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ARTICLE

Single nucleotide polymorphisms in immune response genes in acute Q fever cases with differences in self-reported symptoms

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Abstract Genes involved in human immune response are well recognized to influence the clinical course of infection. The association of host genetics with susceptibility to and severity of clinical symptoms in acute Q fever was investigated. Single nucleotide polymorphisms (SNPs) in the *IFNG* (rs2430561/rs1861493), *STAT1* (rs1914408), and *VDR* (rs2228570) genes were determined in 85 patients from the 2007 Dutch acute Q fever outbreak, and a symptom score was calculated. *IFNG* rs1861493 showed a significant association with the symptom score; *IFNG* rs2430561 showed a similar trend. These SNPs were then used to reproduce results in a 2009 outbreak population (n=123). The median symptom score differed significantly in both populations: 2 versus 7. The significant association of *IFNG* rs1861493 with symptom score in the first population was not reproduced in the second population. We hypothesize that individuals in the second outbreak were exposed to a higher *Coxiella burnetii* dose compared to the first, which overruled the protection conferred by the A-allele of *IFNG* rs1861493 in the first population.

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Introduction

From 2007 through 2010, the Netherlands faced the largest Q fever outbreak recorded to date (>4,000 notified cases) [1, 2]. The causative agent of Q fever is the intracellular bacterium *Coxiella burnetii*. *C. burnetii*-infected individuals can remain asymptomatic or develop a flu-like illness, pneumonia, or hepatitis, known as acute Q fever [3]. Approximately 2 % of symptomatic acute Q fever cases progress to chronic Q fever [4].

Genes involved in human immune response, and also other genes, are well recognized to influence the clinical course of infection, especially in case of pathogens with cell dependency similar to that of *C. burnetii*. Significant immunogenetic differences have been found comparing patients suffering from chronic sequelae due to Q fever [Q fever endocarditis or prolonged post-infection fatigue (i.e., Q fever fatigue syndrome)] to patients who had an uncomplicated recovery from acute Q fever or subjects from the general population [5, 6]. Polymorphic variations in individual "candidate genes" [single nucleotide polymorphisms (SNPs)] assumed to be of direct importance in pathogenesis may help clarify the relation between immune response genes and varying degrees of disease severity or susceptibility of subpopulations [7–12].

The aim of this study was to assess whether SNPs in several immune response genes influence susceptibility to and severity of an acute *C. burnetii* infection, based on self-reported symptoms. We also compared the allelic frequencies observed in these individuals to frequencies in the general population. Four SNPs in genes involved in innate and adaptive immunity were selected to study this association.

Materials and methods

Study design

Study population 1

The first notified cases in the Dutch Q fever outbreak (2007–2010) were residents of a village in the southern parts of the Netherlands (Herpen), where a case–control study was performed for source identification and risk factor analysis in 2007 [13]. This outbreak was likely caused by abortions on a single dairy goat farm in close vicinity to the village. Acute Q fever cases were selected from participants of this study [at least IgM phase II and/or IgG phase II antibodies against *C. burnetii* antigens ≥1:64 (indirect immunofluorescence assay, IFA; Focus Diagnostics, Cypress, CA, USA)], based on available questionnaire data (self-reported symptoms), including no more than one person per household, and giving consent for the investigation of genetic differences. This study, including our analysis, was approved by the Medical Ethical Committee of the University Medical Center Utrecht (reference number: 07-241).

Study population 2

An abortion wave on a dairy goat farm led to another single-point source outbreak in the southernmost part of the Netherlands in 2009 (Voerendaal). Farm residents, employees, and visitors were serologically screened for *C. burnetii* and assessed for self-reported symptoms by means of a questionnaire almost identical to the one used in study population 1 and administered immediately following laboratory notification [14]. Laboratory-confirmed acute Q fever cases were defined as: a positive *C. burnetii* DNA polymerase chain reaction (PCR) test and/or the presence of IgM phase II and/or IgG phase II antibodies [screening by enzyme-linked immunosorbent assay (ELISA; Serion ELISA classic, Institut Virion\Serion GmbH, Würzburg, Germany) and confirmation by IFA ≥1:32 (Fuller Laboratories, Fullerton, CA, USA)] [14]. In addition, laboratory-confirmed community cases of acute Q fever subsequently notified to the regional Public Health Service between March 2009 and April 2010 received a questionnaire with questions on age, sex, and specific symptoms within days following the laboratory diagnosis. In March 2012, all farm and community cases who had earlier returned the questionnaire were selected for participation in our study, following approval by the Medical Ethical Committee of the Maastricht University Medical Centre (reference number: 10-4-034).

