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Cytokine profiles in patients with Q fever fatigue syndrome

Ruud P.H. Raijmakers[a,b,*], Valerie A.C.M. Koeken[b], Anne F.M. Jansen[a,b], Stephan P. Keijmel[a,b], Megan E. Roerink[b], Leo A.B. Joosten[a,b,c], Mihai G. Netea[a,b,c], Jos W.M. van der Meer[a,b], Chantal P. Bleeker-Rovers[a,b,c]

[a] Radboud Expertise Center for Q Fever, Department of Internal Medicine, Division of Infectious Diseases, Radboud University Medical Center, P.O. Box 9101, 6500 HB, Nijmegen, the Netherlands
[b] Department of Internal Medicine, Radboud University Medical Center, P.O. Box 9101, 6500 HB, Nijmegen, the Netherlands
[c] Radboud Center for Infectious Diseases, Radboud University Medical Center, P.O. Box 9101, 6500 HB, Nijmegen, the Netherlands

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SUMMARY

Background: Q fever fatigue syndrome (QFS) is a state of prolonged fatigue following around 20% of acute Q fever cases. It is thought that chronic inflammation plays a role in its etiology. To test this hypothesis we measured circulating cytokines and the *ex-vivo* cytokine production in patients with QFS and compared with various control groups.

Materials/methods: Peripheral blood mononuclear cells (PBMCs), whole blood, and serum were collected from 20 QFS patients, 19 chronic fatigue syndrome (CFS) patients, 19 Q fever seropositive controls, and 25 age- and sex-matched healthy controls. *Coxiella*-specific *ex-vivo* production of tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6, and interferon (IFN) was measured, together with a total of 92 circulating inflammatory proteins.

Results: PBMCs of QFS patients produced more IL-6 ($P = 0.0001$), TNFα ($P = 0.0002$), and IL-1β ($P = 0.0005$) than the various control groups when stimulated with *Coxiella* antigen. QFS patients had distinct differences in circulating inflammatory markers compared to the other groups, including higher concentrations of circulating IL-6 and IFNγ.

Conclusion: QFS patients showed signs of chronic inflammation compared to asymptomatic Q fever seropositive controls, CFS patients, and healthy controls, of which the monocyte-derived cytokines TNFα, IL-1β, and especially IL-6, are likely crucial components.

Introduction

Between 2007 and 2011, the Netherlands experienced the largest Q fever outbreak ever reported which led to at least 32,200 infected individuals and over 4000 notified cases of symptomatic disease, i.e., acute Q fever.[1,2] Taking into account that around 60% of initial infections with *C. burnetii* remain asymptomatic,[3] these numbers are probably an underestimation of the actual magnitude of this outbreak.

Q fever fatigue syndrome, or QFS, is a debilitating postinfective fatigue syndrome that occurs in around 20% of acute Q fever patients. QFS is characterized by a state of prolonged fatigue that lasts for at least 6 months and often coincides with various other complaints.[4] Contrary to the less common, but more notorious, other long-term sequella following infection with *C. burnetii*, i.e., chronic Q fever, or persistent localized infection,[5–7] no indications of active or persistent infection were convincingly found in QFS.[8,9]

Although the etiology of QFS remains unknown, complaints such as fatigue, musculoskeletal pain, headache, night sweating, and recurrent upper respiratory tract infections suggest that an inflammatory component might contribute to its pathogenesis. In 1998, Pentilla *et al.* reported that peripheral blood mononuclear cells (PBMCs) of QFS patients produced significantly more interleukin (IL)−6 than cells of various control groups when exposed to Q fever antigens.[10] Our group has demonstrated that QFS patients exhibit *Coxiella*-specific enhanced interferon-(IFN)

* Corresponding author at: Ruud P.H. Raijmakers, Department of Internal Medicine 463, Radboud university medical center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.
E-mail addresses: Ruud.Raijmakers@radboudumc.nl (R.P.H. Raijmakers), Valerie.Koeken@radboudumc.nl (V.A.C.M. Koeken), Anne.FM.Jansen@radboudumc.nl (A.F.M. Jansen), Stephan.Keijmel@radboudumc.nl (S.P. Keijmel), Megan.Roerink@radboudumc.nl (M.E. Roerink), Leo.Joosten@radboudumc.nl (L.A.B. Joosten), Mihai.Netea@radboudumc.nl (M.G. Netea), Jos.vanderMeer@radboudumc.nl (J.W.M. van der Meer), Chantal.Bleeker-Rovers@radboudumc.nl (C.P. Bleeker-Rovers).

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γ-production compared to Q fever seropositive controls.[11,12] These findings urged us to further explore inflammation in QFS.

In this study, we investigated signs of a long-lasting inflammatory component in QFS by conducting stimulation experiments on both PBMCs and whole blood, and collecting serum of; QFS patients, CFS patients, asymptomatic Q fever seropositive controls, and healthy controls, all matched for age and sex. By measuring the production of key cytokines, e.g. IFNγ and IL-6, and concentrations of a total of 92 circulating inflammatory markers, we explore and validate previous immunologic findings and further broaden our scope on chronic inflammatory processes in QFS.

Methods

Study population

The study population consisted of QFS patients (n = 20), CFS patients (n = 19), asymptomatic Q fever seropositive controls (n = 19), and healthy controls (n = 25), matched for age (± 10 years) and sex.

All QFS patients were diagnosed at the Radboud Expertise Center for Q fever, Nijmegen, the Netherlands, after a uniform work-up according to the Dutch guideline on QFS.[13] All QFS patients met the following diagnostic criteria: (1) fatigue lasting ≥ 6 months; (2) sudden onset of severe fatigue (defined as a score ≥ 35 on the subscale fatigue severity of the Checklist Individual Strength (CIS) questionnaire), or significant increase in fatigue, both related to a symptomatic acute Q fever infection; (2) chronic Q fever, or persistent localized infection, and other causes of fatigue, somatic or psychiatric, being excluded; and (4) fatigue resulted in significant functional impairment (defined as a total score ≥ 450 on the Sickness Impact Profile-8 (SIP-8) questionnaire).[14]

All CFS patients were diagnosed with CFS at the Department of Internal Medicine and Expert Center for Chronic Fatigue (ECCF) of the Radboud university medical center, Nijmegen, the Netherlands, after a uniform work-up according to the Centers for Disease Control (CDC) criteria for CFS. All CFS patients tested negative on Q fever serology (Immunofluorescence assay, or IFA; Focus Diagnostics, Cypress, CA, USA) and, additionally, had a score ≥35 on the subscale fatigue severity of the CIS questionnaire and a score ≥450 on the SIP-8 questionnaire.

Asymptomatic Q fever seropositive controls consisted of known former acute Q fever patients and Q fever seropositives who were asked to participate by the primary investigator (RR). These individuals tested positive on Q fever serology ≥ 5 years after the Q fever outbreak (IgG phase I or II ≥1:16, but IgG phase I <512 on IFA), and reported no complaints of fatigue or functional impairment.

