Increased Activation of Innate Immunity and Pro-Apoptotic CXCR3B in Normal-Appearing Skin on the Lesional Site of Patients with Segmental Vitiligo

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TO THE EDITOR

The mechanisms involved in the pathogenesis of segmental vitiligo (SV) are poorly understood. Owing to its characteristic asymmetric unilateral distribution of depigmentation, proposed hypotheses include melanocytes and/or keratinocytes carrying mosaic mutation and being intrinsically abnormal and/or more susceptible to stress. Moreover, a subclinical inflammatory response is now well-demonstrated also in segmental forms of vitiligo (Speeckaert et al., 2020) and the involvement of $CD8^+$ melanocyte-specific T-cell–mediated immunity as is observed in generalized vitiligo (van Geel et al., 2010). We have recently shown the importance of innate immunity in triggering early immune responses against melanocytes in generalized vitiligo and have shown a role of a newly discovered chemokine receptor on melanocytes, CXCR3B, in triggering melanocyte death and subsequent T-cell autoreactivity (Tulic et al., 2019). There are increasing data emphasizing the key role of innate immunity, especially in the initial steps and the flares of vitiligo (Boniface et al., 2021). However, the role of innate immunity and of CXCR3B in SV has not been previously studied. The objective of this study was to better understand the mechanisms involved in SV and to assess the potential role of innate immunity in this subtype of the disease.

Five adult patients with SV affecting a location outside the face were included. All the patients exhibited disease onset >3 years before, and none had active disease at the time of inclusion. From each patient with vitiligo, we obtained two pigmented 4-mm skin punch biopsies. One was from the lesional (L) or segmental site not yet depigmented and the other from the nonlesional (NL) contralateral site where no lesions were apparent. We also took one biopsy from healthy patients (Supplementary Figure S1). The groups were matched for sex, age, and biopsy location (Supplementary Table S1). Each biopsy was cut in half, one for qPCR analysis and the other half for immunofluorescence. The study was approved by the National ethics committee (N12.034). Methods are detailed in the Supplementary Materials and Methods. The raw data that support the findings of this study are available from the corresponding author on reasonable request.

We first measured the mRNA expression of *IFNg*, *CXCL9*, and *CXCL10* chemokines, which are implicated in the recruitment of T cells in vitiligo (Maouia et al., 2017; Rashighi et al., 2014). Our results show increased expression of all markers in both NL and L skin of patients with SV compared with the expression of those in healthy controls **(**Figure 1a–c**).** We then assessed the markers of extracellular matrix disruption implicated in melanocyte loss in SV and non-SV (NSV), matrix metalloproteinase 9 and E-cadherin (Boukhedouni et al., 2020). Our results show a significant increase in metalloprotease 9 and a loss of E-cadherin in both NL and L sites of patients with SV compared with those of the healthy controls (Figure 1d and e). Together, these results suggest that in SV, changes in innate and adaptative immunity and chemokine expression and changes in skin integrity are not only increased in L but also in normal-appearing NL skin of patients with SV. These early events precede depigmentation and are similar to early innate mechanisms we recently described in NSV (Tulic et al., 2019).

Heightened innate immunity may be induced by local damage-associated molecular pattern–induced stress/inflammation in the skin of these patients (van Geel et al., 2012). To try and understand the mechanisms of increased immunity in SV skin, we measured the targets of cellular stress and damage, including CXCL-16 (produced by stressed keratinocytes, which induce the migration of $CXCR6^+CD8^+$ T cells derived from patients with vitiligo) (Li et al., 2017), inducible HSP70 (well-known stress mediator in melanocytes) (Mosenson et al., 2014), and mtDNA, as a marker of cellular damage (Vaseghi et al., 2017). Our results have shown a significant increase in *CXCL16* mRNA (Figure 1f) and the number of inducible HSP70-positive (Figure 1g) and mtDNA-positive (Figure 1h) cells in the L skin of patients with SV. This increase was not seen on the NL site. To examine whether there is an inherent default in melanocyte function from L sites of patients with SV, which may explain their increased responses to chemokines, we set out to examine their CXCR3B expression. We found a significant increase in *CXCR3B* mRNA levels in both NL and L sites of SV skin (Figure 2a) and a significantly increased number of CXCR3B-positive melanocytes in the L but not in the NL sites of patients compared with those of the healthy controls (Figure 2b and c).