SNP selection

Four SNPs in three candidate genes involved in innate and adaptive immunity to infection were selected based on previously published associations with various diseases: two SNPs in the *IFNG* gene [interferon-γ (IFNγ); rs1861493 and rs2430561], one in *STAT1* (signal transducer and activator of transcription factor 1; rs1914408) involved in the IFNγ-mediated signal transduction, and one in *VDR* (vitamin D receptor; rs2228570).

The defense against intracellular *C. burnetii* mainly depends on cell-mediated immunity, including IFNγ-mediated macrophage activation [15–17]. The IFNγ pathway is believed to be crucial for the host defense against this intracellular pathogen [16, 18], as well as other intracellular bacteria, like *Mycobacterium* spp. [10, 19–21], but has also been associated with outcomes of, for example, severe acute respiratory syndrome (SARS) [11], respiratory syncytial virus infection [22, 23], and gastroenteritis episodes [24]. Consequently, it is reasonable to assume that polymorphisms in the *IFNG* gene and that of its receptor will modulate the IFNγ host pathogen (*C. burnetii*) interaction process [6]. A previous study by Vollmer-Conna et al. showed a significant influence of functional polymorphisms in the *IFNG* loci (rs2430561) on the severity and duration of illness after infection with several pathogens, including *C. burnetii* [12]. Vitamin D stimulates the innate immune response, but suppresses the adaptive immune response and is important in immunity to tuberculosis, diabetes, and respiratory syncytial virus [25–27].

The association of these SNPs with other diseases increases the chance of selecting SNPs that are actually of functional importance. Allelic frequencies of the four selected SNPs were investigated in study population 1. Only those SNPs that showed a statistically significant association or a trend in association with disease severity were used for reproduction in population 2. The allelic frequencies for a Dutch community control group were also available ("Regenboog" study, a large Dutch population health examination survey in 1998 including randomly selected individuals of all ages [28]).

Sample acquisition, DNA isolation, and genotyping

Blood samples from population 1 were collected and analyzed late 2007 and early 2008 for those patients that gave permission for the genetic analysis. Subjects from population 2, selected for participation based on a returned questionnaire, received a self-administrable buccal swab kit accompanied by

an information folder and an informed consent form in March 2012.

For both populations, DNA was isolated as described by Hoebee et al. [29] using the QIAamp DNA Blood Mini Kit (Qiagen NV, Venlo, the Netherlands). Polymorphisms were genotyped using predesigned or custom TaqMan SNP genotyping assays (Life Technologies, Bleiswijk, the Netherlands). For each sample, 2.5 μL of TaqMan Fast Universal PCR Master Mix (Life Technologies) and 10 ng of genomic DNA were used in a total volume of 5 μL. Primer and probe sequences and assay numbers are as described by Doorduyn et al. [24]. The protocol for amplification was 20 s at 95 °C and 40 cycles of 3 s at 95 °C and 30 s at 60 °C. All genotyping assays were performed on a 7500 Fast Real-Time PCR System (Life Technologies).

Questionnaire

Both questionnaires included demographic information and the following 14 Q fever-related symptoms: fever (>38 °C), malaise, headache, cough, severe fatigue, shortness of breath or respiratory difficulties, pain or pressure on the chest, diarrhea, joint pain, night sweating, loss of weight, itch, clinical diagnosis of jaundice/hepatitis, and clinical diagnosis of pneumonia. Hospitalization at the time of the acute *C. burnetii* infection was also recorded.

Statistical analysis

Participants with missing self-reported symptom data were excluded. For each participant, a symptom score was calculated as the sum of all reported symptoms (range 0–14). The median score and corresponding interquartile range (IQR) were calculated for both study populations, and the Mann–Whitney *U*-test was used to assess statistical significance for the difference in the median score between both populations. Multiple regression analysis was used to confirm independence of the symptom score from age and gender.