Colleagues from the Department of Internal Medicine at the Radboud University Medical Center, Nijmegen, who lived in areas previously endemic for Q fever during the Dutch outbreak between 2007 and 2011, were asked to participate by the primary investigator (RR) as healthy controls. They all tested negative on Q fever serology (IFA), and reported no complaints of fatigue or functional impairment.

PBMC stimulation

PBMC isolation was performed by dilution of blood in PBS (1:1) and fractions were separated by density centrifugation over Ficoll-Paque (Ficoll-Paque Plus; GE healthcare, Zeist, The Netherlands). Cells were washed three times with cold PBS and resuspended in RPMI 1640 Dutch modification culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) supplemented with 50 μg/mL gentamicin, 2 mM Glutamax™, and 1 mM pyruvate (Life Technologies). PBMCs were then plated in 96-well round-bottom plates (Corning) at a concentration of 5×10^5/mL in a total volume of 200 µL. The samples were exposed to heat-inactivated *C. burnetii* Nine Mile (NM) RSA 493 phase I (1×10^7/mL) for 24 hours at 37°C with 5% CO_2. After stimulation, supernatants were collected and stored at −20°C until cytokine assays were performed.

In-vitro whole blood stimulation

Whole blood stimulation and subsequent measurement of IFNγ production was done as previously described.[15] In brief, venous blood was drawn into 5 mL endotoxin-free lithium-heparin tubes (Vacutainer, BD Bioscience). Incubation of samples was done as previously described.[15] Heat-inactivated *C. burnetii* Nine Mile (NM) RSA 493 phase I was used as a stimulus at a concentration of 1×10^7/mL.[16] After incubation, supernatants were collected and stored at −20°C until the cytokine assay was performed.

Cytokine assays

By determining *C. burnetii*-specific production of cytokines TNFα, IL-1β, and IL-6, but also of IFNγ, we aimed to validate previous results from our group and revisit the theory that QFS patients exhibit a long-lasting altered immune response. We compared them to asymptomatic Q fever seropositive controls, but also CFS patients and healthy controls.[10-12] Tumor necrosis factor (TNF)α, Il-1β, IL-6, and IFNγ were measured using enzyme-linked immune sorbent assay (ELISA) according to the manufacturer's protocol (IL6 and IFNγ: Sanquin, Amsterdam, the Netherlands; and TNFα, IL1β: R&D Systems, Minneapolis, USA).

Proximity extension assay (PEA)

To further substantiate differences in inflammatory profiles between the groups, circulating inflammatory proteins were determined. Inflammation biomarker profiles were analyzed by the analysis service of Olink Proteomics AB (Uppsala, Sweden), using their PEA based Proseek© Multiplex Inflammation panel$^{96 \times 96}$.[17] This analysis simultaneously measures 92 selected inflammatory proteins using 1 μL of serum. For each protein, there are two separate antibodies connected to one oligonucleotide each. After binding by the antibody pair to its target, the 3′ ends of the oligonucleotides hybridize, priming a DNA polymerization reaction that forms a protein-specific reporter DNA sequence for each detected protein molecule. The reporter DNA strands are then quantified using qPCR. Four internal controls and two external controls were included in each assay.

Statistical analysis

Patient characteristics

Data were analyzed using Graphpad Prism (Graphpad Software Inc., version 5.03) and SPSS (Version 22.0, SPSS, Inc). ANOVA was used to determine differences between groups.

Cytokine assays

For cytokine production analysis, the differences between groups were analyzed using the Kruskal-Wallis test in GraphPad Prism (Graphpad Software Inc., version 5.03). The level of significance was defined as a P value ≤ 0.05.

PEA analysis

The raw Cq values were normalized for variation between and within runs and converted into Normalized Protein Expression Units (NPX). The NPX values are expressed on a Log2 scale where one unit higher NPX values represent a doubling of the measured

Table 1
Characteristics of healthy controls, chronic fatigue syndrome (CFS) patients, Q fever fatigue syndrome (QFS) patients, and asymptomatic Q fever seropositive controls.

Characteristics	Healthy controls(n = 25)	CFS(n = 19)	QFS(n = 20)	Q fever seropositives(n = 19)
Male sex, number (%)	11 (44)	6 (32)	10 (50)	11 (58)
Age, years Median (IQR)	40 (26–60)	46 (38–52)	47 (41–60)	57 (28–60)
Duration of symptoms, months[a] Median (IQR)	-	108 (39 - 240)	83 (69–86)	-
CIS subscale fatigue severity score, mean ± SD	-	51 ± 4.1	52 ± 3.7	-
SIP-8 total score, mean ± SD	-	1398 ± 631.8	1416 ± 505.6	-

Abbreviations: *QFS* = Q fever fatigue syndrome; CFS = chronic fatigue syndrome; *IQR* = interquartile range; *CIS* = Checklist Individual Strength; *SD* = standard deviation; *SIP-8* = Sickness Impact Profile-8.
[a] Symptom duration: time onset of symptoms until blood sampling.

protein concentrations. This arbitrary unit can be used for relative quantification of proteins and comparing the fold changes between groups. Inflammatory markers showing ≥35% of values under the detection limit in all groups were excluded from analysis. Excluded markers had an even distribution between groups. Protein values under the detection threshold were replaced with the proteins' lower limit of detection. All computational analyses were performed in R 3.3.3. Global proteins signatures were analyzed using Spearman's Rank-Order correlations and clustered using average hierarchical clustering, visualized using the R package "corrplot". Then, individual protein levels were compared between subgroups using Kruskal-Wallis tests, and post hoc Mann-Whitney U tests. Additional packages used for the analysis included "ggplot2," "devtools, " and "ggbiplot. " Statistical significance was attained if $P < 0.05$.

Ethical statement

All participants provided written informed consent and the study was approved by the Medical Ethical Review Committee of the Arnhem-Nijmegen region (NL52893.091.15).

Results

Patients and controls

At the time of blood collection, the median symptom duration of QFS and CFS did not differ significantly ($P = 0.10$), nor did the median age of all groups ($P = 0.42$) (Table 1). As there is a female preponderance in CFS, the proportion of females in the CFS group was higher than in the other groups. All QFS patients and asymptomatic Q fever seropositive controls had IgG phase I or phase II titers ≥1:16, but IgG phase I ≤ 1:512, and none of them showed serological signs of an acute or recent Q fever infection, reflected by IgM antibodies in absence of IgG antibodies.

