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Association of the CXCL9-CXCR3 and CXCL13-CXCR5 axes with B-cell trafficking in giant cell arteritis and polymyalgia rheumatica

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ABSTRACT

Objective: B-cells are present in the inflamed arteries of giant cell arteritis (GCA) patients and a disturbed B-cell homeostasis is reported in peripheral blood of both GCA and the overlapping disease polymyalgia rheumatica (PMR). In this study, we aimed to investigate chemokine-chemokine receptor axes governing the migration of B-cells in GCA and PMR.

Methods: We performed Luminex screening assay for serum levels of B-cell related chemokines in treatment-naïve GCA (n = 41), PMR (n = 31) and age- and sex matched healthy controls (HC, n = 34). Expression of chemokine receptors on circulating B-cell subsets were investigated by flow cytometry. Immunohistochemistry was performed on GCA temporal artery (n = 14) and aorta (n = 10) and on atherosclerosis aorta (n = 10) tissue.

Results: The chemokines CXCL9 and CXCL13 were significantly increased in the circulation of treatment-naïve GCA and PMR patients. CXCL13 increased even further after three months of glucocorticoid treatment. At baseline CXCL13 correlated with disease activity markers. Peripheral CXCR3+ and CXCR5+ switched memory B-cells were significantly reduced in both patient groups and correlated inversely with their complementary chemokines CXCL9 and CXCL13. At the arterial lesions in GCA, CXCR3+ and CXCR5+ B-cells were observed in areas with high CXCL9 and CXCL13 expression.

Conclusion: Changes in systemic and local chemokine and chemokine receptor pathways related to B-cell migration were observed in GCA and PMR mainly in the CXCL9-CXCR3 and CXCL13-CXCR5 axes. These changes can contribute to homing and organization of B-cells in the vessel wall and provide further evidence for an active involvement of B-cells in GCA and PMR.

1. Introduction

Giant cell arteritis (GCA) and polymyalgia rheumatica (PMR) are frequently overlapping inflammatory diseases occurring at an older age [1]. PMR is diagnosed in up to 60% of GCA patients [2,3], whereas the prevalence of GCA in PMR varies between 5 and 30% [4]. GCA is characterized by inflammation of the medium- and large arteries, while PMR is characterized by inflammation primarily of the synovial tissue of shoulders and hips. Dependent on which arteries are involved, GCA leads to cranial symptoms like headache (temporal artery; TA), or more systemic symptoms like weight loss and low-grade fever (aorta and its proximal branches) [5]. A complication of GCA is the formation of an aortic aneurysm. PMR is characterized by pain and stiffness mainly in the shoulder and hip girdle [2,3].

The pathogenesis of both diseases is incompletely understood. When GCA is suspected, the TA is easily accessible for a diagnostic biopsy, while the aorta or other large arterial branches are usually not approached for histological investigation. Based on studies of the TA, GCA is regarded as a granulomatous disease mainly mediated by T-cells and macrophages [6]. So far, the inflammatory processes in the TA and aorta are considered to be the same, but studies in GCA aorta are scarce.

B-cells have long been neglected in GCA research because so far no disease-specific auto-antibodies have been found and because early studies showed a relative low number of B-cells in the TA [7,8]. In more recent studies we and others have clearly documented the presence and organization of B-cells in both the TA and aorta of patients with GCA [9–11]. Notably, the inflamed aorta of GCA patients contained high numbers of B-cells, even outnumbering T-cells [11]. Scant information is

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available about B-cells in PMR synovial tissue. One study reported that B-cells were mostly absent in synovium biopsies of the shoulder in active PMR patients [12]. Also, it has been clearly demonstrated that circulating B-cells are altered during the disease course in both GCA and PMR [13–16]. B-cell numbers were reduced during active disease and correlated inversely with disease activity markers. Glucocorticoid (GC) treatment, which remains standard of care in both GCA and PMR, normalized B-cell numbers [14] [–] [16] without repopulation by newly produced B-cells or compensatory hyperproliferation [13], indicating B-cell extravasation or marginalization during active disease and redistribution to circulation upon treatment. In addition, the returning effector B-cells demonstrated an enhanced capacity to produce the pro-inflammatory cytokine Interleukin (IL)-6 [13]. Collectively, these data clearly document B-cell presence in GCA arteries, B-cell trafficking between the tissues and blood in GCA and PMR, and the capacity of B-cells to produce inflammatory cytokines in these two related diseases.

The specific mechanisms governing migration and organization of B-cells in GCA are largely unknown but leukocyte homing to tissues is generally mediated through chemotactic gradients of chemokines. Chronic inflammatory and autoimmune diseases have been associated with disturbed production of chemokines [17–19]. B-cells express multiple chemokine receptors and can therefore migrate in response to several chemokines. Chemokines C-X-C motif receptor (CXCR)3 is expressed on the surface of B-cells and binds to the CXC ligands (CXCL)9, -10, and-11. This axis regulates migration towards the site of inflammation and is also involved in cell activation and differentiation [20]. Another chemokine, CXCL13, was originally called B-cell chemo-attractant because together with its receptor, CXCR5, it exerts important functions in lymphoid neogenesis, B-cell homing and B-cell differentiation [21].

Here we aimed to characterize the chemokine-chemokine receptor axes involved in the migration of B-cells in GCA and PMR in order to understand their migration patterns and to gain further insights into the role of B-cells in the pathogenesis of these diseases. To this end, various chemokines, chemokine receptor expression by B-cells and B-cell differentiation subsets were measured in the circulation of both GCA and PMR patients and compared to age- and sex-matched healthy controls (HC). In addition, TA biopsies and aorta tissue of GCA patients, and aorta tissue of atherosclerosis patients were studied for local chemokine and chemokine receptor expression with a focus on the CXCL9-CXCR3 and CXCL13-CXCR5 axes.

