

# CXCL8 and CXCL11 chemokine secretion in dermal fibroblasts is differentially modulated by vanadium pentoxide

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Received November 1, 2017; Accepted April 16, 2018

DOI: 10.3892/mmr.2018.9121

**Abstract.** An increase in skin rashes or atopic dermatitis has been observed in individuals working with vanadium. However, to the best of our knowledge no *in vivo* or *in vitro* studies have evaluated the effect of exposure to vanadium in dermal fibroblasts. Cells viability and proliferation were assessed by WST-1 assay, cells were treated with increasing concentrations of V<sub>2</sub>O<sub>5</sub> (1, 10 and 100 nM). CXCL8 and CXCL11 concentrations were measured in the supernatants using an ELISA assay. V<sub>2</sub>O<sub>5</sub> was not observed as having a significant effect on dermal fibroblast's viability and proliferation. However, it was revealed that V<sub>2</sub>O<sub>5</sub> was able to induce the secretion of CXCL8 and CXCL11 chemokines into dermal fibroblasts. V<sub>2</sub>O<sub>5</sub> synergistically increased the effect of interferon (IFN)γ on CXCL11 secretion. In addition, V<sub>2</sub>O<sub>5</sub> synergistically increased the effect of the tumor necrosis factor α on CXCL8 secretion and abolished the inhibitory effect of IFNγ. V<sub>2</sub>O<sub>5</sub> induction of CXCL8 and CXCL11 chemokines may lead to the appearance and perpetuation of an inflammatory reaction into the dermal tissue. Further studies are required to evaluate dermal integrity and manifestations in subjects occupationally exposed, or living in polluted areas.

## Introduction

Vanadium is a grey metal that exists in different states of oxidation (ranging from -1 to +5) of which vanadium pentoxide (V<sub>2</sub>O<sub>5</sub>) is the most usual form.

All vanadium compounds have been considered toxic. The exposure limit to V<sub>2</sub>O<sub>5</sub> dust and fumes in workplace

air (8 h work day/40 h work week) has been fixed by the Occupational Safety and Health Administration in 0.05 and 0.1 mg/m<sup>3</sup>, respectively (1).

The National Institute for Occupational Safety and Health (NIOSH) sets to 35 mg/m<sup>3</sup> the dose of vanadium exposure that may cause seriously health issues up to death (1).

Toxic effects of vanadium are reflected mainly on respiratory system, while the effect on the gastrointestinal system is less relevant because of the minimal gut absorption rate of the substance (2-4). Unfortunately, no sufficient data are available in order to determine the reference range of a subchronic or chronic inhaled dose.

Studies conducted on rat models showed the toxic effects (resulted from an oral, or inhaled, vanadium exposures) on serum parameters (5,6), liver (7), nervous (8) and other tissues development (9).

Vanadium workers (NIOSH 1983) showed an increased prevalence of skin rashes, such as atopic dermatitis.

Until now no *in vivo*, or *in vitro*, studies were carried out to evaluate the effect of exposure to vanadium in dermal fibroblasts.

Here, we evaluate the effect of V<sub>2</sub>O<sub>5</sub> on viability and proliferation, and secretion of chemokine (C-X-C motif) ligand (CXCL)8, or CXCL11 [an interferon (IFN)γ dependent chemokine, of the same class of CXCL9, and CXCL10] in dermal human fibroblasts.

## Materials and methods

**Fibroblast cell cultures.** We have obtained fibroblasts from derma of six patients who underwent an operation for thyroid nodular goiter (discard dermal material; all females, age range 57-76 years, euthyroid, without other disorders or diseases, and not treated with any kind of drugs).