Production of TNFα, IL-1β, IL-6, and IFNγ

Stimulation of PBMCs with *C. burnetii* NM phase I antigen for 24 hours resulted in a significantly higher median TNFα production in the QFS group (2178 pg/mL; interquartile range, or IQR, 1288–3474), than the CFS group (485 pg/mL; IQR 351–964) and healthy controls (723 pg/mL; IQR 397–1140) ($P = 0.0002$). No significant difference was found with the asymptomatic Q fever seropositive control group (1457 pg/mL; IQR 513–2073) (Fig. 1A). Median IL-1β production was slightly, albeit significantly, higher in the QFS group (283 pg/mL; IQR 196–525) than in the CFS group (195 pg/mL; IQR 195–195) and asymptomatic Q fever seropositive control group (195 pg/mL; IQR 195–216) ($P = 0.0005$), but not the healthy control group (195 pg/mL; IQR 195–305) (Fig. 1B). The median IL-6 production was significantly higher in the QFS group (4062 pg/mL; IQR 2984–5999), than in the CFS group (2216 pg/mL; IQR 1729–2820), asymptomatic Q fever seropositive control group (2162 pg/mL; IQR 1383–2696), and healthy control group (2320 pg/mL; IQR 1644–2990) ($P = 0.0001$) (Fig. 1C). Stimulation of whole blood with *C. burnetii* NM phase I antigen for 24 hours revealed no significant difference in median IFNy production between the QFS group (116 pg/mL; IQR 46–186) compared to CFS (53 pg/mL; IQR 34–93), asymptomatic Q fever seropositive control (115 pg/mL; IQR 45–171), and healthy control group (33 pg/mL; IQR 19–55) ($P = 0.392$) (Fig. 2).

Circulating inflammatory markers

After employing a cut-off of ≥35% of inflammatory marker values under the detection limit, a total of 81 inflammatory markers were analyzed. Global correlation patterns by means of average clustering were analysed for each group (Fig. 3), after which we used the healthy control group clustering pattern as a reference for the other groups, exposing a differential correlation in inflammatory proteins for QFS patients, CFS patients, and asymptomatic Q fever seropositive controls, compared to healthy controls (Fig. 4). A similar analysis was performed using CFS patients and asymptomatic Q fever seropositive controls as a reference for QFS patients (Fig. 5A and B, respectively). Inflammatory proteins with concentrations that were significantly different compared to healthy controls were identified in CFS patients (CXCL9 ($P = 0.001$), Caspase 8 ($P = 0.001$), HGF ($P = 0.001$), TGFα ($P = 0.002$), CST5 ($P = 0.005$), OSM ($P = 0.007$), AXIN1 ($P = 0.022$), ST1A1 ($P = 0.026$), TNFRSF9 ($P = 0.028$), CXCL10 ($P = 0.042$), and CX3CL1 ($P = 0.048$)), QFS patients (CDCP1 ($P = 0.005$), ARTN ($P = 0.012$), IL-6 ($P = 0.026$), and IL-20 ($P = 0.028$)), and asymptomatic Q fever seropositive controls (ST1A1 ($P = 0.002$), IL-7 ($P = 0.003$), Flt3L ($P = 0.006$), AXIN1 ($P = 0.010$), STAMPB ($P = 0.013$), FGF 21 ($P = 0.017$), SIRT2 ($P = 0.017$), CXCL5 ($P = 0.023$), IFNγ ($P = 0.041$), CSF1 ($P = 0.045$), and CXCL9 ($P = 0.050$)) (Fig. 6A, B, and C). Additionally, inflammatory proteins were identified that differed significantly in CFS patients (Caspase 8 ($P = 4.070^{-7}$), CCL28 ($P = 0.001$), CXCL9 ($P = 0.003$), TNFRSF9 ($P = 0.007$), IL-17C ($P = 0.009$), CCL20 ($P = 0.013$), CST5 ($P = 0.018$), CCL23 ($P = 0.026$), OPG ($P = 0.028$), CDCP1 ($P = 0.046$), and TNFSF14 ($P = 0.020$)) and asymptomatic Q fever seropositive controls (CCL28 ($P = 0.002$), TGF21 ($P = 0.003$), IL-7 ($P = 0.005$), IFNγ ($P = 0.008$), CCL19 ($P = 0.011$), OPG ($P = 0.011$), ARTN ($P = 0.016$), Flt3L ($P = 0.018$), CCL20 ($P = 0.018$), IL-20 ($P = 0.020$), AXIN1 ($P = 0.023$), and ST1A1 ($P = 0.041$)), compared to QFS patients (Fig. 6D and E, respectively).

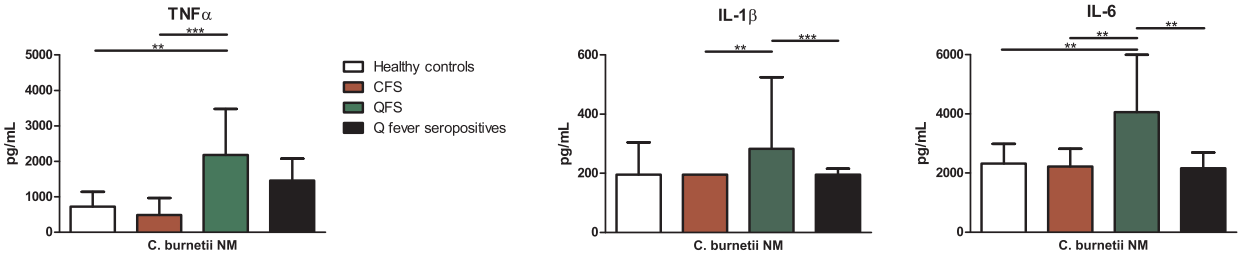

Fig. 1. Production of IL-6, TNFα, and IL-1β after 24 h incubation of PBMCs with *C. burnetii* Nine Mile.
C. burnetii NM-induced median cytokine production after 24 h incubation of PBMC's of healthy controls (n = 25), CFS patients (n = 19), QFS patients (n = 20), and asymptomatic Q fever seropositive controls (n = 19). (A) Median TNFα production, (B) median IL-1β production, and (C) median IL-6 production. Data are depicted as median with IQR.
Abbreviations: *IL* = interleukin; *TNF* = tumor Necrosis Factor; *CFS* = chronic fatigue syndrome; *QFS* = Q fever fatigue syndrome; *Q fever seropositives* = Q fever seropositive controls; *C. burnetii NM* = *Coxiella burnetii* Nine Mile 493 phase I strain; *PBMC* = peripheral blood mononuclear Cell; *IQR* = interquartile range.
*** $P \leq 0.001$
** $P \leq 0.01$.

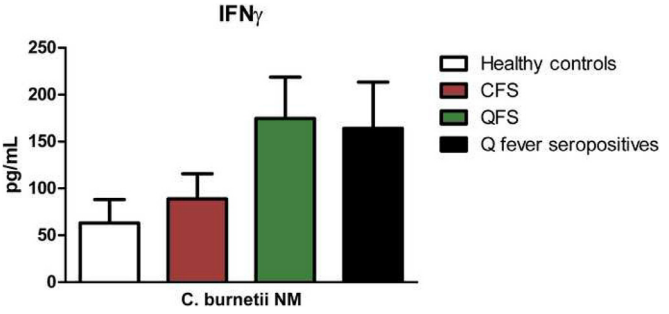

Fig. 2. Production of IFNγ after 24 h incubation of whole blood with *C. burnetii* Nine Mile.
C. burnetii NM-induced median IFNγ production after 24 h incubation of whole blood of healthy controls (n = 25), CFS patients (n = 19), QFS patients (n = 20), and asymptomatic Q fever seropositive controls (n = 19), showing no significant difference between QFS patients (116 pg/mL; IQR 46–186), CFS patients (53 pg/mL; IQR 34–93), asymptomatic Q fever seropositive controls (115 pg/mL; IQR 45–171), and healthy controls (33 pg/mL; IQR 19–55) (P = 0.392).
Abbreviations *IFN* = interferon; *CFS* = chronic fatigue syndrome; *QFS* = Q fever fatigue syndrome; *Q fever seropositives* = Q fever seropositive controls; *C. burnetii NM* = *Coxiella burnetii* Nine Mile 493 phase I strain; *IQR* = interquartile range.