Altogether, these data emphasize that patients with SV have altered expression of the key proteins involved in vitiligo pathogenesis, not only in their L site but also in the skin located outside their segmental zone. This general involvement of the skin might explain the significantly increased risk (approximatively 10 times) of developing NSV in patients affected by SV (Ezzedine et al., 2011). However,

Abbreviations: L, lesional; NL, nonlesional; NSV, nonsegmental vitiligo; SV, segmental vitiligo

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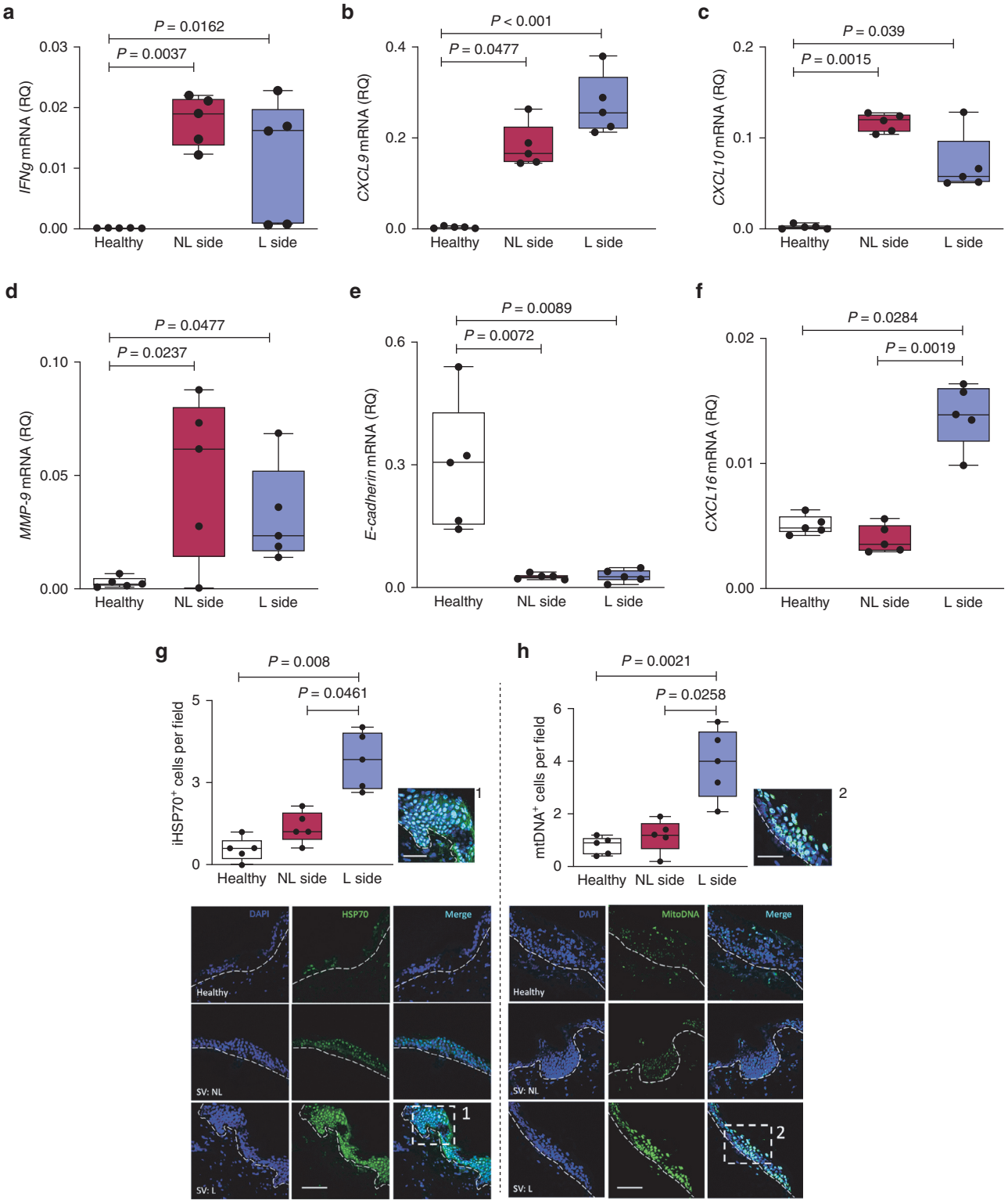