Involved subjects gave their informed consent and the study was approved by the University of Pisa (Pisa, Italy) Ethics Committee. Tissue explants were firstly minced and then placed in culture dishes, allowing the fibroblasts proliferation (as previously described) (10). Fibroblasts were propagated in 199 medium [with 20% FBS (Gibco; Thermo Fisher Scientific,

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**Key words:** vanadium, CXCL8, CXCL11, chemokines, dermal fibroblasts, environmental exposure, occupational exposure

Waltham, MA, USA), gentamycin (20 µg/ml), penicillin (100 U/ml)], in a 37°C humidified incubator with 5% CO<sub>2</sub>; and maintained subsequently in a 199 medium with 10% FBS (and antibiotics) (11). The cells were all used at the 4th passage, and were tested for purity by immunocytochemistry (12).

**Proliferation and viability.** We have done the WST-1 (Roche Diagnostics, Almere, The Netherlands) assay (that uses 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, in the MTT assay) to evaluate cell viability and proliferation (13-16).

Firstly fibroblasts were seeded in each well of 96-well plates at a concentration of 35,000 cells/ml (in a final volume of 100 µl).

Subsequently V<sub>2</sub>O<sub>5</sub> effect on fibroblasts viability and proliferation was determined exposing cells for 24 h with increased concentrations of the compound (1, 10, 100 nM).

Fibroblasts were plated and treated with V<sub>2</sub>O<sub>5</sub> or with its vehicle alone (for 24 h), performing all experiments in triplicate for each cell preparation.

As the cell viability and proliferation WST-1 assay may have limitations on evaluating cellular proliferation (17), fibroblasts proliferation was determined also by cell number counting (13-16).

**Chemokine secretion assay and ELISA.** To perform the CXCL8 and CXCL11 secretion assays, 30,000 cells/ml were seeded in 96-well plates, in a final volume of 100 µl per well, in growth medium, that was removed after 24 h. After cells were washed in PBS, and incubated (24 h) in phenol red and serum-free medium containing IFNγ (500, 1,000, 5,000, 10,000 IU/ml) and/or 10 ng/ml TNFα (all R&D Systems, Minneapolis, MN, USA), alone or in combination (10). The TNFα concentration to obtain the highest secretion was selected in preliminary experiments. After 1 day the supernatants were collected and then kept frozen at -20°C (until chemokine assay).

We treated fibroblasts, for 24 h, with increasing concentrations of V<sub>2</sub>O<sub>5</sub> (1, 10, 100 nM), in presence/absence of IFNγ (1,000 IU/ml), and/or TNFα (10 ng/ml), in order to evaluate the effect of V<sub>2</sub>O<sub>5</sub> on the chemokine secretion induced by IFNγ.

CXCL8 and CXCL11 concentrations were measured in the supernatants using the ELISA assay. The experiments were carried out three times, for each different cell preparation.

Chemokines levels were measured in culture supernatants, using commercially kits (R&D Systems). The mean minimum detectable dose was 2.7 pg/ml for CXCL8 and 3.2 pg/ml for CXCL11; the intra- and inter-assay coefficients of variation were 3.5 and 6.5% for CXCL8, 4.7 and 8.5% for CXCL11. Quality control pools of low, normal, or high concentration for all parameters were included in each assay.

**Statistical analysis.** For normally distributed variables values are given in text as mean (±SD), or mean (±SEM) in figures, otherwise as median [and interquartile range]. Mean group values are compared by one-way analysis of variance (ANOVA) for variables normally distributed, or with the Kruskal-Wallis test, or Mann-Whitney U test. Proportions are compared by the Chi-Square. We have used the Bonferroni-Dunn test for post hoc comparison of normally distributed variables.

## Results

**Cell proliferation of dermal fibroblasts.** Cell counting shows that V<sub>2</sub>O<sub>5</sub> (1, 10, 100 nM) does not change viability or proliferation of dermal fibroblasts (Fig. 1). The results of WST-1 assay in dermal fibroblasts with V<sub>2</sub>O<sub>5</sub> (1, 10, 100 nM) confirmed the cell counting data: with V<sub>2</sub>O<sub>5</sub> 1 nM it was 99% with respect to the control; with V<sub>2</sub>O<sub>5</sub> 10 nM it was 97% with respect to the control; and with V<sub>2</sub>O<sub>5</sub> 100 nM it was 98% with respect to the control.

