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Research article

Genetic variants in the *IFNGR2* locus associated with severe chronic Q fever

Susana David [a,b,*], Liliana Castro [a], Elsa Duarte [c], Ulisses Gaspar [a], Maria Rosário da Costa Rodrigues [a,d,e], Maria Vanessa Cueto-Rojo [a], Joana Mendonça [a], José Ferrão [a], Miguel Machado [a], José Poças [f], João Lavinha [a], Luís Vieira [a], Ana Sofia Santos [g,h]

[a] Departamento de Genética Humana, Instituto Nacional de Saúde Doutor Ricardo Jorge, I. P. (INSA), Lisboa, Portugal
[b] Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmacia, Universidade de Lisboa, Portugal
[c] MED – Instituto Mediterrâneo para a Agricultura, AmbientePortugal e Desenvolvimento, Escola de Ciências e Tecnologia, Universidade de Evora, Portugal
[d] Patologia Clínica – Lab. Bioquímica Genética/Endocrinologia Especial, Hosp. D. Estefânia, Unidade Local de Saúde São José, Lisboa, Portugal
[e] BioISI—Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisbon, Lisboa, Portugal
[f] Serviço de Infeciologia, Hospital de São Bernardo, Centro Hospitalar de Setúbal, Setúbal, Portugal
[g] Centro de Estudos de Vectores e Doenças Infecciosas Dr. Francisco Cambournac (CEVDI), Departamento de Doenças Infeciosas (INSA), Águas de Moura, Portugal
[h] Instituto de Saúde Ambiental, Faculdade de Medicina, Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal

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ABSTRACT

Q fever is a highly contagious zoonosis capable of causing large outbreaks of important health and economic consequences. Host genetic factors are believed to influence the development of severe chronic Q fever following the infection by the etiological agent, *Coxiella burnetii*. Targetted next generation sequencing (NGS) was performed in a case-control genetic association study on 53 confirmed Q fever cases, including 38 compatible with acute and 15 with chronic disease, and 29 samples from the general Portuguese population. Four SNPs in the *IFNGR2* locus, rs78407108 G > A, rs17879956 C > T, rs7277167 C > T, and rs9974603 C > A, showed a statistically significant association to chronic Q fever, resisting the Bonferroni correction. These belonged to haplotypes significantly associated with chronic Q fever. The individual SNPs are referenced in the GTEx database as possible eQTLs. Given the direct bearing of IFNGR2 on IFN-γ signaling, the possible involvement of the associated variants with higher *IFNGR2* expression could be in line with observations suggesting that IFN-γ production in chronic Q fever patients is significantly higher than in healthy controls. Further investigations are required to clarify the role of IFNGR2 signaling in association with chronic Q fever.

1. Introduction

Q fever is a highly contagious zoonosis caused by *Coxiella burnetii* bacteria. The disease is endemic in virtually every country in the world. However, its clinical unspecificity and weak epidemiological context result in an underestimation of Q fever prevalence in many countries [1–3].

The disease is characterized by a wide spectrum of clinical manifestations [4,5]. Almost 60% of Q fever cases are asymptomatic. Symptomatic forms, either classified as acute or chronic Q fever, are characterized by different evolution, serological profiles, and treatment outcomes [6]. Presentations of acute Q fever include a primarily self-limited often-unspecific febrile syndrome, usually not requiring hospitalization. In patients requiring hospitalization, Q fever is frequently expressed as an atypical pneumonia or acute hepatitis. Infection progresses to chronic Q fever in 1%–5% of the patients infected by *C. burnetii,* months to years after the primary infection [4,6,7]. Endocarditis is the most common presentation of chronic Q fever. There are numerous risk factors conditioning this evolution amongst which host related factors prevail [5–10]. Cardiovascular risk factors and, in particular, preexisting valvular disease, are amongst the principal risk factors for chronic Q fever endocarditis. The potential for severe hypersensitivity reactions of the only commercially available vaccine, Q-Vax®, has restricted its widespread use. Extensive efforts are underway to develop a non-reactive next-generation human Q fever vaccine, affording potent and durable protection against *C. burnetii* [11]. Given the recognizable threat of *C. burnetii* infection to public health, the limitations of current antibiotic treatments, the difficulty incurred in establishing vaccination campaigns, and the risk of persistent infection, there is an urgent need for early diagnostic and prognostic markers.