Discussion

This study set out to investigate the question whether there is chronic inflammation in QFS. We found that PBMCs from QFS patients produced higher amounts of monocyte-derived cytokines IL-6, TNFα and IL-1β, when stimulated with Q fever antigen, compared to various control groups. Especially the higher production of IL-6 and IL-1β in QFS patients compared to asymptomatic Q fever seropositive controls is of great interest and suggestive for a more persistent immune response to *Coxiella* antigens in QFS. Additionally, different profiles of circulating inflammatory proteins were found in QFS patients, CFS patients, and asymptomatic Q fever seropositive controls, compared to healthy controls. QFS patients also exhibited a profile that differed from asymptomatic Q fever seropositive controls and CFS patients. These results suggest that chronic inflammation is a standing characteristic of QFS, most likely acting through monocyte-derived derived cytokines TNFα, IL-1β, and especially IL-6, together with the IFNγ-axis.

Our findings of enhanced IL-6 production by PBMCs of QFS patients after *in-vitro* exposure to *Coxiella* corroborate those of Pentilla *et al.*[10] It is of interest that circulating IL-6 concentrations were also significantly increased in QFS patients compared to healthy controls (Supplementary Figure 1A). It has been suggested that QFS patients experience chronic immune stimulation through persisting non-viable *C. burnetii* antigens.[8] If such antigens indeed persist, this could in turn lead to increased concentrations of circulating IL-6 through chronic stimulation of cytokine-producing cells. Additionally, it should be noted that high concentrations of IL-6, and subsequently CRP, were found to correlate with disease severity during an acute Q fever infection.[18] Unfortunately, no correlation has been found between acute phase IL-6 concentrations and development of fatigue. It would be interesting to investigate the longitudinal IL-6 concentration in these patients in order to try and predict which patients will develop QFS and which patients recover normally from their acute Q fever infection.

Recent studies have shown that QFS patients show a higher *Coxiella*-specific IFNγ response compared to Q fever seropositive controls.[11,12] This response is, however, lower than in patients with chronic Q fever, or persistent localized infection. These studies underscore that there is a degree of *Coxiella*-specific immune activation in QFS. The question remains whether this response could be a diagnostic marker of QFS in individual patients. In the present study no difference in *Coxiella*-specific whole blood IFNγ production was observed between QFS patients and asymptomatic Q fever seropositive controls, although levels of circulating IFNγ tended to be higher in QFS patients (Supplementary Figure 1B). It is of interest that asymptomatic Q fever seropositive controls showed lower levels of circulating IFNγ than healthy controls. It is possible that this is part of the recovery process of acute Q fever. The fact that we did not find differences in *Coxiella*-specific whole blood IFNγ production between QFS patients and asymptomatic Q fever seropositive controls in the present study, as compared with previous studies,[11] could be explained by the observation that patients with more severe and longer-lasting acute Q fever produce more IFNγ.[19] In the current study, 13 out of 19 asymptomatic Q fever seropositive controls suffered from a symptomatic initial infection, i.e., acute Q fever, whereas the Q fever seropositive controls of the previous study all had had an asymptomatic initial infection.

Mege *et al.* recently published a group of Q fever patients who developed B-cell non-Hodgkin lymphomas. The investigators stimulated PBMCs of acute Q fever patients, patients with persistent focal infection, and Q fever seropositives with concomitant lymphoma, and found that the latter group produced less TNFα but not IL-10. Interestingly, unstimulated PBMC's of this group produced significantly more IL-10 but not TNFα. Unfortunately, the groups that were used are not comparable to the ones used in this study as patients with lymphoma are by definition not asymptomatic and often experience complaints of fatigue.

With regard to the chronic immune stimulation hypothesis in QFS,[8] persisting non-viable *C. burnetii* antigens could activate effector and memory T-cells, resulting in increased production of

