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Study of the comparative expression of CXCL9, CXCL10 and IFNγ in vitiligo and alopecia areata patients

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Dear Editor,

Vitiligo and Alopecia areata (AA) are autoimmune T cell–mediated diseases of the skin that share the implication of the IFNγ pathway in their pathophysiology. C-X-C motif chemokine receptor 3 (CXCR3) and its ligands, C-X-C motif chemokine ligand 9 (CXCL9), CXCL10

and CXCL11, are linked to the Th1 pattern and have been suggested as one of the most relevant chemokine axes that promote T cell migration in different autoimmune and inflammatory processes (Lacotte et al., 2009). Chemokines play an important role in regulating the homing of immune cells (Vazirinejad et al., 2014). CXCL10 and CXCR3 have been shown to be elevated in vitiligo patients with an increased expression in active phase (Rashighi et al., 2014; Wang et al., 2016). However studies have assessed their level in serum or only on active borders of vitiligo lesions that are known to be the elective area of the CD8+ infiltrate. Recent transcriptome analysis of vitiligo patients compared to controls corroborates these results with an increased expression of CXL10 in non lesional and perilesional skin compared to controls but surprisingly no significant increase of CXCL10 expression was observed in already depigmented skin (Regazzetti et al., 2015). In this context, we investigated the respective serum levels of CXCL9 and CXCL10 in vitiligo patients compared to AA and controls and molecular expressions of CXCL10 and IFNγ in lesional, perilesional and non-lesional in clinically active or non-active vitiligos compared to controls.

This cross-sectional study included 15 patients with vitiligo, 15 patients with AA attending the dermatology outpatient department of Fattouma Bourguiba hospital of Monastir in Tunisia. Controls were 15 healthy subjects matched for their age and sex. Written informed consent was obtained from all patients. The study protocol, patient information sheet and consent form were approved by the Institutional Ethics Committee. A detailed history and clinical examination was recorded for each patient. Heparinized blood samples were obtained from all study subjects and were objected to ELISA measures. The expressions of serum CXCL9 and CXCL10 were measured by Human Chemokine kits (Peprotech, France) according to the manufacturer's instruction. Expression of CXCL10 and IFNγ mRNA in skin

samples of our subjects was studied using quantitative real-time reverse transcriptase-PCR on lesional, peri-lesional and non-lesional skin from 6 vitiligos and healthy skin from 5 controls matched for age, sex and location. Real-time PCR was conducted with complimentary DNA (cDNA), according to manufacturer recommendations. Gene expression is reported relative to the average expression in control after normalization to expression of GAPDH. The characteristics of the study population are summarized in Table S1. To assess the clinical relevance of serum CXCL9 and CXCL10 in vitiligo and AA patients, we calculated Vitiligo Area Severity Index (VASI) (Hamzavi et al., 2004) and Severity of ALopecia Tool score (SALT) (Olsen et al., 2004) scores, respectively. SPSS version 20.0 was used to analyze the results. Student's t-test and Pearson correlation test were used for Means comparison test. Odds ratios and their 95% confidence interval (CI) were determined. $p<0.05$ was considered statistically significant. Additional methodological information is provided in Methods S1.

CXCL10 serum level was significantly elevated in vitiligo and in AA patients compared to healthy controls ($p>0.05$ in both cases), but was not significantly different between vitiligo and AA patients. CXCL9 was slightly but significantly elevated in vitiligo patients compared to controls ($p<0.05$). The CXCL9 level was significantly higher in AA compared to controls ($p<1.5\ 10^{-4}$) and to vitiligo ($p<0.026$) (Figure 1 A-B). We next compared these concentrations between patients in progressive and stable stages of vitiligo and found that the expression levels of both serum CXCL9 and CXCL10 were higher in patients in the progressive stage than in those in the stable stage, suggesting a mechanistic role of these chemokines in vitiligo (Table S2). We did not found any correlation between the serum level of CXCL10 and CXCL9 and the VASI and the SALT scores. CXCL10 and IFNγ mRNA expression was significantly higher in non-lesional and perilesional skin compared to healthy controls; however, the level of expression of CXCL10 and IFNγ in depigmented vitiligo lesions was

not different than in healthy skin (Figure 1 C-D). Interestingly, the relative mRNA expression of CXCL10 and more notably of IFNγ was higher in the non lesional skin of active vitiligo patients compared to the skin of stable patients (active patients 12.77 / stable 9.10 and active 16.59 / stable 2.41 for CXCL10 and IFNγ, respectively).