2. Methods

2.1. Study populations

Forty-one GCA patients and 31 PMR patients participated in the cohort study (Table 1). All were newly-diagnosed patients who did not take GC or other disease modifying anti-rheumatic drugs at pre-treatment assessment. The diagnosis of GCA was based on a positive TA biopsy and/or a positive 18F-fluorodeoxyglucose-positron emission computer tomography (FDG-PET-CT) scan. Twenty-seven of the GCA patients fulfilled the 1990 ACR criteria as these criteria are mainly useful for GCA with temporal artery involvement. The remaining 14 GCA patients did have a positive FDG-PET-CT scan. Twenty-three of the 31 PMR patients fulfilled the Chuang criteria and 29 PMR patients had a positive FDG-PET-CT scan. The 2 PMR patients without an FDG-PET-CT scan did fulfil the Chuang criteria. The PMR patients had neither GCA symptoms and signs nor vasculitis on imaging. Cross-sectional data of 34 age- and sex-matched healthy controls (HCs) selected from our SENEX cohort was included in this study. These are healthy individuals older than 50 years of age recruited from the general population of the north part of the Netherlands. At inclusion, the HCs had no relevant past and present diseases and did not take any immunosuppressive drugs. All subjects provided informed consent, and samples were obtained in compliance with the Declaration of Helsinki. The study was approved by the institutional review board of the University Medical Center Groningen (METc2012/375 for HC and METc2010 for GCA and PMR). PBMC samples of subpopulations, consisting of 18 GCA patients, 10 PMR patients and 25 age- and sex-matched HC, were used for flow cytometric analysis/experiments. Detailed characteristics of these subpopulations are described in supplementary Table 1.

2.2. Luminex for measurement of chemokines in circulation

CXCL9 and CXCL13 were quantified in serum as part of a human premix magnetic Luminex screening assay kit (R&D systems), which also included CCL19, CCL21, CXCL10, CXCL11, and CXCL12. The assay was read on a Luminex Magpix instrument (Luminex) and data were analysed with xPONENT 4.2 software (Luminex).

Table 1
Pre-treatment characteristics of newly diagnosed giant cell arteritis (GCA), polymyalgia rheumatica (PMR) patients and age-matched healthy controls (HC).

	HC	GCA	PMR	KW/Chi-square	p-value HC vs GCA	p-value HC vs PMR	p-value GCA vs PMR
n	34	41	31				
age in years; median (range)	**71** (52–85)	**71** (52–84)	**73** (54–82)	NS			
Females (%)	23 (68)	28 (70)	17 (55)	NS			
TA positive/performed	NA	19/25	0/4				
FDG-PET-CT positive for GCA/PMR/GCA + PMR	NA	18/0/11	0/29/0				
Fulfilled ACR criteria (GCA); Yes/No	NA	27/14	NA				
Fulfilled Chuang criteria (PMR); Yes/No	NA	NA	23/8				
CRP mg/L; median (range)	**5** (1,7–11)	**49** (2,2–215)	**32,5** (3,2–127)	<0,0001	<0,0001	<0,0001	NS
ESR mm/h; median (range)	**11** (2-24)	**81** (7–121)	**57** (7–109)	<0,0001	<0,0001	<0,0001	<0,05
Hb mmol/L; median (range)	**8,7** (7,3–10)	**7,3** (5,5–8,5)	**7,8** (6,2–9,3)	<0,0001	<0,0001	<0,0001	<0,05
Lymphocytes 10^9/L; median (range)	**1,96** (0,69-2,72)	**1,67** (0,76-3,19)	**1,5** (0,8–2.85)	NS			
$CD19^+$ B-cells 10^9/L; median (range)	**0,20** (0,07-0,49)	**0,15** (0,03-0,54)	**0,17** (0,06-0,44)	NS			

2.3. Flow cytometry for the analysis of circulating B-cell subsets and chemokine receptor expression

Absolute numbers of circulating B-cells were measured in fresh EDTA blood samples using the Beckton Dickinson Multitest Trucount method, as described by the manufacturer. Data were acquired on a FACS Canto-II (BD) and analysed with FACSCanto Clinical Software (BD). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood with Lymphoprep (Axis-shield) and frozen in 10% DMSO/FCS medium. The PBMCS were stored in liquid nitrogen for future analysis. For flow cytometry analysis, cryopreserved PBMCs were thawed and 2 million cells were labelled with fluorochrome-conjugated antibodies for B-cell subset markers and chemokine receptor expression (see supplementary Table 2 for a list of used antibodies). Samples were measured using an LSR-II flow cytometer (BD) and data were analysed using Kaluza software (BD). B-cells were defined as $CD19^{+}$ cells and divided into seven subsets: transitional ($CD38^{High}CD27^{-}$), naïve ($CD38^{Low/-}CD27^{-}$), unswitched memory ($CD27^{+}IgD^{+}IgM^{+}$), switched memory ($CD27^{+}IgD^{-}IgM^{-}$), IgM only memory ($CD27^{+}IgD^{-}IgM^{+}$), plasmablasts ($CD27^{High}CD38^{High}$) and double negative B-cells ($CD27^{-}CD38^{-}$) (See supplementary data, Fig. 1 for gating strategy). Dead cells were excluded by using fixable viability dye and expression of chemokine receptors was determined for total B-cells and for each aforementioned specific subset. Gates were based on Fluorescence minus one controls and isotype controls.

2.4. Immunohistochemistry for detection of arterial B-cells, chemokines and chemokine receptors

TA (n = 14) and aorta tissue (n = 10) of GCA patients was formalin fixed, paraffin embedded and sectioned at 3 μm thickness. For comparison, aorta tissue (n = 10) of atherosclerotic patients with an aortic aneurysm were also stained. Consecutive sections were deparaffinized and rehydrated after which antigen retrieval and endogenous peroxidase block were performed. Sections were incubated with anti-human antibodies for the detection of the following markers: CD20, CXCL9, CXCL13, CXCR3, and CXCR5 (supplementary Table 3). Subsequently, tissues were incubated with secondary antibodies, 3,3′-Diaminobenzidine (DAKO, Denmark) for peroxidase activity detection and counterstained with hematoxylin (MERCK, Kenilworth, NJ, USA). Tonsil