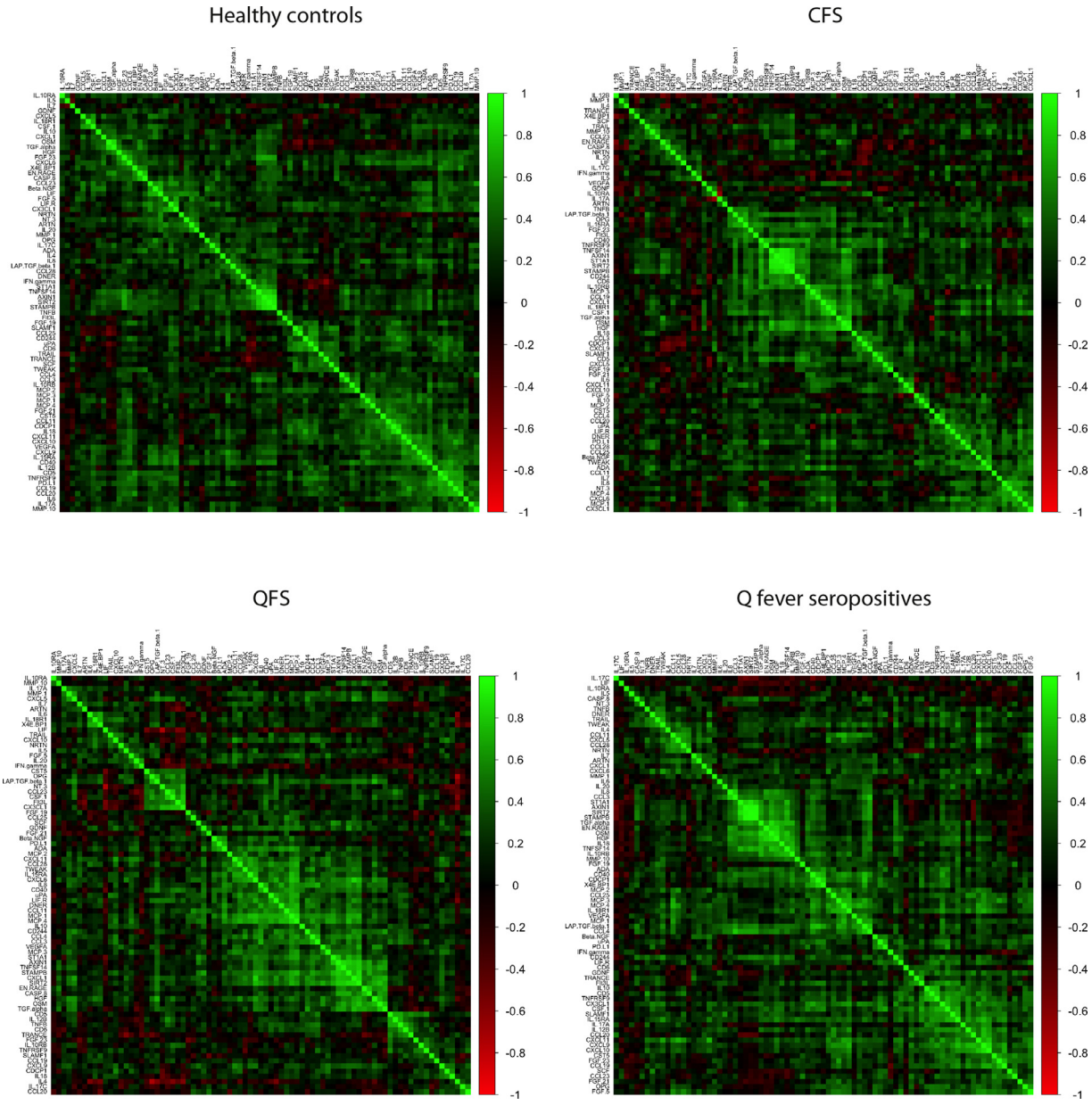

Fig. 3. Global correlation structures of healthy controls, chronic fatigue syndrome (CFS) patients, Q fever fatigue syndrome (QFS) patients, and asymptomatic Q fever seropositive controls.
Global correlation structures of healthy controls (n = 25), CFS patients (n = 19), QFS patients (n = 20), and asymptomatic Q fever seropositive controls (n = 19). Pearson's correlation coefficients in 81 serum proteins of each group.
Abbreviations: *CFS* = chronic fatigue syndrome; *QFS* = Q fever fatigue syndrome; *Q fever seropositives* = asymptomatic Q fever seropositive controls.

IFNγ, which also appears in the circulation. An important question is whether the enhanced immune response could also be due to epigenetic reprogramming, a phenomenon called trained immunity.[20,21] The observation that monocyte-derived cytokines IL-6, TNFα, and IL-1β are produced in significantly higher amounts than in control groups would be compatible with such a mechanism. Recent non-specific stimulation experiments by our group however showed a lower production of these cytokines in QFS patients who experience longer lasting and more severe upper respiratory tract infections compared to healthy controls. This observation could be explained by the counterpart of trained immunity, i.e. tolerance, suggesting a diminished immune response due to epigenetic reprogramming.[22] The fact that we found an altered epigenetic profile on the promoter regions of various cytokines in these patients, suggests that such a mechanism might indeed play part in the altered immune activation found in QFS. This altered activation could for instance result from the acute Q fever infection itself or the persistence of non-viable Q fever antigens.

When we scrutinize the profiles of circulating inflammatory proteins in QFS patients, CFS patients, and asymptomatic Q fever seropositive controls and compared to healthy controls, there are clear differences. In QFS, both Artemin (ARTN) and IL-20 appear to be increased compared to both asymptomatic Q fever seropositive controls and healthy controls. In CFS patients, a differential expression of several inflammatory proteins is found compared to healthy controls. Although previous studies have shown similar expression of CXCL9, Caspase 8, TGFα, and CX3CL1,[23-25] the decreased expression of CXCL10 is in conflict with existing literature which indicates towards an increased expression of this chemokine, compared to healthy controls.[24,25] As CXCL9 and CXCL10 are closely related, one

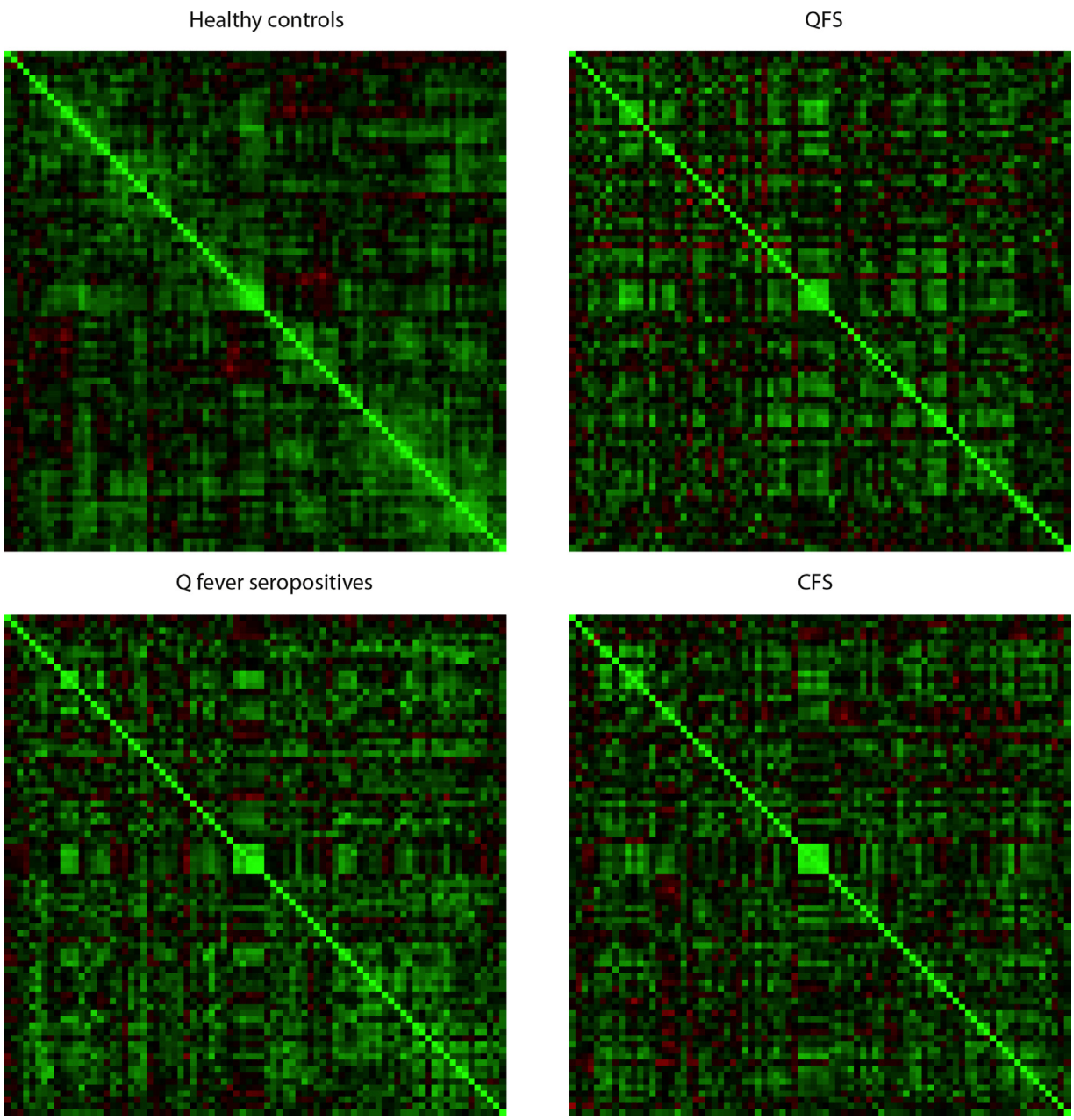

Fig. 4. Global correlation structures of chronic fatigue syndrome (CFS) patients, Q fever fatigue syndrome (QFS) patients, and asymptomatic Q fever seropositive controls, compared to healthy controls.
Global correlation structures of CFS patients (n = 19), QFS patients (n = 20), and asymptomatic Q fever seropositive controls (n = 19), compared to healthy controls (n = 25). Pearson's correlation coefficients in 81 serum proteins, the healthy control group is used as a reference and the proteins in the CFS, QFS, and asymptomatic Q fever seropositive controls groups are ordered accordingly.
Abbreviations: *CFS* = chronic fatigue syndrome; *QFS* = Q fever fatigue syndrome; *Q fever seropositives* = asymptomatic Q fever seropositive controls.