**Fibroblast secretion of CXCL8.** In basal conditions, the secretion of CXCL8 (range, 51-213 pg/ml) was measured in all preparations of cultured dermal fibroblasts (Fig. 2).

CXCL8 secretion increased in a dose-dependent manner using different concentrations of TNFα (1, 5, 10 ng/ml), with the highest response reached with 10 ng/ml TNFα (basal 156±46 pg/ml vs. TNFα 1154±321 pg/ml; P<0.01) (Fig. 2).

The basal CXCL8 secretion was significantly inhibited by IFNγ in a dose-dependent manner (CXCL8: 84±37, 34±25, pg/ml; respectively, with IFNγ 500 or 1,000 IU/ml; ANOVA, P<0.05), while TNFα alone (10 ng/ml) significantly stimulated the CXCL8 secretion (P<0.01) (Fig. 3). Combining IFNγ with TNFα led to a significant reversal of the stimulating effect of TNFα (TNFα+IFNγ 661±176 pg/ml vs. TNFα 1154±321 pg/ml; P<0.05) (Fig. 3). However, the stimulating effect of TNFα on the secretion of CXCL8 was not completely reversed by IFNγ, because the concentration of this chemokine was still significantly higher than in basal conditions (TNFα+IFNγ vs. basal; P<0.01).

When fibroblasts were treated with increased V<sub>2</sub>O<sub>5</sub> concentrations (1, 10, 100 nM) the CXCL8 release was dose-dependently stimulated (P<0.0001, by ANOVA) (Fig. 4).

When treating dermal fibroblasts with V<sub>2</sub>O<sub>5</sub> (100 nM), together with IFNγ, CXCL8 release was not significantly changed with respect to the basal condition, and IFNγ suppressed the V<sub>2</sub>O<sub>5</sub> stimulating effect, but it stills increased it compared to IFNγ alone (Fig. 5).

V<sub>2</sub>O<sub>5</sub> (100 nM) *plus* TNFα elicited a synergistic effect on CXCL8 secretion (P<0.0001, by ANOVA), compared to TNFα alone (Fig. 6).

The CXCL8 release synergistically increased (P<0.0001, by ANOVA), when fibroblasts were treated with V<sub>2</sub>O<sub>5</sub> (100 nM) with the combination of IFNγ and TNFα, abolishing the inhibitory effect of IFNγ (Fig. 7).

**Fibroblast secretion of CXCL11.** CXCL11 release was induced by IFNγ in a dose-dependent manner (CXCL11: 0, 31±17, 87±35, 123±47, 187±52 pg/ml; respectively, with IFNγ 0, 500, 1,000, 5,000, 10,000 IU/ml; ANOVA, P<0.001).

TNFα alone had no effect (chemokine remaining undetectable), while the combination of IFNγ and TNFα had a significant synergistic effect on the CXCL11 secretion (CXCL11, 1724±252 vs. 87±35 pg/ml with IFNγ alone, ANOVA, P<0.0001).

When fibroblasts were treated with increased V<sub>2</sub>O<sub>5</sub> concentrations (1, 10, 100 nM) the CXCL11 release was dose-dependently stimulated (ANOVA, P<0.0001) (Fig. 8).

CXCL11 release was not significantly changed treating cells with V<sub>2</sub>O<sub>5</sub> (100 nM), together with TNFα, with respect to V<sub>2</sub>O<sub>5</sub> alone (data not shown).

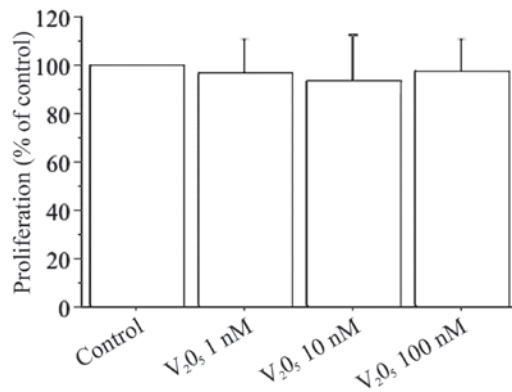


Figure 1. V<sub>2</sub>O<sub>5</sub> treatment does not significantly change the proliferation of dermal fibroblasts (mean group values are compared using one-way analysis of variance; the Bonferroni-Dunn test was used for post-hoc comparison; P>0.05, for all comparisons). V<sub>2</sub>O<sub>5</sub>, vanadium pentoxide.