The genotype data of all tested SNPs were used to estimate Hardy–Weinberg equilibrium by the comparison of genotype frequencies within population 1, population 2, and the community controls by a Chi-square test [30]. To study the association of the determined SNPs with the symptom score, allele frequencies of each SNP in subjects who indicated no symptoms at all were compared with allele frequencies in subjects who reported at least one symptom ("symptomatic") by using cross-tabulations and Chi-square tests. Odds ratios (ORs) and 95 % confidence intervals (95 % CIs) were calculated as well. The same type of comparisons were performed for subjects with a symptom score up to and including the median number of symptoms ("symptomatic") compared with subjects with a symptom score higher than the median score ("severe symptomatic"). Allele frequencies of the "symptomatic" and "severe symptomatic" groups were also compared with the allele frequencies of a Dutch community control group. The distribution of the symptom score for the different genotypes was assessed as well. Data were analyzed using IBM SPSS Statistics version 19.0.0 (SPSS Inc.).

Results

General

All tested polymorphisms were in Hardy–Weinberg equilibrium ($p>0.05$) in all three groups (population 1, population 2, and community controls). There was no substantial relationship between symptom score and the demographic factors age and sex.

Study population 1

Study population 1 consisted of 85 acute Q fever cases after excluding three subjects with missing symptom data. The median age of the patients was 49 years (IQR: 41–58) and 54 (64 %) were male. The self-reported symptom frequency

Table 1 Frequencies of reported symptoms and hospitalization of study populations 1 and 2

	Study population 1 (n=85)			Study population 2 (n=123)		
	Total	Yes	Percentage (%)	Total	Yes	Percentage (%)
Symptoms						
Fever (>38 °C)	84	23	27	122	98	80
General malaise	83	35	42	121	104	86
Headache	82	33	40	121	86	71
Cough	84	31	37	123	72	59
Severe fatigue	85	35	41	120	83	69
Shortness of breath of respiratory difficulties	84	20	24	120	43	36
Pain of pressure on the chest	84	12	14	122	41	34
Diarrhea	84	19	23	122	42	34
Joint pain	84	23	27	121	63	52
Night sweating	84	28	33	122	80	66
Loss of weight	84	9	11	122	39	32
Itch	83	9	11	120	27	23
Jaundice/hepatitis[a]	83	1	1	121	4	3
Pneumonia[a]	84	7	8	120	30	25
Hospitalization	85	2	2	120	25	21

[a] As clinical diagnosis

ranged between 1 % for hepatitis/jaundice and 42 % for malaise, and 2 % of the cases had been hospitalized (Table 1).

The median symptom score was 2 (IQR: 0–6). Table 2 presents the allele distribution of each SNP by using the two different classifications of "symptomatic" (at least one symptom and "severe symptomatic", i.e., a symptom score above the median score). The Dutch community control group consisted of 1,008 persons, of which 55 % was male. A statistically significant difference was observed for both *IFNG* SNPs when subjects who did not report any symptoms were compared to subjects who reported at least one symptom (Table 2) (*IFNG* rs1861493: OR: 3.53; 95 % CI: 1.39–8.99; p=0.006; *IFNG* rs2430561: OR: 2.13; 95 % CI: 1.09–4.19; p=0.027). A similar result was found for one of the *IFNG* SNPs (rs1861493) when using the median symptom score as the cut-off (the A-allele was less frequently observed among the participants with the above median symptom score), while the other *IFNG* SNP (rs2430561) showed a trend in association in the same direction (the T-allele was less frequently observed among the participants with the above median symptom score), though it was no longer statistically significant. The GG genotype (rs1861493) and AA genotype (rs2430561) seemed to increase the symptom score (Fig. 1), though the combination of both genotypes was not significantly associated with the two symptom classifications used. The other SNPs did not show an association with the symptom score.

Table 2 Allele frequencies of four different single nucleotide polymorphisms (SNPs) in study population 1 (n=85; n=170 alleles per SNP) by using two different classifications of the symptom score and a Dutch population control group (n=1,008; n=2,016 alleles per SNP)