would expect to see a similar trend in these chemokines. In our cohort, we find a decreased expression of both CXCL9 and CXCL10 in CFS patients compared to healthy controls. No increased concentrations of TNFα were found and, while circulating IL-1β is notoriously difficult to measure, it was excluded from this PEA.[26] With regard to CFS, measurements of circulating IL-6 show conflicting results.[25] Like in the present investigation, a recent study of our group in 50 CFS patients and neighborhood controls, matched for age and sex, did not find significant differences in IL-6 concentrations using PEA.[27] This study by Roerink *et al.* used the same PEA and further investigated a set of markers predictive for CFS. Possible explanations for the different results in both studies could lie in differences in gender (Roerink *et al.* only included female participants), age, group size, and matching to controls. These differences in results again highlight the importance of developing universal and adequate inclusion criteria and use of comparable control groups.

Looking at comparisons between QFS patients and both asymptomatic Q fever seropositive controls and CFS patients, interesting observations can be made. For one, several inflammatory markers appear to be less expressed in CFS patients compared to QFS patients. It was previously described that, despite a striking overlap in symptoms, QFS and CFS are not the same entity and show differences in perpetuating factors for fatigue.[28] This can now be supplemented by a concomitant difference in inflammatory profiles with QFS patients exhibiting more of an inflammatory profile

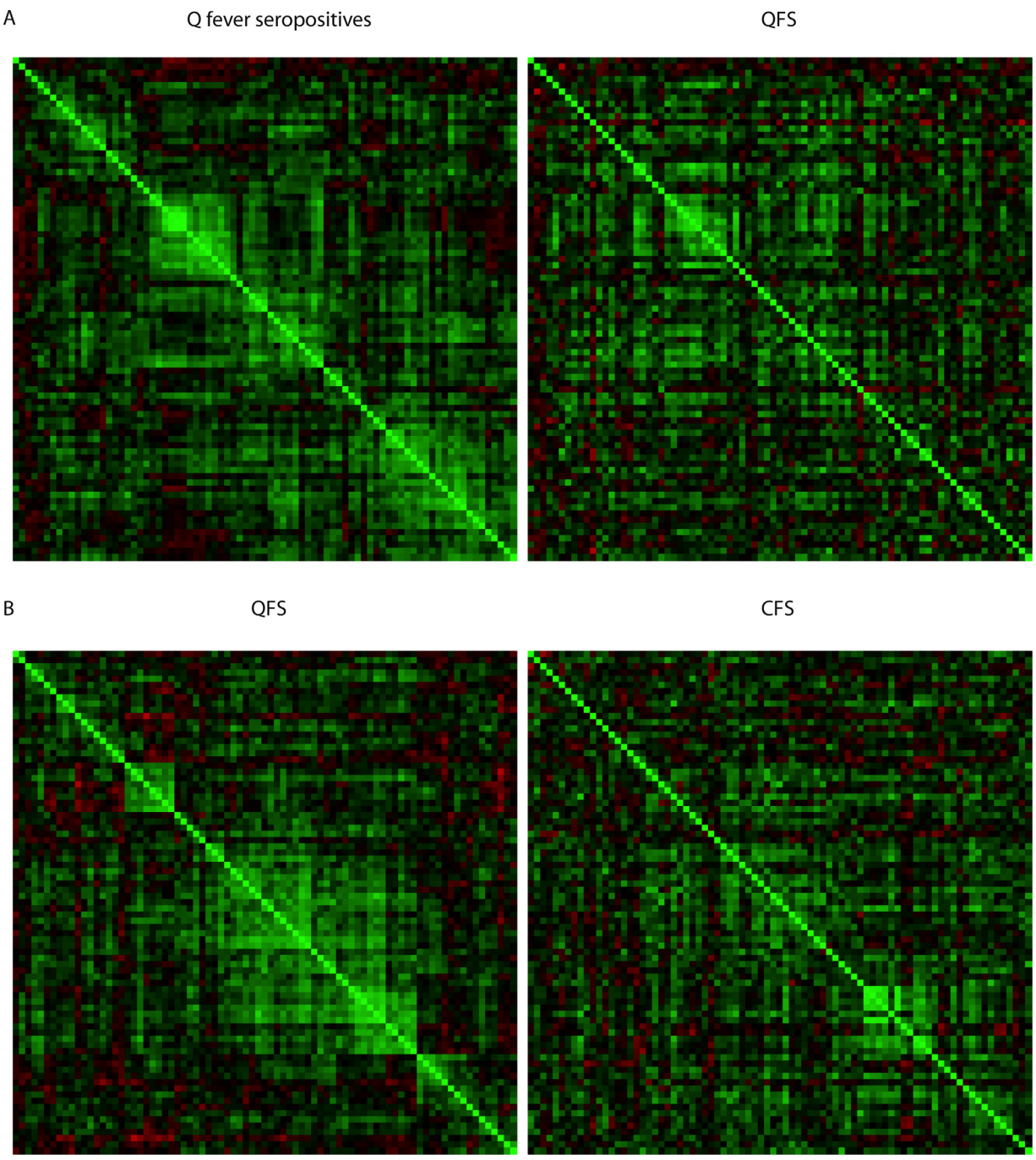

Fig. 5. Global correlation structures of asymptomatic Q fever seropositive controls and chronic fatigue syndrome (CFS) patients, compared to Q fever fatigue syndrome (QFS) patients.
Global correlation structures of asymptomatic Q fever seropositive controls (n = 19) and CFS patients (n = 19), compared to QFS patients (n = 20). Pearson's correlation coefficients in 81 serum proteins, the asymptomatic Q fever seropositive control and CFS patient groups are used as a respective references and the proteins in the QFS patient group are ordered accordingly.
Abbreviations: *CFS* = chronic fatigue syndrome; *QFS* = Q fever fatigue syndrome; *Q fever seropositives* = asymptomatic Q fever seropositive controls.

than CFS patients. One could speculate that the infectious trigger that initiated complaints in QFS plays part in this difference. The observation that asymptomatic Q fever seropositive controls exhibit less of an inflammatory profile than QFS patients strengthens the theory that QFS might partly be driven by an inflammatory component that would normally wane off following an acute Q fever infection.