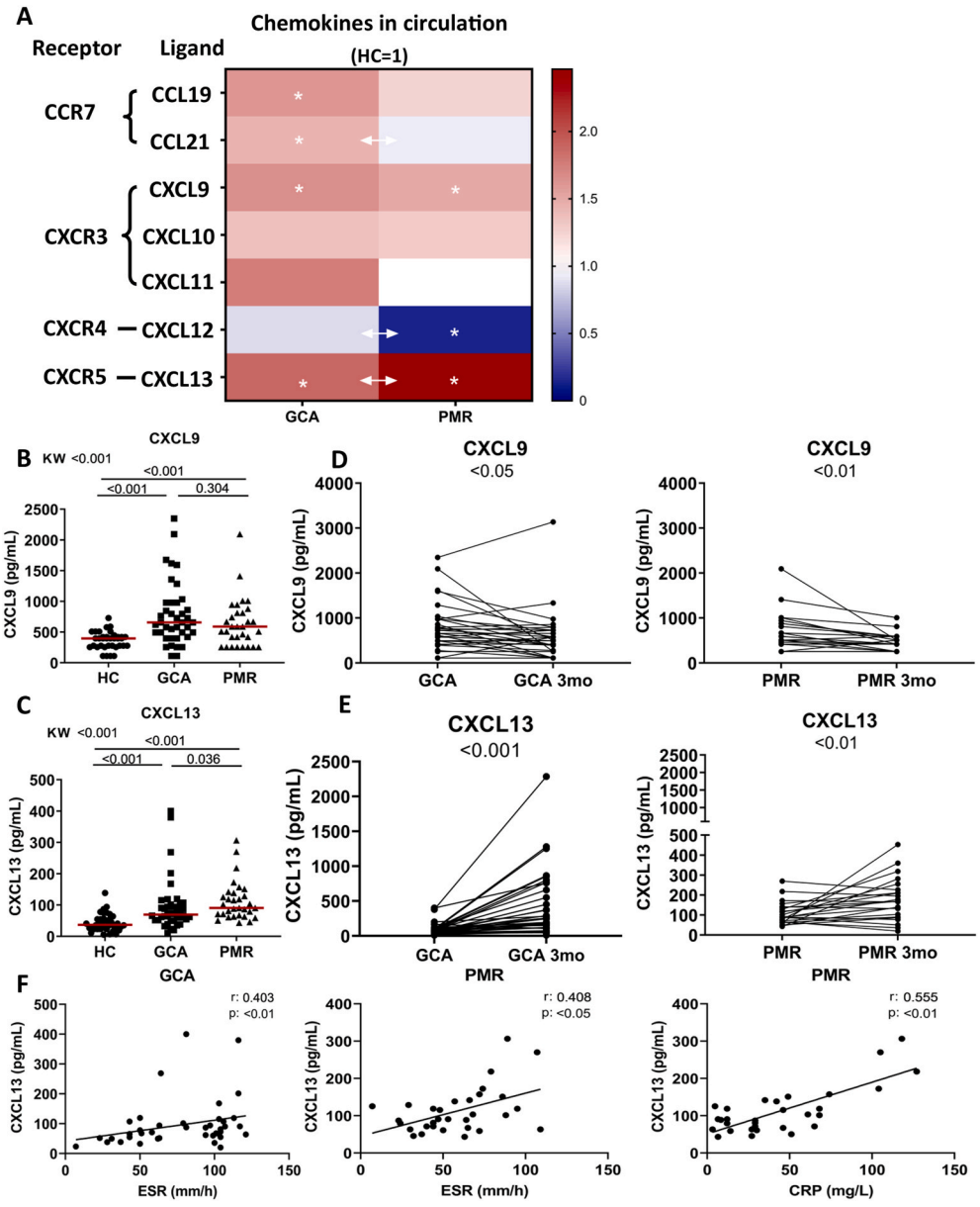

Fig. 1. Chemokine expression in the circulation of GCA (n = 41), PMR (n = 31) and HC (n = 34). (**A**) Heatmap depicting median fold-change compared to HC with * = $p < 0.05$ compared to HC, ↔ = $p < 0.05$ between GCA and PMR. (**B–C**), Baseline (pre-treatment) levels of respectively CXCL9 and CXCL13 for HC, GCA and PMR. (**D-E**), Change in circulating chemokine levels between baseline and 3 months (GC-treated) for respectively CXCL9 and CXCL13 in both GCA (n = 35) and PMR (n = 23). (**F**) Correlations of CXCL13 with disease activity markers ESR (GCA and PMR) and CRP (PMR).

was used as positive control tissue and appropriate isotype control stainings were performed. All stained tissue sections were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U 10074-01, Hamamatsu Photonics K·K.) before being analysed. Representative areas, containing infiltrating cells and if present B-cells, were semi-quantitatively scored on a five-point scale (0–4) with 0 = no positivity, 1 = occasional positivity (0–1%), 2 = mild positivity (>1–20%), 3 = moderate positivity (>20–50%), 4 = high positivity (>50%).

2.5. Statistical analysis

Non-parametric tests were used for data analysis as data was not normally distributed. For comparison of the three groups the Kruskal-Wallis test was performed and, where appropriate, for comparison of two groups the Mann-Whitney *U* test was performed. Paired samples (pre-treatment and after 3 months of treatment) were compared with the Wilcoxon signed rank test. Correlations were assessed with the Spearman's rank correlation coefficients. GraphPad Prism 8.0 software was used for analysis and p-values of less than 0.05 (2-tailed) were considered statistically significant.

3. Results

3.1. Serum chemokine levels in GCA and PMR patients compared to HC

First, serum levels of several chemokines involved in B-cell migration were measured in order to identify which chemokine pathways are altered and thus are possibly involved in B-cell migration in GCA and PMR. Fig. 1A depicts in a heatmap the differences in serum chemokine levels between the three study groups. In GCA, CCL19 and CCL21 were significantly elevated compared to HC. The levels of CCL21 in GCA were also significantly elevated when compared to PMR patients. The chemokine CXCL9 was significantly elevated in both patient groups (Fig. 1B). Although CXCL10 and CXCL11 were slightly higher, especially in GCA patients, they were not significantly altered compared to HC. PMR patients showed significantly lowered CXCL12 levels compared to both HC and GCA patients. CXCL13 was increased in both patient groups compared to HC and CXCL13 levels in PMR were significantly elevated compared to GCA (Fig. 1C). CXCL13 correlated with baseline levels of disease activity markers ESR (GCA and PMR) and CRP (only in PMR) (Fig. 1F). Serum chemokine levels did not differ between cranial GCA and large-vessel GCA/PMR patients. More specifically, no difference was seen within the GCA group between the patients with and without headache, jaw claudication, vision loss and leg claudication. There was a significant difference (p = 0.04) regarding the arm claudication, whereby the group with arm claudication had a lower level of CXCL9. However the group with arm claudication was small (n = 5). After 3 months of GC treatment, the levels of all chemokines measured either normalized to HC levels or showed a tendency towards normalization (see for example CXCL9, Fig. 1D). The only exception was CXCL13, which was already increased at baseline and further increased after 3 months in both GCA and PMR patients (Fig. 1E).