Allele	Symptom score=0, *n* (%)	Symptom score≥1, *n* (%)	*p*-Value[a]	OR (95 % CI)	Dutch community controls, *n* (%)	*p*-Value[a,b]	OR (95 % CI)[b]
IFNG (rs1861493)			0.006			0.086	
A	44 (88)	81 (68)		Ref	1,503 (75)		Ref
G	6 (12)	39 (33)		3.53 (1.39–8.99)	513 (25)		1.41 (0.95–2.09)
IFNG (rs2430561)			0.027			0.396	
T	31 (62)	52 (43)		Ref	952 (47)[c]		Ref
A	19 (38)	68 (57)		2.13 (1.09–4.19)	1,060 (53)[c]		1.17 (0.81–1.70)
STAT1			0.684			0.706	
G	36 (72)	90 (75)		Ref	1,479 (73)[d]		Ref
A	14 (28)	30 (25)		0.86 (0.41–1.80)	535 (27)[d]		0.92 (0.60–1.41)
VDR			0.179			0.629	
G	27 (54)	78 (65)		Ref	1,265 (63)[d]		Ref
A	23 (46)	42 (35)		0.63 (0.32–1.24)	749 (37)[d]		0.91 (0.62–1.34)
	Symptom score≤2[e], *n* (%)	Symptom score≥3[e], *n* (%)	*p*-Value[a]	OR (95 % CI)	Dutch community controls, *n* (%)	*p*-Value[a,f]	OR (95 % CI)[f]
IFNG (rs1861493)			0.027			0.069	
A	74 (80)	51 (65)		Ref	1,503 (75)		Ref
G	18 (20)	27 (35)		2.18 (1.09–4.36)	513 (25)		1.55 (0.96–2.50)
IFNG (rs2430561)			0.209			0.518	
T	49 (53)	34 (44)		Ref	952 (47)[c]		Ref
A	43 (47)	44 (56)		1.48 (0.80–2.71)	1,060 (53)[c]		1.16 (0.74–1.83)
STAT1			0.676			0.665	
G	67 (73)	59 (76)		Ref	1,479 (73)[d]		Ref
A	25 (27)	19 (24)		0.86 (0.43–1.72)	535 (27)[d]		0.89 (0.53–1.51)
VDR			0.709			0.647	
G	58 (63)	47 (60)		Ref	1,265 (63)[d]		Ref
A	34 (37)	31 (40)		1.13 (0.61–2.09)	749 (37)[d]		1.11 (0.70–1.77)

95 % CI 95 % confidence interval; *OR* odds ratio; *Ref* reference

[a] Chi-square test

[b] Symptom score≥1 vs. Dutch community controls

[c] 2 subjects/4 alleles missing

[d] 1 subject/2 alleles missing

[e] Based on the median symptom score (2) in the study population

[f] Symptom score≥3 vs. Dutch community controls

Therefore, the two *IFNG* SNPs were selected as the most likely candidates for reproduction of our results in study population 2.

Compared with the general Dutch population, the A-allele of the *IFNG* rs1861493 SNP was more often present in participants with a low symptom score and less often in participants with a high symptom score; a borderline significant result was found for participants with a higher symptom score compared with the community controls (OR: 1.55; 95 % CI: 0.96–2.50; p=0.069).

Study population 2

From study population 2, 192 individuals were invited, of whom 129 (67 %) submitted a buccal swab sample. Three participants were excluded because only one member per household could be included and another three because of missing symptom data. In the remaining 123 cases, the median age was 48 years (IQR: 40–60) and 70 (57 %) were male; the self-reported symptom frequency ranged between 3 % for hepatitis/jaundice and 86 % for malaise; hospitalization was reported in 21 % of the cases (Table 1).

The median symptom score was significantly higher than in population 1: 7 (IQR: 5–8; p<0.001). Non-responders from population 2 did not differ from responders according to age, gender, and median symptom score (data not shown). Figure 2 shows the distribution of the symptom scores in both study populations. As there were only four patients who reported no symptoms in this second population, only the "severe symptomatic" classification with above median symptom score could be used for analysis (Table 3). No statistically significant association between SNP allele frequency and severity of symptoms was observed in this population. There is a less clear distinction between the genotypes and symptom score compared to population 1 (Fig. 1), i.e., all genotypes show moderate to high symptom scores.

We also investigated whether fever in combination with other symptoms, hospitalization, pneumonia, or hepatitis was associated with the allele frequencies of the SNPs, but no clear associations were found.

Discussion

This study investigated the association of four SNPs in several immune response genes with susceptibility to and severity of self-reported symptoms in acute *C. burnetii* infection. Both *IFNG* SNPs seemed to be related to the symptom score in population 1. This association remained significant for the *IFNG* rs1861493 SNP irrespective of the cut-offs for symptom severity chosen (protection conferred by the A-allele, G-allele is the risk allele), while a trend in association was found for the *IFNG* rs2430561 SNP (protection conferred by the T-allele, A-allele is the risk allele). The other SNPs did not show an association with the symptom score. The associations found in the *IFNG* SNPs in population 1, however, could not be reproduced in population 2.