Figure 1. Innate immunity, skin integrity, and stress markers in patients with SV. (**a**) *IFNg* mRNA, (**b**) *CXCL9* mRNA, (**c**) *CXCL10* mRNA, (**d**) *MMP-9* mRNA, (**e**) *E-cadherin* mRNA, and (**f**) *CXCL16* mRNA levels in patients with SV taken from their NL (n = 5) and L (n = 5) site and compared with those of the healthy control skin (n = 5). Results are given as RQ of the target gene expressed compared with that of the housekeeping gene *RPLPO*. (**g**) iHSP70 and (**h**) mtDNA staining in patients with SV taken from their NL (n = 5) and L (n = 5) site and compared with those of the healthy control skin (n = 5). The number of HSP70-positive cells or the number of mtDNA-positive cells was counted over 10 nonoverlapping fields along the dermis, and the results were given as the mean number of positive cells per field. Representative immunofluorescence staining is shown below with zoomed area numbered accordingly. Differences between groups were tested using Kruskal–Wallis test followed by Dunn's posthoc analysis. Significance was set at $P < 0.05$. Bar = 30 mm (10 mm in close-up pictures). iHSP70, inducible HSP70; L, lesional; MMP, matrix metalloproteinase; NL, nonlesional; RQ, relative quantity; SV, segmental vitiligo.

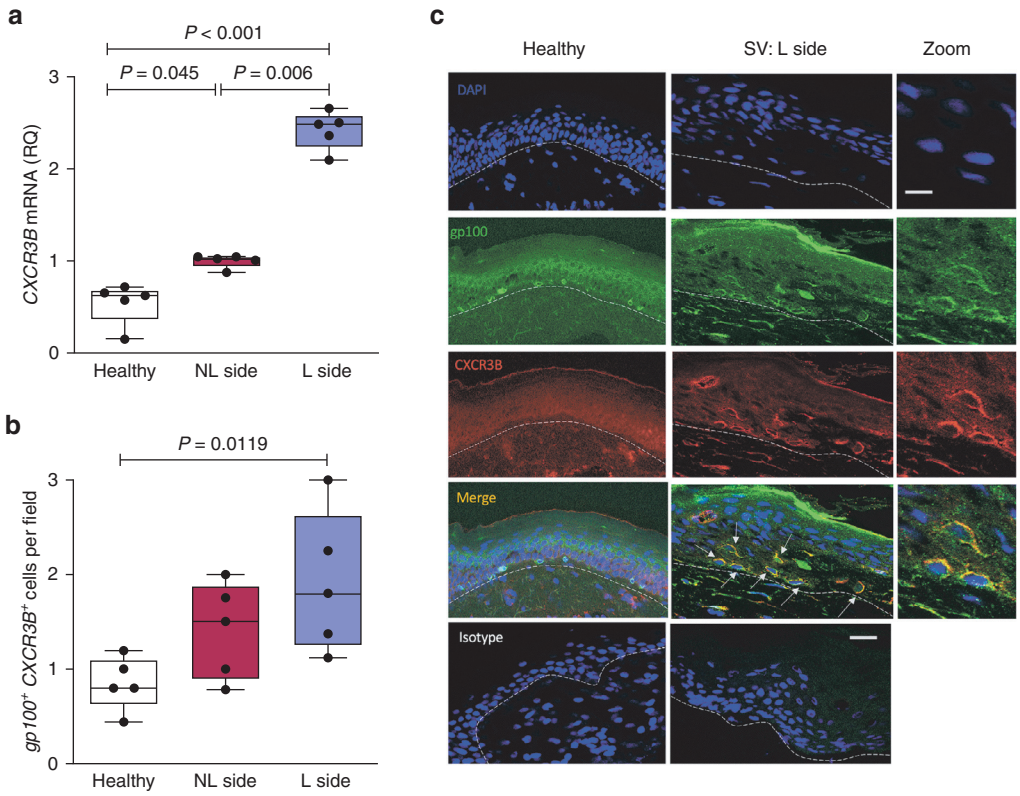

Figure 2. CXCR3B in the skin of patients with SV. (**a**) *CXCR3B* mRNA level and (**b**) the number of CXCR3B$^+$ melanocytes (gp100$^+$) in patients with SV taken from their NL (n = 5) and L (n = 5) site and compared with those of the healthy control skin (n = 5). *CXCR3B* mRNA results are given as RQ of the target gene expressed compared with that of housekeeping gene *RPLPO*. The number of double-positive gp100/CXCR3B-positive cells (depicted in **c** as yellow colocalization) were counted over 10 nonoverlapping fields along the dermis, and results were given as the mean number of positive cells per field. Differences between groups were tested using Kruskal–Wallis test followed by Dunn's posthoc analysis. Significance was set at $P < 0.05$. Bar = 30 mm (10 mm in close-up pictures). L, lesional; NL, nonlesional; RQ, relative quantity; SV, segmental vitiligo.