Although the exact pathophysiology remains elusive and is probably multifactorial, these results show that QFS patients have an altered inflammatory profile compared to various control groups, arguing for a role of chronic inflammation in this clinical condition. This altered status is seen both after stimulation of PBMCs, and in profiles of circulating inflammatory proteins. More insight in the regulation of these responses may aid to find interventions for treating QFS patients. Given these results, hypotheses such as chronic immune stimulation and epigenetic reprogramming of innate immune cells are of particular interest for further investigation.

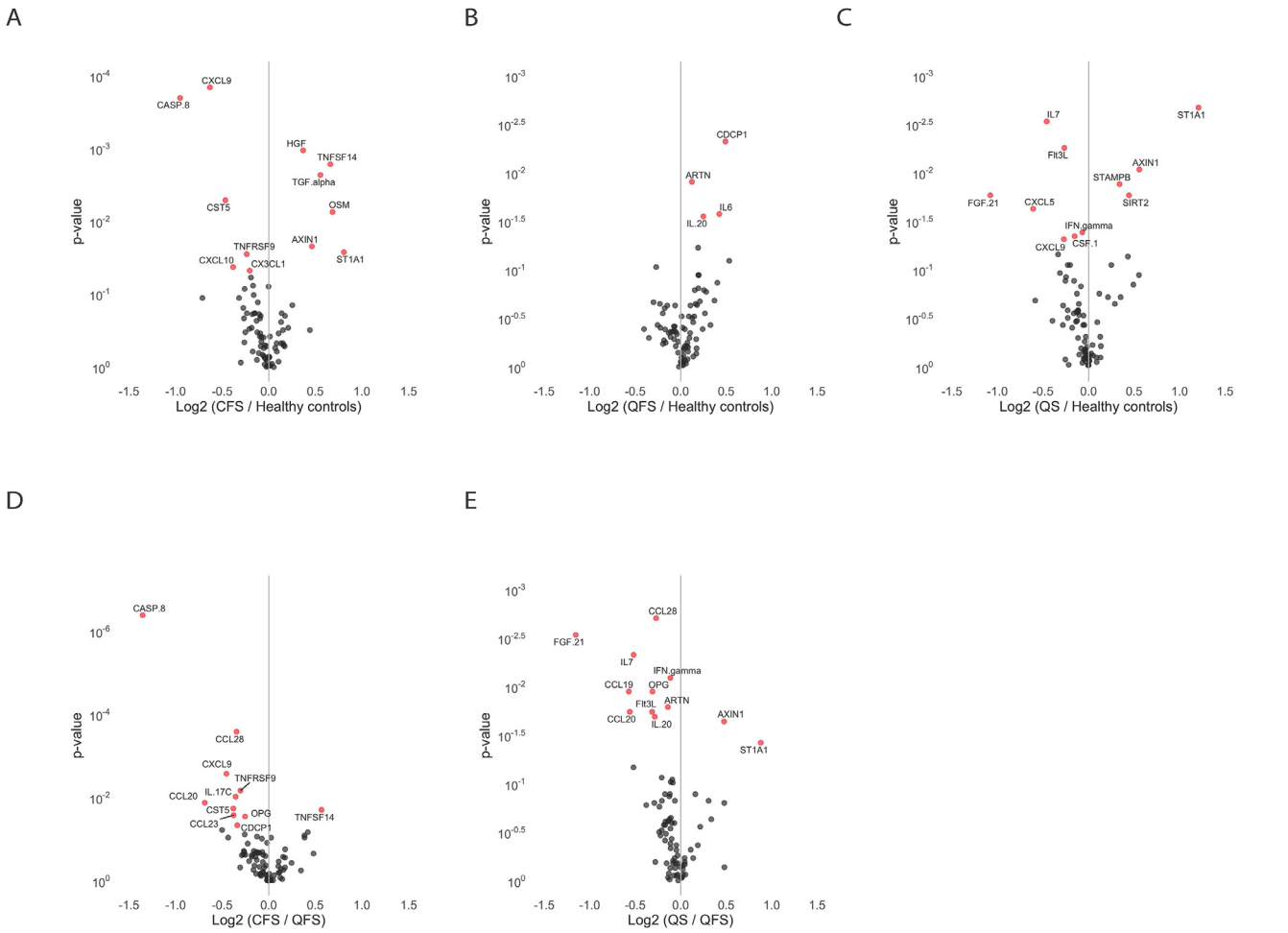

Fig. 6. Volcanoplots showing differential expression of inflammatory proteins in chronic fatigue syndrome (CFS) patients, Q fever fatigue syndrome (QFS) patients, and asymptomatic Q fever seropositive controls, compared to healthy controls, together with differential expression in CFS patients and Q fever seropositive controls compared to QFS patients.

Volcanoplots showing differential expression of various circulating inflammatory proteins in (A) CFS patients (n = 19), (B) QFS patients (n = 20), and (C) asymptomatic Q fever seropositive controls (n = 19), compared to healthy controls (n = 25). Volcanoplots showing differential expression of various circulating inflammatory proteins in CFS patients (D) and asymptomatic Q fever seropositive controls (E), compared to QFS patients.

Abbreviations: *CFS* = chronic fatigue syndrome; *QFS* = Q fever fatigue syndrome; *CXCL* = (C-X-C motif) ligand; *Casp* = caspase; *HGF* = hepatocyte growth factor; *TNFSF* = tumor necrosis factor superfamily; *TGF* = transforming growth factor; *CST* = cystatin-D; *OSM* = oncostatin M; *TNFRSF* = tumor necrosis factor receptor superfamily; *ST1A1* = sulfotransferase 1A1; *CX3CL* = (C-X3-C motif) ligand; *CDCP* = CUB domain-containing protein 1; *ARTN* = artemin; *IL* = interleukin; *Flt3L* = FMS-like tyrosine kinase 3 ligand; *STAMPB* = STAM-binding protein; *FGF* = fibroblast growth factor; *SIRT* = sirtuin; *IFN* = interferon; *CSF* = colony stimulating factor; *CCL* = (C–C motif) ligand; *OPG* = osteoprotegerin.

Possible limitations of this study include the fact that this an exploratory study which did not correct for multiple testing. We would therefore like to point out that type 1 errors might have occurred and urge that these results are interpreted with care and validated in different cohorts. Furthermore, it would be preferred to solely use subject who experienced a symptomatic acute Q fever infection as asymptomatic Q fever seropositive controls. For this study, a more adequate control group, i.e., 19 instead of 13 asymptomatic Q fever seropositive controls who reported having had an acute Q fever infection, could unfortunately not be found. We do however feel that with at least 13 out of 19 subjects having had a symptomatic acute Q fever infection, the group is sufficiently adequate for analysis.

