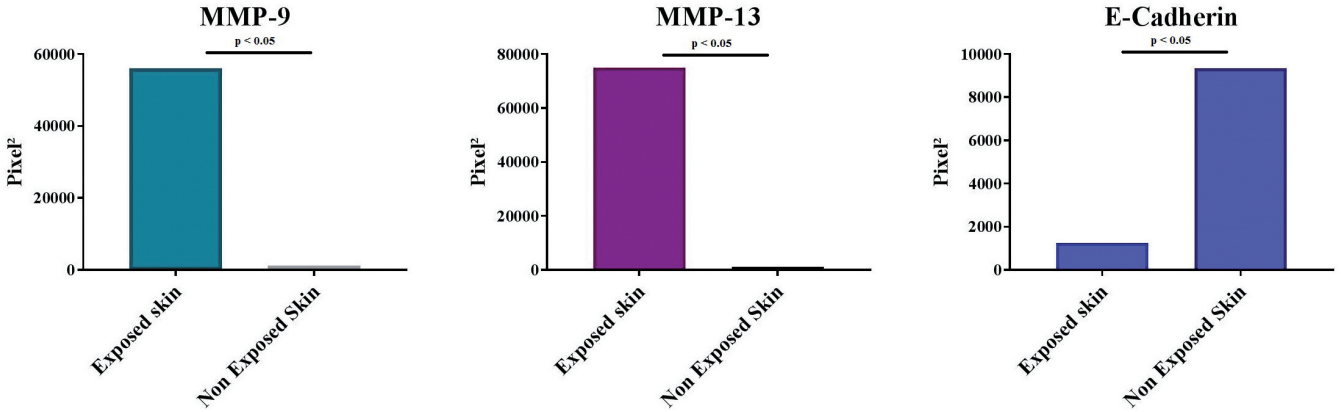

Fig. 3. MMP-9, MMP-13 and E-Cadherin densiometric analysis.

development of promising new MMP inhibitors that target MMPs more selectively.

Acknowledgements. The authors declare no conflict of interest. The authors have no support or funding to report. The participation of workers is this study is gratefully acknowledged.

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Accepted April 11, 2019