The A-allele of the *IFNG* rs2430561 SNP is associated with low IFNγ production [12, 31]. This A-allele has been reported to significantly increase the susceptibility to develop tuberculosis [10, 19–21] and SARS [11]. A dose-dependent association was found for this A-allele with susceptibility to SARS [11]. Pacheco et al. suggested that the increased levels of IFNγ in the early events during infection could probably control the replication and spread of *M. tuberculosis* or other intracellular pathogens [20]. With respect to disease severity, however, significant associations of the T-allele with a more severe acute sickness response to infection have been reported previously [12, 32]. This suggests that high or low levels of IFNγ can have different effects on disease, depending on the outcome under investigation.

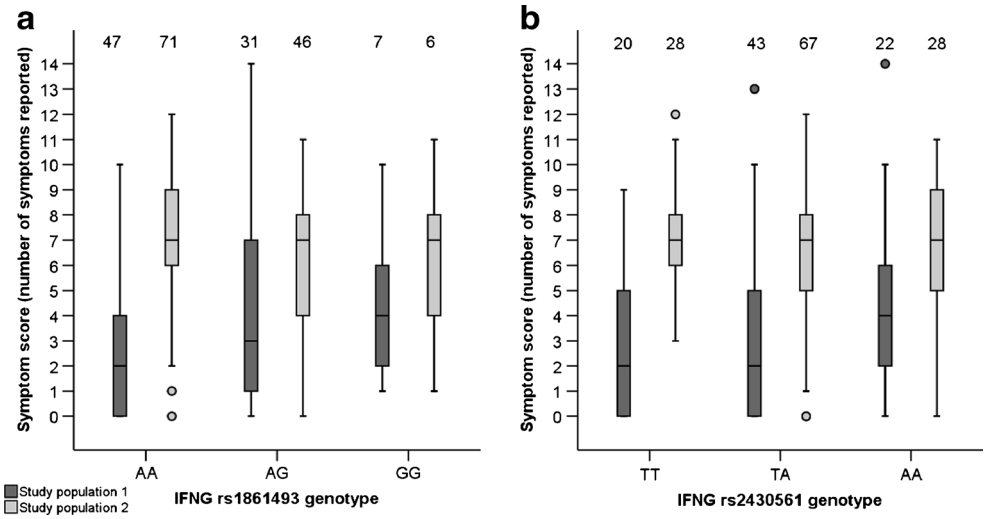

Fig. 1 Box plots of the *IFNG* genotype and symptom score in study populations 1 and 2: **a** *IFNG* rs1861493, **b** *IFNG* rs2430561. The numbers above the bars indicate the number of cases, the horizontal lines within the boxes represent the median symptom score, the lower and upper boundaries of the boxes represent the 25th and 75th percentiles, respectively, and the T-bars represent the 2.5th and 97.5th percentiles. Outliers are indicated by the dots

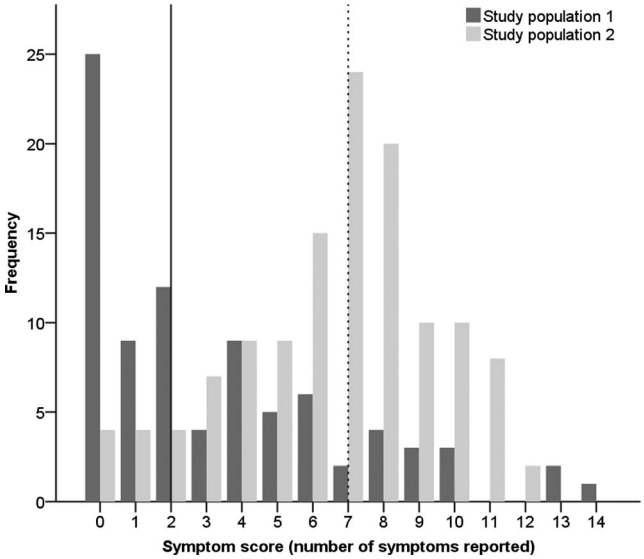

Fig. 2 Symptom score distribution in study populations 1 and 2. The *solid vertical line* represents the median score of population 1 and the *dashed line* represents the median score of population 2