3.2. Peripheral B-cell subset distribution and chemokine receptor expression on circulating B-cells of GCA, PMR and HC

To gain insight into which B-cells subsets migrate in GCA and PMR we analysed the distribution of B-cell subsets in the circulation in conjunction with chemokine receptor expression on these specific B-cell subsets. In circulation and before treatment, the total B-cell number in GCA and PMR tended to be lower compared to HC although this difference did to reach statistical significance (see Table 1 and individual values included in supplementary figure 2). Analysis of the different B-cell differentiation subsets revealed altered distribution patterns compared to HC (Fig. 2A). GCA patients showed lowered switched memory B-cells while PMR patients showed elevated double negative B-cells and plasmablasts. After 3 months of GC-treatment, transitional B-cells were almost depleted in both patient groups (figure 2B and 2C)

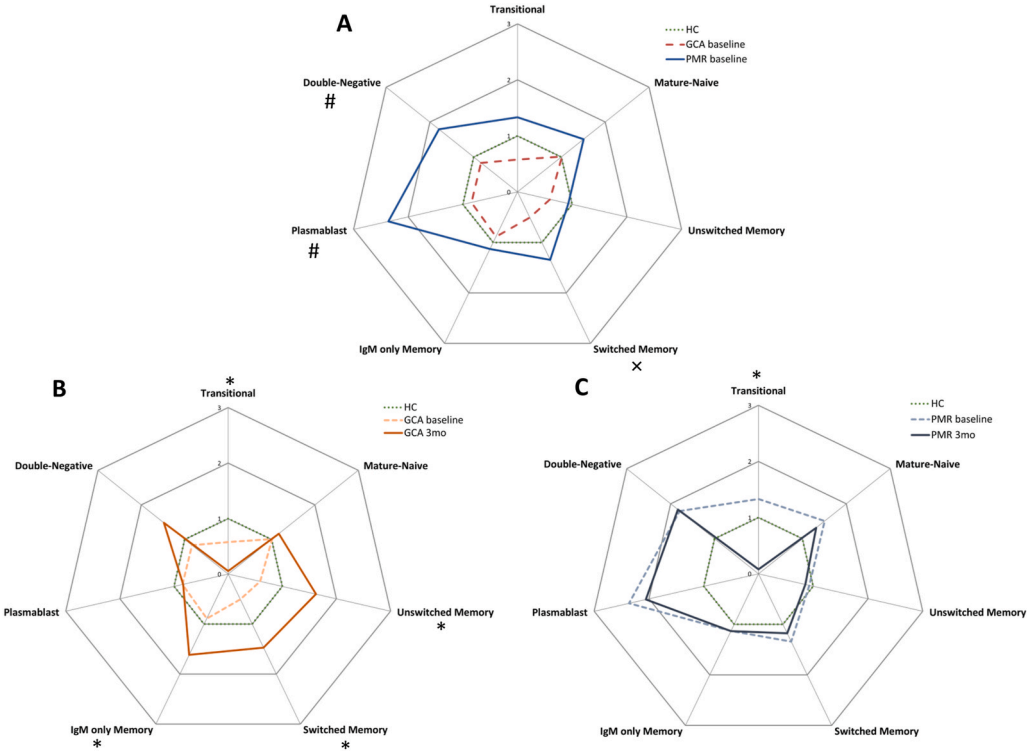

Fig. 2. Dynamics in B-cell subsets before (**A**) and during 3months follow-up after GC treatment for GCA (n = 18) (**B**) and PMR (n = 10) (**C**). B-cell subset cell counts are expressed in radar plots as median fold-change compered to healthy control (n = 25). # = sign. difference between HC and PMR, × = sign. difference between HC and GCA, * = sign. difference between baseline and 3 months.

while, specifically for GCA, all memory B-cells subsets increased.

Next, we focused on the expression of the chemokine receptors by circulating B-cells for which the corresponding chemokines were altered in the sera of GCA or PMR patients. The percentage of B-cells expressing CXCR3, CXCR4, CXCR5 or CCR7 was lowered in GCA and PMR patients compared to HC (Fig. 3A). This decrement only reached statistical significance for the PMR patients and here a negative correlation between CXCR5+ B-cells and CRP (Fig. 3B) was observed. Similar negative correlations were also observed in PMR between CXCR4+ B-cells and CRP ($r = -0.77$, $p < 0.01$) and CCR7+ B-cells with CRP ($r = -0.82$, $p < 0.01$). Looking into specific B-cell subsets, we found that the percentage and absolute number of CXCR3+ switched memory B-cells were significantly lowered in both patient groups compared to HC (Fig. 3C). This finding was also true for the percentage of CXCR5+ switched memory B-cells (Fig. 3D). There was a large variation especially in the CXCR3+ switched memory B-cells, not only in the patient groups but also in the healthy controls. In de healthy controls a negative correlation was found between the CXCR3+ switched memory B-cells and age for both the absolute numbers ($r = -0.70$, $p < 0.01$) and frequencies ($r = -0.41$, $p = 0.05$). This was also the case for the CXCR5+ switched memory B-cells and age for both the absolute numbers ($r = -0.50$, $p = 0.02$) and frequencies ($r = -0.46$, $p = 0.03$). However, no such correlation was documented in the GCA and PMR patient groups. We found inverse correlations between CXCL9 and CXCR3+ B-cells, and between CXCL13 and CXCR5+ B-cells in GCA patients (Fig. 3E and F). The same inverse correlations were observed in GCA patients for CXCL9 with CXCR3+ memory B-cells (Fig. 3G), CXCL9 with switched memory B-cells (Fig. 3H), and for CXCL13 with CXCR5+ switched memory B-cells (Fig. 3I). These correlations were only observed for the GCA patients and not found in the PMR patient group.