additional local stress or cellular susceptibility must trigger the development of a specific autoimmune response against melanocytes at the L sites. Interestingly, we found a significant increase in mitochondrial stress and an increased expression of inducible HSP70 and CXCL16 only in the skin from the site where lesions were located. We recently reported a significant increase in mtDNA in the L biopsies of patients with NSV compared with those of their NL sites and with those of the healthy controls (Bzioueche et al., 2021). Inducible HSP70 has been reported to activate dendritic cells, to accelerate depigmentation in a mouse model of vitiligo, and to be secreted by melanocytes under stress conditions (Mosenson et al., 2014). CXCL16 is also known to be a key chemokine secreted by keratinocytes under stress conditions that further attracts CD8$^+$ T cells (Li et al., 2017). The significant increase of these stress-induced markers only at the L sites of patients with SV suggests that a local susceptibility to stress might promote depigmentation only in one specific area, whereas the entire skin showed an increase in inflammatory markers. These data support the theory of mosaic mutations in epidermal cells that render them more susceptible to stress. Meanwhile, these data do not explain why the CD8$^+$ T cells that are attracted by CXCL16 target specifically melanocytes when most of the attractive chemokines are secreted by keratinocytes. Our findings may hold a key. In this study, we have shown that melanocytes from patients with SV have a higher expression of CXCR3B than those from healthy controls (Figure 2b) but also higher than melanocytes from the skin of the same patients with SV located outside of the segmental zone (Figure 2a). Together, this evidence suggests that CXCR3B could be one of the suspected melanocytic defects in patients with SV, which is responsible for initiating the local autoimmune response. Our previous findings in NSV showing increased CXCR3B expression in vitiligo melanocytes (Tulic et al., 2019) has now been extended to SV, suggesting that fighting against the local stress or targeting more specifically the CXCR3B activation could provide effective approaches for halting the progression of the disease in these patients.

Data availability statement

No datasets were generated or analyzed during this study.

Ethics statement

Patients were recruited from the Department of Dermatology, Archet 2 Hospital, Centre Hospitalier Universitaire de Nice, France. Five patients accepted to participate in the study and were included after informed written consent was obtained. The study was approved by the National ethics committee SUD MEDITERRANEE V (N12.034).

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AUTHOR CONTRIBUTIONS

Conceptualization: TP, MKT; Data Curation: TP, VEAM, HB, SM, MKT; Formal Analysis: TP, VEAM, HB, MKT; Funding Acquisition: TP; Investigation: TP, VEAM, HB, MKT; Methodology: TP, MKT; Project Administration: TP, MKT, SR; Resources: TP, MKT, SR; Supervision: TP, MKT; Validation: TP, MKT, SR; Visualization: TP, VEAM, HB, SM, MKT; Writing - Original Draft Preparation: TP, VEAM, HB, MKT; Writing - Review and Editing: TP, MKT, SM, SR

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.07.157.

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SUPPLEMENTARY MATERIALS AND METHODS

Subjects

Patients were recruited from the Department of Dermatology, Archet 2 Hospital, Centre Hospitalier Universitaire de Nice, France. Inclusion criteria were patients aged >18 years suffering from segmental vitiligo. Exclusion criteria included segmental vitiligo affecting only the face (excluded to avoid a skin biopsy in this area), mixed vitiligo, presence of other autoimmune diseases (except autoimmune dysthyroiditis), or previous treatment (phototherapy, topical or systemic corticosteroids, systemic antibiotics, and/or other immunosuppressive agents) for up to 3 months before skin sampling. Among the 1,721 patients with vitiligo in our database, only 14 fulfilled all the criteria. Five patients accepted to participate in the study and were included after informed written consent was obtained. All had their disease for >3 years and had no sign of disease activity when they were included. Five healthy control patients without any history of vitiligo or any other autoimmune disease were recruited from the same clinic. These skin samples were taken from surgical waste from the nonlesional area of benign skin tumors. The groups were matched for sex, age, and biopsy location. From each patient with vitiligo, we obtained two 4-mm skin punch biopsies (one from lesional and one from nonlesional side), and for each healthy patient, one skin sample was obtained. Importantly, lesional biopsies were taken from normal-appearing (nondepigmented) skin but localized on the segmental side of the body affected by vitiligo (Supplementary Figure S1). All the skin samples were taken from the dry locations (Supplementary Table S1). Each biopsy was cut in half; one was used for qPCR analysis, and the other half was embedded in optimal cutting temperature medium, snap frozen, and used for immunofluorescence staining. Both were stored at −80 °C until analysis. The study was approved by the National ethics committee (N12.034).