Conclusion

QFS patients show signs of altered immunity and inflammatory profile compared to asymptomatic Q fever seropositive controls, CFS patients, and healthy controls. As was previously shown, monocyte-derived cytokines TNFα, IL-1β, and especially IL-6, together with the IFNγ-axis, are likely components of this altered inflammatory reaction and warrant further investigation. It is of interest to further investigate underlying processes of these observations as they could show potential for intervention. Based on these results, we recommend focusing on the possible epigenetic reprogramming of innate immune cells and chronic immune stimulation through persisting non-viable Q fever antigens.

Conflict of Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.01.006.

References

1. Kampschreur L, Hagenaars J, Wielders C, Elsman P, Lestrade P, Koning O, et al. Screening for Coxiella burnetii seroprevalence in chronic Q fever high-risk groups reveals the magnitude of the Dutch Q fever outbreak. *Epidemiol Infect* 2013;**141**:847–51. doi:10.1017/S0950268812001203.
2. Van der Hoek W, Hogema B, Dijkstra F, Rietveld A, Wijkmans C, Schneeberger P, et al. Relation between Q fever notifications and Coxiella burnetii infections during the 2009 outbreak in The Netherlands. *Euro Surveill* 2012;**17**(3):20058.
3. Parker NR, Barralet JH, Bell AM. Q fever. *Lancet* 2006;**367**(9511):679–88.
4. Morroy G, Keijmel SP, Delsing CE, Bleijenberg G, Langendam M, Timen A, et al. Fatigue following acute Q-fever: a systematic literature review. *PLOS ONE* 2016;**11**(5):e0155884.
5. Million M, Raoult D. No Such Thing as Chronic Q Fever. *Emerg Infect Dis* 2017;**23**(5):856–7.
6. Kampschreur LM, Delsing CE, Groenwold RHH, Wegdam-Blans MCA, Bleeker-Rovers CP, de Jager-Leclercq MGL, et al. Chronic Q fever in the Netherlands five years after the start of the Q fever epidemic: results from the Dutch Chronic Q Fever Database. *J Clin Microbiol* 2014.
7. Eldin C, Melenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, et al. From Q fever to *Coxiella burnetii* infection: a paradigm change. *Clin Microbiol Rev* 2017;**30**(1):115–90.
8. Marmion BP, Sukocheva O, Storm PA, Lockhart M, Turra M, Kok T, et al. Q fever: persistence of antigenic non-viable cell residues of Coxiella burnetii in the host–implications for post Q fever infection fatigue syndrome and other chronic sequelae. *QJM* 2009;**102**(10):673–84.
9. Hussain-Yusuf H, Islam A, Healy B, Lockhart M, Nguyen C, Sukocheva O, et al. An analysis of Q fever patients 6 years after an outbreak in Newport, Wales, UK. *QJM* 2012;**105**(11):1067–73.
10. Penttila IA, Harris RJ, Storm P, Haynes D, Worswick DA, Marmion BP. Cytokine dysregulation in the post-Q-fever fatigue syndrome. *QJM* 1998;**91**(8):549–60.
11. Keijmel SP, Raijmakers RP, Bleeker-Rovers CP, van der Meer JW, Netea MG, Schoffelen T, et al. Altered interferon-gamma response in patients with Q-fever fatigue syndrome. *J Infect* 2016;**72**(4):478–85.
12. Raijmakers RPH, Jansen AFM, Keijmel SP, Schoffelen T, Scholzen A, van der Meer JWM, et al. Interferon-gamma and CXCL10 responses related to complaints in patients with Q fever fatigue syndrome. *Eur J Clin Microbiol Infect Dis* 2018;**37**(7):1385–91.
13. Timen A, Beaujean D, Bleijenberg G, Bleekers-Rovers CP, Van Dam S, Dekker JAW, et al. In: Milieu RvVe, editor *Multidisciplinaire LCI-richtlijn Q-koorts-vermoeidheidssyndroom (QVS).* Rijksinstituut voor Volksgezondheid en Milieu; 2011. Editor p. 57.
14. de Bruin AF, de Witte LP, Stevens F, Diederiks JP. Sickness impact profile: the state of the art of a generic functional status measure. *Soc Sci Med* 1992;**35**(8):1003–14.
15. Schoffelen T, Joosten LA, Herremans T, de Haan AF, Ammerdorffer A, Rumke HC, et al. Specific interferon gamma detection for the diagnosis of previous Q fever. *Clin Infect Dis* 2013;**56**(12):1742–51.
16. Seshadri R, Paulsen IT, Eisen JA, Read TD, Nelson KE, Nelson WC, et al. Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc Natl Acad Sci U S A* 2003;**100**(9):5455–60.
17. Assarsson E, Lundberg M, Holmquist G, Bjorkesten J, Thorsen SB, Ekman D, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One* 2014;**9**(4):e95192.
18. Kremers MN, Janssen R, Wielders CC, Kampschreur LM, Schneeberger PM, Netten PM, et al. Correlations between peripheral blood *Coxiella burnetii* DNA load, interleukin-6 levels, and C-reactive protein levels in patients with acute Q fever. *Clin Vaccine Immunol* 2014;**21**(4):484–7.
19. Vollmer-Conna U, Piraino BF, Cameron B, Davenport T, Hickie I, Wakefield D, et al. Cytokine polymorphisms have a synergistic effect on severity of the acute sickness response to infection. *Clin Infect Dis* 2008;**47**(11):1418–25.
20. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe* 2011;**9**(5):355–61.
21. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* 2012;**12**(2):223–32.
22. Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 2007;**447**(7147):972–8.
23. Landi A, Broadhurst D, Vernon SD, Tyrrell DL, Houghton M. Reductions in circulating levels of IL-16, IL-7 and VEGF-A in myalgic encephalomyelitis/chronic fatigue syndrome. *Cytokine* 2016;**78**:27–36.
24. Montoya JG, Holmes TH, Anderson JN, Maecker HT, Rosenberg-Hasson Y, Valencia IJ, et al. Cytokine signature associated with disease severity in chronic fatigue syndrome patients. *Proc Natl Acad Sci U S A* 2017;**114**(34):E7150–8.
25. Blundell S, Ray KK, Buckland M, White PD. Chronic fatigue syndrome and circulating cytokines: a systematic review. *Brain Behav Immun* 2015;**50**:186–95.
26. Roerink ME, van der Schaaf ME, Dinarello CA, Knoop H, van der Meer JW. Interleukin-1 as a mediator of fatigue in disease: a narrative review. *J Neuroinflammation* 2017;**14**(1):16.
27. Roerink ME, Knoop H, Bronkhorst EM, Mouthaan HA, Hawinkels L, Joosten LAB, et al. Cytokine signatures in chronic fatigue syndrome patients: a case control study and the effect of anakinra treatment. *J Transl Med* 2017;**15**(1):267.
28. Keijmel SP, Saxe J, van der Meer JW, Nikolaus S, Netea MG, Bleijenberg G, et al. A comparison of patients with Q fever fatigue syndrome and patients with chronic fatigue syndrome with a focus on inflammatory markers and possible fatigue perpetuating cognitions and behaviour. *J Psychosom Res* 2015;**79**(4):295–302.