There are several explanations for our observation that findings from population 1 could not be reproduced in population 2. The first observation is that, surprisingly, symptom severity appeared to be much higher in population 2 compared to population 1, based on symptom scores and hospital admission rates (21 vs. 2 %). A possible explanation could be recall bias in study population 1, as participants were asked in September 2007 about their symptoms between 7 May 2007 and 8 July 2007. Nevertheless, the difference in symptom scores seems too large to be solely explained by recall bias in population 1. Furthermore, self-reported hospital admission rates seem highly unlikely to be affected by recall bias.

Higher symptom severity in population 2 could possibly be explained by higher environmental exposure dose, as reflected by the large numbers of abortions in goats in the 2009 outbreak and extremely high attack rates in farm contacts [14]. A high *C. burnetii* dose in the environment is likely to lead to a higher probability of symptoms (i.e., a higher attack rate), even in less susceptible individuals. A dose-dependent attack rate has earlier been described for *Salmonella* infections in humans [33, 34]. A similar relationship for *C. burnetii* is suggested by experimental animal data [35], and a human dose–response model for *C. burnetii* was recently published as well [36]. Evidence from these studies suggests that higher doses may be likely to overrule the immune system of the exposed person, resulting in an increased probability of illness. This hypothesis, in the light of the findings from our study, leads us to presume that a high dose will not only cause symptoms in the genetically susceptible individuals, but also in those genetically less prone to symptoms. In other words, the effects of heterogeneity in host susceptibility are diminished or even extinguished. Although we cannot exclude some influence of recall bias on our results of population 1, we presume that a higher *C. burnetii* dose was present in population 2 in 2009 compared to population 1 in 2007. Modeling studies investigating human exposure might give more insight in this hypothesis.

An attempt to rule out dose effects in population 2 by excluding subjects who we assumed had the highest degree of exposure [farm residents, employees, visitors, and people living close (<3 km) to the farm as a measure of exposure dose] still did not enable us to reproduce our results from population 1. This may be due to a loss in statistical power because of excluding a large number of subjects from our analysis. Besides, the question remains as to whether the criteria we used for exclusion was a good measure for high exposure, even though it was the best available option in our study.

Symptoms associated with Q fever are non-specific and are also common in other respiratory tract infections, such as

Table 3 Allele frequencies of four different SNPs in study population 2 (n=123; n=246 alleles per SNP) and a Dutch population control group (n=1,008; n=2,016 alleles per SNP)

Allele	Symptom score≤7[b], *n* (%)	Symptom score≥8[b], *n* (%)	*p*-Value[a]	OR (95 % CI)	Dutch community controls, *n* (%)	*p*-Value[a,c]	OR (95 % CI)[c]
IFNG (rs1861493)			0.417			0.309	
A	112 (75)	76 (79)		Ref	1,503 (75)		Ref
G	38 (25)	20 (21)		0.77 (0.41–1.43)	513 (25)		0.77 (0.47–1.28)
IFNG (rs2430561)			0.794			0.753	
T	76 (51)	47 (49)		Ref	952 (47)[d]		Ref
A	74 (49)	49 (51)		1.07 (0.64–1.79)	1,060 (53)[d]		0.94 (0.62–1.41)

95 % CI 95 % confidence interval; *OR* odds ratio; *Ref* reference

[a] Chi-square test

[b] Based on the median symptom score (7) in the study population

[c] Symptom score≥8 vs. Dutch community controls

[d] 2 subjects/4 alleles missing

influenza. It can, therefore, be questioned whether the reported symptoms can be ascribed to other respiratory tract infections. Although the two outbreaks took place in different years, both occurred as seasonal peaks in spring (April–June), which corresponds to the main lambing season in goats [37, 38]. Influenza, however, mostly occurs in winter and the incidence in spring was low in both years in the Netherlands [39]. Therefore, it is highly unlikely that other respiratory diseases played a major role in the two populations used for this study.

In conclusion, a significant difference was found for the *IFNG* rs1861493 SNP between persons with a mild or more severe presentation of acute Q fever, which was not confirmed in a second study population. Such an effect could not be observed in the second outbreak due to the observed high rates of severe symptoms, possibly saturating the effects of host susceptibility factors.

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Conflict of interest None declared.

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