RNA extraction and quantitative real-time reverse transcriptase–PCR

RNA was extracted from homogenized biopsies using the RNeasy kit (Qiagen, Hilden, Germany). The cDNA was synthesized from 1 μg mRNA using the Reverse Transcription System (Promega, Madison, WI). Relative quantification of gene expression was determined by real-time qPCR with SYBR Green reagent (Life Technologies, Carlsbad, CA), and specific primers were directed against human *IFNg*, *CXCL9*, *CXCL10*, *CXCL16*, matrix metalloproteinase gene *MMP-9*, E-cadherin, and *CXCR3B*. All measurements were performed in triplicate, and results were normalized to those of the *RPLPO* housekeeping gene (Wang et al., 2012).

Immunofluorescence

Biopsies were cut into 7-mm sections and mounted on Superfrost slides. Tissue was fixed with 4% paraformaldehyde, permeabilized for 10 minutes with 0.3% Triton X-100, and blocked for 1 hour at room temperature with PBS 5% BSA containing 10% normal goat serum and 0.05% Triton. Afterward, tissue was incubated overnight at 4 °C with primary antibody directed against human HSP70 (HSPA1A/B, 1:50, Enzo Life Sciences, Farmingdale, New York), mtDNA (1:10, Progen, Heidelberg, Germany), or CXCR3B (1:50, ProteinTech, Rosemont, IL) and later in presence or absence of gp100 (1:200, PMEL, Abcam, Cambridge, United Kingdom). The next day, slides were incubated with appropriate secondary antibodies conjugated to fluor chromogens (1:1,000, Invitrogen, Waltham, MA) for 1 hour and then incubated for 5 minutes with Hoesch DAPI stain (1:1,000) before mounting the slides with Prolong Gold Antifade Reagent (Thermo Fisher Scientific, Waltham, MA). Red or green immune-positive cells were counted over 10 nonoverlapping fields along the dermis at ×40 objective using a confocal microscope (Nikon A1R, Nikon, Tokyo, Japan). Results are represented as the mean number of positive cells per field.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA). Differences between groups were analyzed using Kruskal–Wallis nonparametric test followed by Dunn's posthoc analysis to adjust for multiple testing. Differences were considered significant at $P < 0.05$.

SUPPLEMENTARY REFERENCE

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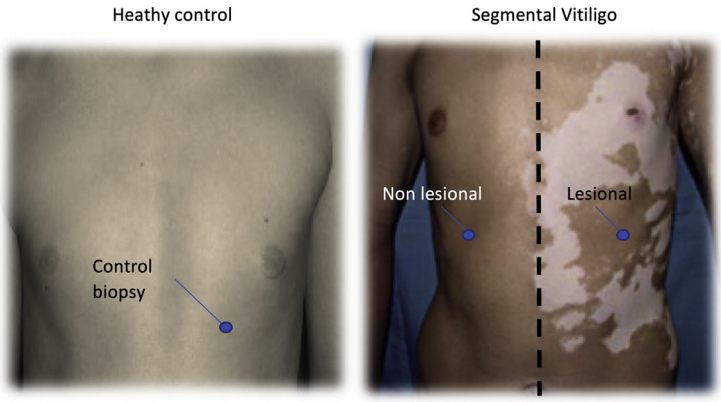

Supplementary Figure S1. Location of samples biopsied. Representation of the sites taken for skin biopsy in patients with segmental vitiligo and healthy controls. Note that the lesional sites in patients with segmental vitiligo were in the segment involved in the depigmentation process but in an area not depigmented.

Supplementary Table S1. Patient Characteristics

Characteristics	Healthy Controls	Segmental Vitiligo
Subjects (n)	5	5
Age (y), median (IQR))	37.9 (8.6)	25.6 (5.8)
Gender (male/female)	3/5	3/5
Disease duration (y)	—	11.6 (3.4–18.6)
Family history of vitiligo (%)	—	1 (20%)
Location of biopsy (n)	Arm (2), chest (2), leg (1)	Chest (3), leg (1), arm (1)

Abbreviations: IQR, interquartile range.