Chemokine receptor expression on circulating B-cells did not significantly differ between cranial GCA and large vessel GCA at baseline. Also, there were no significant correlations between specific CXCR3+ B cells and CXCR5+ B-cells subsets, and these clinical parameters. An inverse correlation was observed between total B-cell counts and CXCL9, as well as between total B-cell counts and CXCL13 in large vessel-GCA only (data not shown).

3.3. Local expression of migration axes CXCL9-CXCR3 and CXCL13-CXCR5 in inflamed GCA arteries and co-localization with B-cells

We next investigated local arterial expression of the B-cell related chemokines and chemokine receptors that were altered in the circulation of GCA and PMR patients. $CD20^+$ B-cell presence was confirmed by immunohistochemistry in TA and aorta of GCA patients (Fig. 4A and 4F). The chemokines CXCL9 and CXCL13 were both highly expressed in the TA and aorta tissues of GCA patients (Fig. 4B and 4D). In TA tissue the chemokines were equally expressed in all three layers of the vessel wall (adventitia, media and intima) while in the aorta the chemokine expression was mostly observed in the media and adventitia (figure 4G and 4I). The expression of both chemokines was higher in GCA than in atherosclerotic aorta. The accompanying receptors for CXCL9 and CXCL13, CXCR3 and CXCR5 respectively, were also highly expressed in the inflamed GCA arteries (figure 4C and 4E). Notably, the expression of the chemokines and their receptors was detected in the same regions of the vessel wall (Fig. 4G–J). Moreover, almost all B-cell clusters were located in areas with high chemokine and chemokine receptor expression. Co-expression of CXCL9 and CXCR3 was detected in all of the B-cell clusters in TA and in the vast majority of the B-cell clusters in aorta. Co-expression of CXCL13 and CXCR5 was also detected in all of the TA B-cell clusters and in the vast majority of the B-cell clusters located in the aorta. Because CXCR3 and CXCR5 can also be expressed by other inflammatory cell types, we confirmed expression of these chemokine receptors by B-cells with double immunostainings using PAX5 (nuclear B-cell marker) together with CXCR3 (Fig. 4K) or CXCR5 (Fig. 4L).

4. Discussion

B-cells represent a dynamic cell population in GCA and PMR as demonstrated by changes of B-cells in both peripheral blood of GCA and PMR patients as well as in the inflamed arterial wall in GCA. In search for the mechanisms governing the trafficking of B-cells in GCA and PMR,

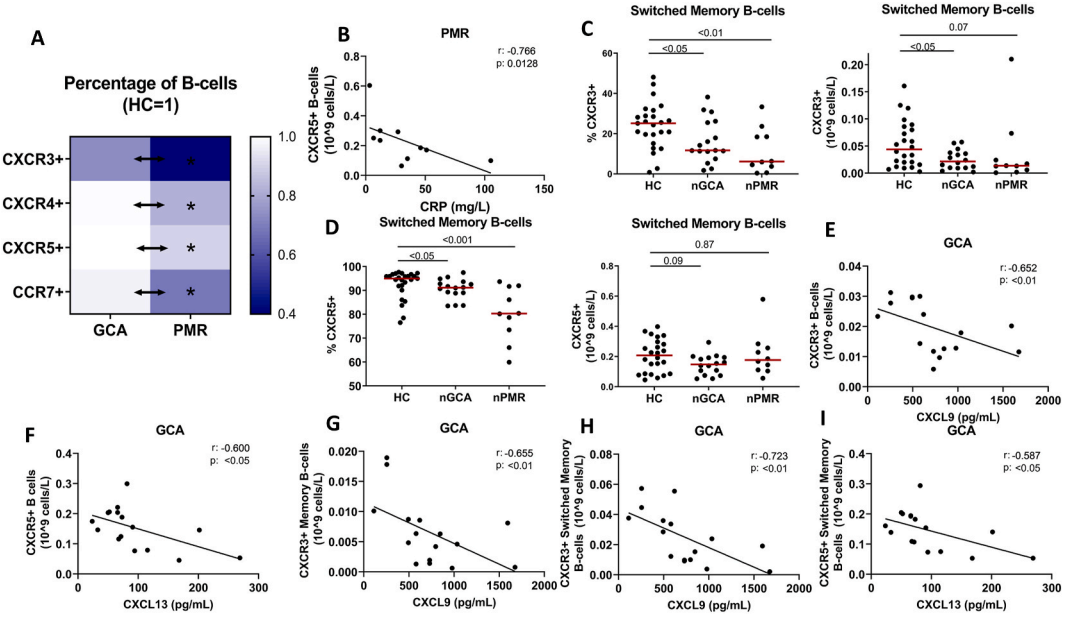

Fig. 3. Chemokine receptor expression on circulating B-cells and correlations with their corresponding chemokines. (**A**) heatmap depicting median fold-change compared to HC with * = $p < 0.05$ compared to HC, ↔ = $p < 0.05$ between GCA and PMR. (**B**) negative correlation in PMR patients between CXCR5+ B-cells and disease activity marker CRP. (**C**) Percentage and absolute number of CXCR3+ switched memory B-cells for GCA and PMR patients. (**D**) Percentage and absolute number of CXCR5+ switched memory B-cells for GCA and PMR patients. Negative correlation in GCA patients between CXCR3+ B-cells and CXCL9 (**E**), CXCR5+ B-cells and CXCL13 (**F**), CXCR3+ memory B-cells and CXCL9 (**G**), CXCR3+ switched memory B-cells and CXCL9 (**H**) and CXCR5+ switched memory B-cells and CXCL13 (**I**).