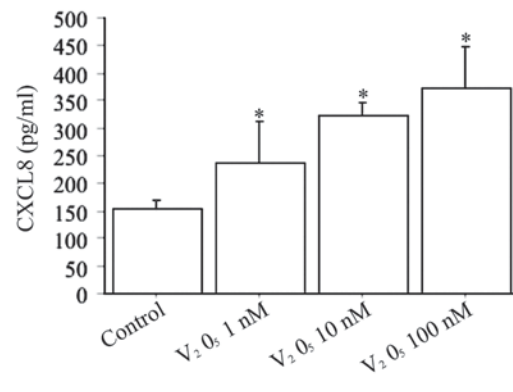


Figure 4. CXCL8 release was dose-dependently stimulated by treating dermal fibroblasts with V<sub>2</sub>O<sub>5</sub> (1, 10 and 100 nM). \*P<0.05 vs. control (the Bonferroni-Dunn test was used for post-hoc comparison). V<sub>2</sub>O<sub>5</sub>, vanadium pentoxide.

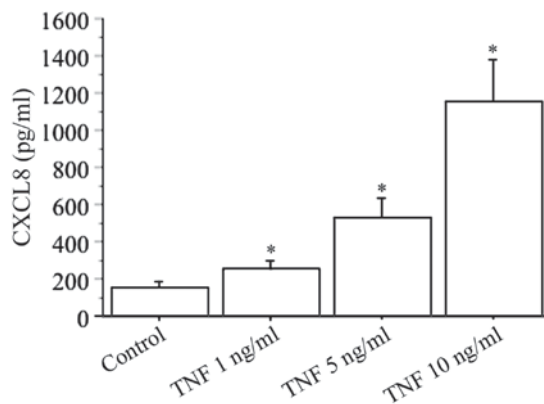


Figure 2. CXCL8 secretion was measured in all cultured dermal fibroblasts preparations in basal conditions (control); its secretion increased significantly and dose-dependently with different concentrations of TNF $\alpha$  (1, 5 and 10 ng/ml) (the Bonferroni-Dunn test was used for post-hoc comparison). \*P<0.05 vs. control. TNF, tumor necrosis factor.

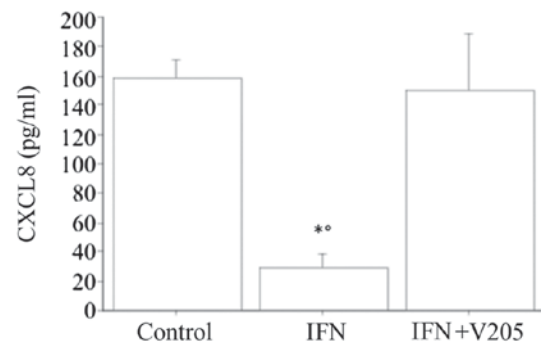


Figure 5. Treatment of dermal fibroblasts with V<sub>2</sub>O<sub>5</sub> (100 nM) abolished the inhibitory effect of IFN $\gamma$  (the Bonferroni-Dunn test was used for post-hoc comparison; \*P<0.05 vs. Control; °P<0.05 vs. IFN+V<sub>2</sub>O<sub>5</sub>). V<sub>2</sub>O<sub>5</sub>, vanadium pentoxide; IFN, interferon.