The identification of host genetic factors of susceptibility to infectious diseases or their more severe forms is a major tool for infection control strategies. Helbig and colleagues reported the existence of

* Corresponding author.

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immunogenetic differences supporting the concept of different immune states in Q fever [12,13]. However, the greatest contribution in this area has been made by the Dutch groups in response to the important outbreaks suffered in the country (Supplementary Table 1). These investigators associated the development of chronic Q fever with genetic variants implicated in the immune response to infection [14,15].

Genetic factors play a recognizable role in the clinical presentations and severity of infections caused by intracellular bacteria. However, with the exception of mycobacterial infections, little is known about the role of host related genetic factors [16]. As has been observed with *Mycobacterium tuberculosis* infection, natural immunity to *C. burnetii* infection leads to granuloma formation, providing some guidance in the choice of candidate genes for Q fever genotype-phenotype association studies. We purpose to further contribute to this area by exploring the coding and non-coding regions of seven candidate genes, *IL12RB1*, *IFNGR1*, *IFNGR2*, *NRAMP1*, *TLR1*, *TLR2* and *TIMP1* in both acute and chronic Q fever, using a retrospective case-control candidate gene association study (CGAS) approach.

2. Materials and methods

2.1. Study population

The study population for the CGAS consisted in 60 unrelated Q fever patients, diagnosed at the Instituto Nacional de Saúde Doutor Ricardo Jorge, I. P. (INSA), from 2005 to 2018 (except one case from 1995).

Acute Q fever was diagnosed in 43 individuals presenting a blood sample with positive PCR or agent's *in-vitro* isolation and/or anti- *C. burnetii* phase II antibodies with IgM ≥ 50 and IgG ≥ 200 [17]. The average age was 44.5 ± 18.3 years, and the male-to-female ratio was 4:1 (34:9). Clinical manifestations included febrile illness, 77% (33/43), hepatitis, 26% (11/43), heart disease, 9% (4/43) and acute respiratory distress syndrome (ARDS), 5% (2/43). Knowledge of pre-existing conditions for the progression to chronic Q fever was not available for 77% (33/43) of these patients. The remaining had clinical signs of renal disease (renal insufficiency), 7% (3/43), immunosuppression (pregnancy related, HIV infection), 7% (3/43), obesity and/or diabetes, 5% (2/43), and valve disease, 7% (3/43).

Chronic Q fever was diagnosed following Duke´s modified criteria [18,19]. Seventeen chronic Q fever patients were included in the study. Mean age was 52.0 ± 19.7 years and the male-to-female ratio 3:1 (13:4). Endocarditis was the main clinical manifestation present in 65% (11/17) of the patients. Recurrent febrile illness was also observed in 24% (4/17) and osteomyelitis was only observed in two children under the age of 10 years, 12% (2/17). Predisposing conditions were present in 76% (13/17) of the patients, while 24% (4/17) had no known pre-existing condition. Pre-existing conditions included valvular disease, 71% (12/17), and neoplastic disease, 6% (1/17).

For comparative purposes, 34 convenience samples from an anonymous collection, representing the general Portuguese population was also analysed. No information concerning previous exposure to *C. burnetii* was available for this group.

The procedures of the present study were authorized by the Ethics Committee of the INSA and carried out in accordance with the Declaration of Helsinki of 1975.

2.2. Amplification and sequencing of target sequences

Candidate genes were genotyped targeting the exons, intronic regions close to the splice sites and non-coding regions at 2 Kb upstream and in the 5′UTR region (Table 1). Genomic DNA was extracted from peripheral leukocytes using MagNA Pure LC (Roche Diagnostics GmbH, Mannheim, Germany).