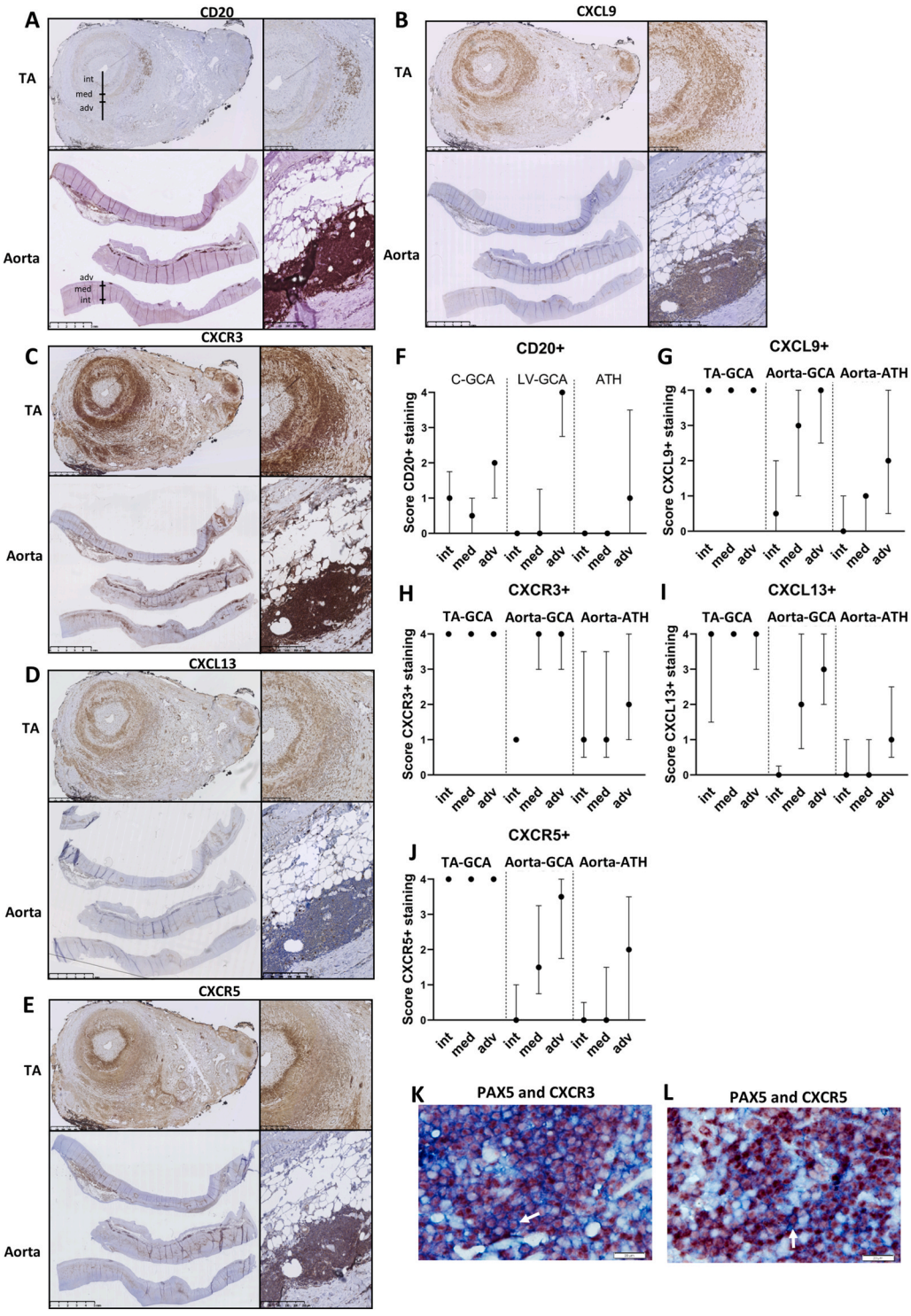

Fig. 4. Local expression of B-cells, chemokines and chemokine receptors in inflamed GCA arteries. Single-staining representative immunohistochemistry showing expression of (**A**) CD20 (B-cells), (**B**) CXCL9, (**C**) CXCR3, (**D**) CXCL13 and (**E**) CXCR5 in both TA and aorta with an overview image (left panel) and a higher magnification picture (right panel). For references, the different layers of the vessel wall are indicated in the CD20 staining, with int = intima, med = media and adv = adventitia. (**F-J**), semi-quantitative scoring of the TA (n = 14) and aorta (n = 10) of GCA patients in comparison to the atherosclerotic aorta (n = 10) for all stainings. Median (line) and IQR are shown (**K**), double-staining of PAX5 (B-cells, red) and CXCR3 (blue). (**L**), double-staining of PAX5 (B-cells, red) and CXCR5 (blue). The white arrows point towards CXCR3+ and CXCR5+ B-cells.

we document here modulation of several B-cell-related chemokines, including CXCL9 and CXCL13, in the serum of GCA and PMR patients before treatment and during follow-up. Accordingly, we observed changes in B-cell subsets expressing the chemokine receptors CXCR3 and CXCR5 in the peripheral blood of patients with GCA and PMR before treatment. Moreover, we document the presence of the B-cell related chemokines CXCL9 and CXCL13 at the site of vascular infiltration with CXCR3+ and CXCR5+ B-cells, respectively, in GCA. A graphical representation of the main study findings is given in Fig. 5.

B-cells respond and migrate towards the chemokines CXCL9 and CXCL13 produced at the site of inflammation by virtue of the CXCR3 and CXCR5 receptors, respectively. We observed high expression of CXCL9 and CXCL13 protein in all layers of the TA and in the medial and adventitial layer of the aorta as assessed by immunohistochemistry. The expression of these chemokines coincided with expression of the corresponding CXCR3 and CXCR5 receptors throughout the entire tissue, including the areas with B-cell clusters. Thus B-cells in GCA arteries express the CXCR3 and CXCR5 receptors and are mostly located in the adventitia and media where the corresponding chemokines are also present, supporting an ongoing tissue-infiltrating migratory process. In