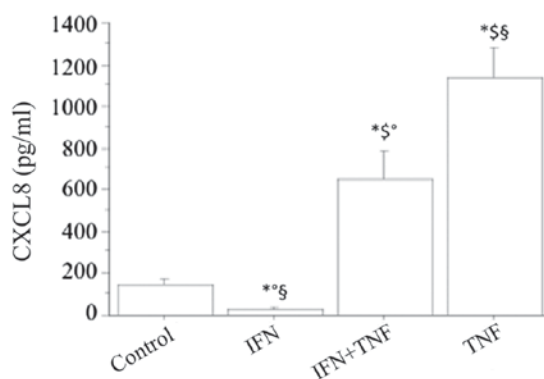


Figure 3. IFN $\gamma$  (1,000 IU/ml) significantly inhibited the basal CXCL8 secretion. The stimulating effect of TNF $\alpha$  was significantly reversed after the addition of IFN $\gamma$  (the Bonferroni-Dunn test was used for post-hoc comparison; °P<0.05 vs. Control; \*P<0.05 vs. TNF; §P<0.05 vs. IFN+TNF; §P<0.05 vs. IFN). IFN, interferon; TNF, tumor necrosis factor.

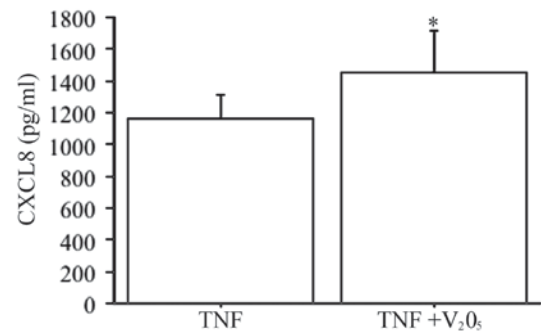


Figure 6. CXCL8 release was significantly increased by treating dermal fibroblasts with V<sub>2</sub>O<sub>5</sub> (100 nM) and TNF $\alpha$ . \*P<0.05 vs. the TNF group. V<sub>2</sub>O<sub>5</sub>, vanadium pentoxide; TNF, tumor necrosis factor.

When treating fibroblasts with V<sub>2</sub>O<sub>5</sub> (100 nM), *plus* IFN $\gamma$ , CXCL11 release synergistically increased (P<0.0001, by ANOVA), compared to both IFN or V<sub>2</sub>O<sub>5</sub> alone (Fig. 9).

CXCL11 release was synergistically increased (ANOVA, P<0.0001) when fibroblasts were treated with V<sub>2</sub>O<sub>5</sub> (100 nM), together with IFN $\gamma$  and TNF $\alpha$  stimulation, compared to IFN $\gamma$ +TNF $\alpha$  (Fig. 10).

## Discussion

Our results demonstrate that V<sub>2</sub>O<sub>5</sub> stimulates the secretion of the CXCL8 chemokine, and of the IFN $\gamma$  dependent chemokine CXCL11, in dermal fibroblasts, without altering their viability

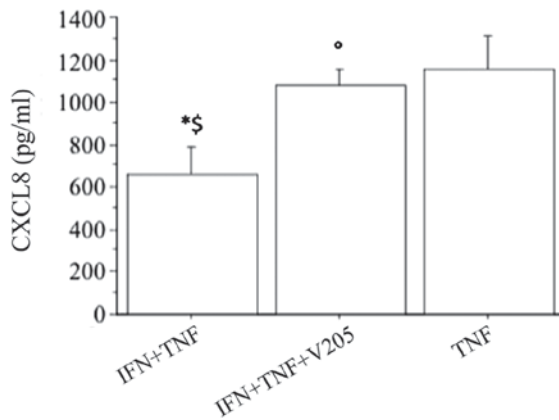


Figure 7. Treatment of dermal fibroblasts with  $V_2O_5$  (100 nM) + IFN $\gamma$  + TNF $\alpha$  stimulation significantly increased CXCL8 release (the Bonferroni-Dunn test was used for post-hoc comparison; \* $P < 0.05$  vs. IFN+TNF+ $V_2O_5$ ;  $^{\$}P < 0.05$  vs. TNF;  $^{\circ}P < 0.05$  vs. IFN+TNF).  $V_2O_5$ , vanadium pentoxide; TNF, tumor necrosis factor; IFN, interferon.