Rashaghi et al. found elevated CXCL10 levels in vitiligo patients, whereas CXCL9 was not significantly different from healthy controls (Rashighi et al., 2014). At the contrary, Wang et al; found an increase of both CXCL9 and CXCL10 in vitiligo patients (Wang et al., 2016). Previous studies have found an increased level of CXCL9 and CXCL10 in the serum (Zainodini et al., 2013) or by the mRNA expression in the skin in AA patients compared to controls (Subramanya et al., 2010; Xing et al., 2014). In our series CXCL9 was significantly increased in vitiligo patients compared to healthy controls but the difference was less pronounced compared to the one observed for CXCL10. At the contrary, CXCL9 was markedly increase in AA. These results further support the mechanistic role of the chemokines CXCL10 and CXCL9 in vitiligo and AA, respectively. The different mechanistic role of CXCL9 and CXCL10 in vitiligo was recently supported using a mouse vitiligo model showing that CXCL9 and CXCL10 expression correlate with disease activity, whereas CXCL10 alone correlates with severity (Richmond et al., 2016). IFNγ-dependent chemokines induce T cell homing into peripheral tissues and endothelial adhesion molecules and promote transmigration into tissues (Groom and Luster, 2011). Here, we show that CXCL10 and IFNγ expressions are increased within perilesional skin of vitiligo patients compared to healthy controls but that their expression is even higher in non-lesional skin. At the contrary, they are not increased in already depigmented skin of vitiligo patients. Previous findings suggest that T cells infiltrate the skin during vitiligo and localize to the epidermis where melanocytes reside. In lesional skin, CD8+ T cells are found in close proximity to dying melanocytes

(Rashighi et al., 2014). IFNγ plays a key role in attracting and activating CD8+ T cells (Yang et al., 2015). Our *in situ* analysis of CXCL10 and IFNγ expression reveals that these cytokines are mostly increased in vitiligo skin before the depigmentation occurs with highest levels observed in active vitiligos. This increase of CXCL10 and IFNγ in non lesional skin of vitiligo patients suggests that it is an early event that further attracts the CD8+ cells and ultimately lead to the destruction of melanocytes. This also suggests that targeting these cytokines and chemokines would be of great interest for preventing further depigmentation. The absence of increase of CXCL10 and IFNγ in already depigmented skin compared to control skin also shows that in these areas the therapeutic strategy should probably focus more on promoting the differentiation and the proliferation of melanocyte stem cells. To this respect activating the WNT pathway had been recently showed to be a key target (Regazzetti et al., 2015). To conclude, our results emphasize the key role of IFNγ as the trigger of the immune infiltrate before any depigmentation occurs. The keratinocytes were recently reported to produce CXCL9 and CXCL10 and thus able to attract CD8+ cells (Richmond et al., 2016). The initial source of the production of IFNγ within vitiligo skin remains unknown. Determining the origin of this increased production should provide crucial clues on the early events occurring during vitiligo pathogenesis.

Conflict of interest

None

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Figure legends

Figure 1: Expression of IFNγ, CXCL9 and CXCL10 in vitiligo compared to alopecia areata and controls

The serum levels CXCL10 (**A**) and CXCL9 (**B**) of vitiligo patients are compared with alopecia areata patients and with healthy controls.

The relative mRNA expression of CXCL10 (**C**) and IFNγ (**D**) in lesional, peri-lesional and unaffected skin of vitiligo patients is compared to the expression in controls matched for age, sex and localization.

HS: Healthy subject, LS: lesional skin, PLS: peri-lesional skin, NLS: non lesional skin.

NS: non-significant, * $p<0.05$, ** $p<0.001$

Figure 1