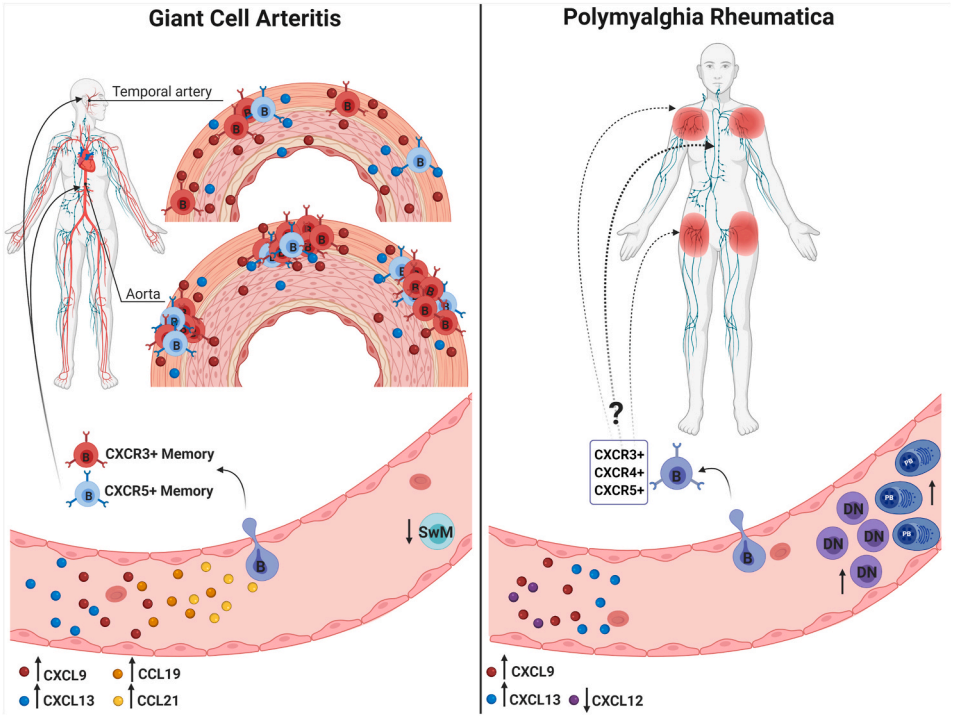

Fig. 5. Graphical representation of the main study findings in GCA (left) and PMR (right). Local expression of the chemokines CXCL9 and CXCL13 was observed with immunohistochemistry in both inflamed temporal artery and aorta of GCA patients. B-cells infiltrated the inflamed arteries and expressed the corresponding CXCR3 and CXCR5 receptors. In the circulation, alterations in chemokine levels were observe in active, untreated GCA and PMR patients. CXCL9 and CXCL13 were increased in the circulation of both patient groups. CCL19 and CCL21 were found increased in GCA patients while CXCL12 was decreased only in PMR patients. B-cell subset distribution was also altered in the circulation of both GCA and PMR patients. Lowered total switched memory B-cells, CXCR3+ memory B-cells and CXCR5+ memory B-cells were detected in the circulation of GCA patients suggesting that these B-cells migrate towards inflamed arteries. In PMR patients elevated double negative B-cells and plasmablasts were observed in the circulation. When focusing on chemokine receptor expression by B-cells, PMR patients had lowered CXCR3+, CXCR4+, and CXCR5+ B-cells. It is still unclear if these B-cells migrate towards inflamed synovia or the lymphatic system in PMR patients.

the most inner part of the neointima there was less CXCL9 and CXCL13 protein expression, which coincided with a paucity of B-cell intimal infiltration.

The ligands of CXCR3, including CXCL9, are commonly produced at peripheral sites to attract lymphocytes [22]. CXCR3 is also expressed on T-cells and the CXCL9-CXCR3 axis is important for the recruitment of $CD4^+$ T-cells and $CD8^+$ T-cells, known to be active in the arteries affected by GCA [23]. Previously, in an ex-vivo TA culture system it was also shown that the CXCR3 receptor ligands, CXCL9, CXCL10 and CXCL11, were increased in GCA biopsies compared to negative biopsies [24]. Blocking or addition of IFNγ to the culture either decreased or increased the production of these chemokines, respectively. Moreover, migration of PBMCs was stimulated by chemokine-rich supernatant of vascular smooth muscle cells exposed to IFNγ and this effect was abated by CXCR3 antagonism. This, together with our data, strongly indicates that the CXCL9-CXCR3 axis is actively contributing to both T- and B-cell migration in GCA.

In accordance with our protein findings in TA, mRNA levels of CXCL13 and CXCR5 were previously found to be increased in the GCA TA that contained B-cells organized into tertiary lymphoid organs (TLOs) [9]. Myofibroblasts were considered as a possible source of the CXCL13 in TA of the GCA patients [9], however we did not observe much CXCL13 expression in the regions with intimal hyperplasia where myofibroblast are present. Another source of CXCL13 is represented by the follicular dendritic cells (FDC), which are present in the TLOs of GCA TA and aorta [11,22]. The main immune function of CXCL13 is inducing trafficking and positioning of CXCR5+ B-cells and CXCR5+ T-cells towards B-cell follicles in lymphoid tissues, including TLOs [22]. The present study demonstrates that there is widespread chemokine expression and chemokine receptor expression by lymphocytes in TA and in the aortic TLOs. This finding supports the notion of an ongoing inflammatory process also in the aorta affected by aneurysm formation in GCA patients. In addition, the TLOs contain high endothelial venules (HEV), which are specialized venules that facilitate lymphocyte extravasation. The presence of HEV in combination with the observed chemokine expression at the TLOs likely aid the ongoing inflammation.

The observed increase in serum CXCL13, before and during GC-treatment, and the correlations between CXCL13 and disease activity markers before treatment in both GCA and PMR are remarkable. CXCL13 and its receptor CXCR5 play a central role in the organization of lymphoid tissue, are essential for the formation of TLOs in multiple autoimmune diseases and can also cause local memory B-cell differentiation [21]. CXCL13 is considered to be a serum biomarker for germinal center activity [25] and it has been associated with unfavorable disease prognosis in multiple diseases [21,26–28]. TLOs with FDCs and germinal centers are indeed observed in the GCA TA and aorta and could contribute to systemic CXCL13 levels. The source of the increased CXCL13 in PMR is obscure in the absence of synovial tissue analysis. The further increase of CXCL13 levels during treatment (3 months) in both diseases is intriguing and spikes interest in CXCL13 level dynamics during a longer follow-up period. In rheumatoid arthritis (RA) patients, B-cell depletion therapy led to a reduction in peripheral and systemic levels of CXCL13 which was followed by a subsequent increase upon B-cell repopulation [29]. Next to CXCL13, the chemokine CCL19, which we also found increased in the circulation of GCA patients, is implicated in TLO formation [30]. Blockade of specific chemokine-chemokine receptor pathways have not been tested so far in GCA/PMR but could be an appealing strategy to diminish B- and T-cell mediated damage in GCA. In the MRL/lpr mice model of systemic lupus erythematosus, treatment with CXCL13-blocking monoclonal antibodies diminished the renal damage, the level of circulating anti-dsDNA antibodies and immune complex deposition in the kidneys [31].