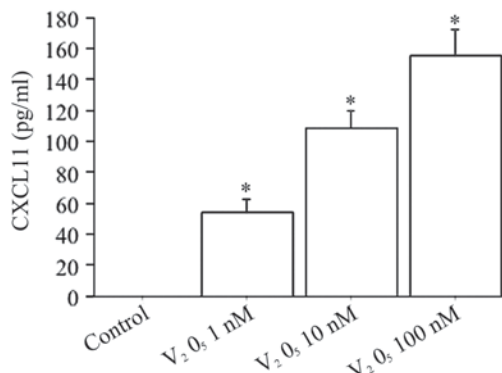


Figure 8. CXCL11 release was dose-dependently stimulated by treating dermal fibroblasts with  $V_2O_5$  (1, 10 and 100 nM). \* $P < 0.05$  vs. the control (the Bonferroni-Dunn test was used for post-hoc comparison).  $V_2O_5$ , vanadium pentoxide.

and proliferation. Moreover, our study confirms that IFN $\gamma$  and TNF $\alpha$  stimulated in a different way, the secretion of CXCL8, or CXCL11, chemokines as expected (18). Interestingly,  $V_2O_5$  can synergize with IFN $\gamma$  and TNF $\alpha$ , furtherly increasing CXCL11 secretion. In addition,  $V_2O_5$  combined with TNF $\alpha$ , elicited a synergistic influence on CXCL8 chemokine production, abolishing the inhibitory effect of IFN $\gamma$ .

These results, on the whole, agreed with the view that  $V_2O_5$  is able to induce and perpetuate an inflammatory disorder in the dermal tissue inducing inflammatory chemokines secretion (13).

Our findings regarding TNF $\alpha$ , and IFN $\gamma$  effect in fibroblasts are in line with the results of another study in a different type of cells. In fact, it has been recently investigated if CXCL8 and CXCL10 chemokines secretion by normal human thyrocytes is dependent upon specific proinflammatory stimuli. CXCL8, but not CXCL10 (an IFN $\gamma$  inducible chemokine, of the same class of CXCL11), was detected in basal conditions. The two chemokines showed differences in their response to proinflammatory cytokines.

Actually, IFN $\gamma$  induced a significant CXCL10 secretion, not obtained with TNF $\alpha$ ; whereas CXCL8 was secreted in response to TNF $\alpha$ , being instead inhibited by IFN $\gamma$ . The

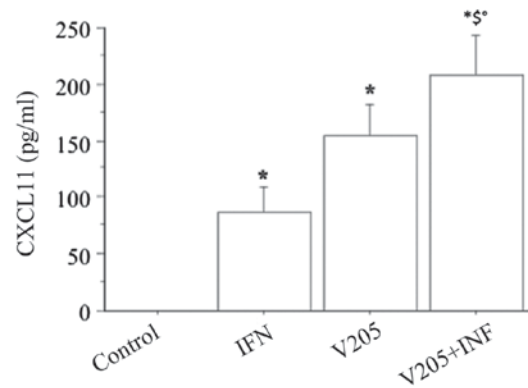


Figure 9. When dermal fibroblasts were treated with  $V_2O_5$  (100 nM) + IFN $\gamma$  CXCL11 release was significantly increased \* $P < 0.05$  vs. the control group.  $^{\$}P < 0.05$  vs. the IFN group.  $^{\circ}P < 0.05$  vs. the  $V_2O_5$  group (the Bonferroni-Dunn test was used for post-hoc comparison).  $V_2O_5$ , vanadium pentoxide; IFN, interferon.