The present study also demonstrates alterations in circulating B-cells in both GCA and PMR. In line with previous reports [13,14,32], the total B-cell numbers were lowered before treatment in GCA and PMR patients although this did not reach statistical significance for the patients included in this study. We did observe an increase in circulating B-cells at the 3-months follow up timepoint as reported before [13,14]. When looking at specific B-cell subsets, total CXCR3+ and CXCR5+ B-cells were significantly lowered in PMR patients but this did not reach statistical significance in GCA. However, the CXCR3+ and CXCR5+ switched memory B-cells were significantly lowered in both diseases, suggesting an increased rate of CXCR3+ and CXCR5+ switched memory B-cell trafficking during active disease in both GCA and PMR as compared to HCs. There was a large variation in the CXCR3+ switched memory B-cells and CXCR5+ switched memory B-cells in all groups.

While in the healthy controls this may be explained by a decrease of specific B-cell subsets with aging, no such negative correlations were found in the patient groups. The variation in the patient groups may be related to heterogeneity in the local chemokine production at the site of inflammation and consequently, a heterogeneity in the migration of the B-cells expressing the corresponding chemokine receptor. We found strong inverse correlations between the serum levels of chemokines and the chemokine receptor expressing total B-cells, $CD27^+$ memory B-cells and switched memory B-cells in GCA. These findings indicate higher chemokine expression levels in tissue compared to circulation allowing migration towards the inflamed vessel wall of specific B-cells subsets according to the chemokine gradient. B-cells were also observed in synovial tissue of RA patients, along with CXCL9, CXCR3, CXCL13 and CXCR5, suggesting that these pathways are also important for B-cells migration and organization in the RA synovium [33]. The lowered switched memory B-cells observed in untreated GCA patients, together with the increase in all memory subsets at three months and simultaneous decrease in transitional B-cells, suggest trafficking specifically of memory B-cells. The sharp decrease of transitional B-cells in both patient groups after three months is likely caused by the GC usage as it was previously shown that especially early B-cell progenitors are sensitive to GC treatment [34]. Double negative (DN) B-cells, which were increased in the circulation of PMR patients, are believed to be related to aging, senescence and the senescence-associated secretory phenotype [35]. Increased DN B-cells were previously documented in several auto-immune disease such as early and established RA [36], multiple sclerosis (MS) [37] and SLE [38]. However, it is unknown whether these cells are also present at the local site of inflammation in PMR or what causes their increase in the circulation of PMR patients but not of GCA patients, as both diseases are associated with older age.

Regarding the clinical phenotype of our patient groups and the B-cell related chemokine-chemokine receptor axes, we found significant lower CXCL9 levels at baseline in the GCA patients with arm claudication. However, as the group with arm claudication was small, this finding should be confirmed and further investigated in a larger group of patients. An inverse correlation was observed between total B cell counts and CXCL9, as well as between total B cell counts and CXCL13 in large vessel-GCA only. Previously, Ciccia et al. [9] documented a positive correlation between the number of organized B cells into tertiary lymphoid organs in the temporal artery and parameters of systemic inflammatory response. There was no correlation with age, duration of symptoms or specific GCA features such as headache or ischemia. Further research into the function of specific B-cell subsets, especially regarding cytokine production is needed to dissect differences between clinical GCA/PMR phenotypes and possible different underlying pathophysiological mechanisms in disease subsets.

Our work further supports an active role of B-cells in the pathogenesis of GCA/PMR. Thus far no disease specific auto-antibodies have been documented in GCA/PMR. There is however ample evidence that the B-cells can also play an antibody-independent, cytokines-mediated role in autoimmune and autoinflammatory diseases. Previous work from our group [13] demonstrated that the circulating effector B-cells (likely returning from the sites of inflammation to the peripheral blood upon glucocorticoid-induced remission) have an enhanced capacity to produce IL-6 in GCA and PMR patients. Given the important role played by IL-6 in the pathogenesis of GCA and the new insights into the B-cell compartment in GCA/PMR, it would be interesting to investigate the therapeutic effect of B-cell depleting therapy in GCA/PMR. We currently investigate the role of B-cells in the production of other cytokines. B-cells are also capable of antigen presentation at the site of tissue inflammation. This mechanism has, to the best of our knowledge, not yet been investigated in GCA and PMR.

There are several limitations of our study. First, chemokine gradients determine the migration of chemokine sensitive cells. As the chemokines levels in biopsies and circulation were not measured with the same technique, a direct chemokine gradient comparison was not feasible. Second, the studied chemokines and chemokine receptors are not exclusive for B-cell trafficking and are also used by other lymphocytes such as T-cells. Third, this study did not include local expression of B-cells, chemokines and chemokine receptors in PMR patients as synovial biopsies of PMR patients are not routinely taken for diagnostic purposes.

5. Conclusion

In conclusion, the data presented here support an important role for the CXCL9-CXCR3 and CXCL13-CXCR5 axes in shaping the peripheral B-cell compartment in GCA and PMR and organization of B-cells in TLOs in GCA. This study provides further grounds for an active involvement of B-cells in the pathogenesis of GCA and PMR.

Author contribution statement

Jacoba Graver: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing- Original Draft, Visualization. Wayel Abdulahad: Methodology, Writing – review & editing, Supervision. Kornelis van der Geest: Writing – review & editing. Peter Heeringa: Writing – review & editing, Supervision. Annemieke Boots: Conceptualization, Writing – review & editing, Supervision. Elisabeth Brouwer: Conceptualization, Writing – review & editing, Supervision. Maria Sandovici: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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Declaration of competing interest

AB was a consultant for Grünenthal Gmbh until 2017. EB as an employee of the UMCG received speaker/consulting fees from Roche paid to the UMCG. PH and EB received funding from the European Union's Horizon 2020 research and innovation program [grant agreement 668036]. The other authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2021.102684.

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