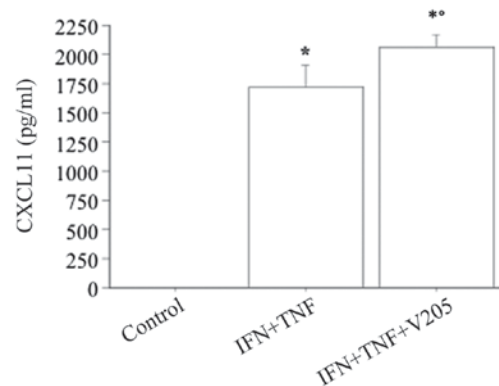


Figure 10. CXCL11 release was significantly increased by treating dermal fibroblasts with  $V_2O_5$  (100 nM) + IFN $\gamma$  and TNF $\alpha$  \* $P < 0.05$  vs. the control group.  $^{\circ}P < 0.05$  vs. the IFN+TNF group (the Bonferroni-Dunn test was used for post-hoc comparison).  $V_2O_5$ , vanadium pentoxide; TNF, tumor necrosis factor; IFN, interferon.

combination of TNF $\alpha$  plus IFN $\gamma$  synergistically increased the IFN $\gamma$ -induced CXCL10 secretion, while reversed the TNF $\alpha$ -induced CXCL8 secretion (19).

IFN $\gamma$ -inducible CXC chemokines can be produced by several types of normal mammalian cells, such as thyrocytes, fibroblasts, colon epithelial cells, islet cells, and others (10,13,14,19-25). However, these cells are not able to produce the CXC chemokines in basal condition, but only when stimulated by cytokines, such as IFN $\gamma$  and TNF $\alpha$ , that are released in a T-helper 1 (Th1) type inflammatory site, such as the thyroid at the beginning of Graves' disease, by Th1 activated lymphocytes. It has been suggested that this process can be involved in the initiation and the perpetuation of the inflammation in several autoimmune diseases (10,13,14,19-25), and considering our results it can be applied to the thyroid, too.

Our findings about vanadium stimulation of chemokines agree with those of other studies conducted in different cell types.  $V_2O_5$  exposure is a cause of occupational bronchitis; a study evaluated gene expression profiles in human lung fibroblasts (in cultures) after  $V_2O_5$  exposure with the aim to identify genes that could be implicated in the bronchial inflammation, repair, and fibrosis in the pathogenesis of bronchitis. Among



the 10 genes overexpressed by  $V_2O_5$ , also *CXCL8*, *CXCL9* and *CXCL10* were induced (26).

Another study reports that fibroblasts have a role in the innate immune response to vanadium-induced oxidative stress through the synthesis of  $IFN\beta$  and the activation of STAT-1 that cause an increase of *CXCL10* levels (27).

Interestingly vanadium can increase chemokine secretion in a dose range, from 1 to 100 nM. It could be observed that normal blood levels of vanadium are ranging from 0.45 to 18.4 nM, and that 100 nM is a dose that might mimic an abnormally high exposure (28). So we could hypothesize that the induction of an inflammatory reaction into the dermal tissue could predispose to the appearance of skin rashes, or atopic dermatitis.

In conclusion our study shows that  $V_2O_5$  can induce *CXCL8*, and *CXCL11* chemokines secretion into the dermal fibroblasts. Interestingly,  $V_2O_5$  synergistically increased the effect of the  $IFN\gamma$  on *CXCL11* secretion. Moreover,  $V_2O_5$  synergistically increased the effect of the  $TNF\alpha$  on *CXCL8* secretion, abolishing the inhibitory effect of  $IFN\gamma$ . Overall *CXCL8*, and *CXCL11* chemokines induction by  $V_2O_5$  could lead to the appearance and perpetuation of an inflammatory reaction into the dermal tissue. Further studies are needed to evaluate dermal integrity, and manifestations in subjects occupationally exposed, or living in polluted areas.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

PF, SB, AA and SMF made substantial contributions to the conception and design of the study and to the acquisition of data. All authors analyzed the data. PF, SB, AA and SMF drafted the manuscript. AA revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Written informed consent was obtained from all study participants and the study was approved by the University of Pisa Ethics Committee.

## Consent for publication

Written informed consent was obtained from all participants for the publication of their data.

## Competing interests

The authors declare that they have no competing interests.

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