For NGS, a PCR amplicon-based target enrichment method was used. The genome version GRCh38.p12 (Genome Reference Consortium Human Build 38) available in Ensembl Genome Browser 96 was used as the reference. The primers were designed using the Primer- BLAST, from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from Thermo Fisher Scientific®. The high-fidelity long-range PCR Taq DNA polymerase TaKaRa LA Taq® Hot Start (Takara Bio Inc., Shiga, Japan) was used for fragment amplification.

Visual quantification of the amplification products was used to standardize the quantity of PCR product first pooled into groups of five size compatible PCR products, then for a final pooling of all 20 PCR products per sample. The pooled products were purified using AMPure XP from Beckman Coulter® (Beckman Coulter Inc., Brea, California).

Table 1
Amplified fragments of the genes *IL12RB1*, *IFNGR1*, *IFNGR2*; *NRAMPI*; *TLR1*, *TLR2* and *TIMP1*.

Gene				Fragment		
Name	Chromosomal location	DNA strand	Genomic coordinates	Name	Size (bp)	Genomic coordinates
TLR1	4p14	Reverse	4:38790677–38856817	T1A1	12,110	4:38793975-38806088
TLR2	4q31.3	Forward	4:153684070–153705702	T2A1	6185	4:153700772-153706956
IL12RB1	19p13.11	Reverse	19:18058995–18098944	LA	10,760	19:18089867-18100631
				LB	13,559	19:18075127-18088684
				LC1	9748	19:18065426-18075173
				LC2	7576	19:18057818-18065393
IFNGR1	6q23.3	Reverse	6:137197484–137219449	G1A	6069	6:137214674-137220742
				G1B4	1415	6:137205750-137207164
				G1B5	2626	6:137203404-137206039
				G1B7	3206	6:137200077-137203282
				G1B8	3330	6:137196715-137200044
IFNGR2	21q22.11	Forward	21:33402896–33479348	G2A3	3790	21:33401040-33404829
				G2B	5587	21:33410124-33415711
				G2C	1821	21:33420534-33422353
				G2D	1793	21:33425848-33427642
				G2E	8034	21:33431071-33439104
				G2F	1636	21:33478213-33479848
NRAMP1	2q35	Forward	2:218382029–218396894	RA	8823	2:218380796-218389008
				RB1	4851	2:218388989-218393840
				RB2	5217	2:218393283-218398499
TIMP1	Xp11.3	Forward	X:47582408–47586789	TIMP1	6726	X:47581095-47587820

The Nextera XT library prep kit combined with Nextera XT Index v2 kit for indexation from Illumina® were used for NGS library preparation, according to the manufacturer's instructions. Sequencing was carried out on the MiSeq™ instrument from Illumina® (Illumina Inc., San Diego, California).

2.3. Data processing and variant analysis

Strict quality control was performed for each phase of the NGS methodology as indicated by the MiSeq instrument (Fig. 1).

The variant calling process comprised the mapping of reads to a reference genome, variant calling, annotation, and filtering. Clean reads (demultiplexed sequences without adapters or indices) were mapped against the reference genome with the Bowtie 2 v2.3.4 software [20]. The alignment was performed using the "very-sensitive-local" mode, for one maximum number of valid alignments per read, a maximum size of insertion for paired-end alignments of 1500 and deterministic behavior. The mapped files were sorted by genomic coordinates using the SAMtools software v1.9, applying the modules view and sort [21]. Duplicated reads were identified and marked by the GATK v4.1.5.0 tool using the MarkDuplicates module [22,23]. Prior to variant calling, base call quality values were corrected for systematic error with the software GATK (tools BaseRecalibrator and ApplyRecalibration) using the known germline variations database downloaded from the Ensembl Release 96 [24]. The resulting alignment file was used for determining variants. Varying positions compared to the reference sequence were initially called for each sample using the tool GATK HaplotypeCaller in the Genomic Variant Call Format (GVCF) mode (StandardAnnotation, StandardHCAnnotation and AS_StandardAnnotation annotation groups were added). Using Ensembl Release 96, known variants were annotated with the corresponding identifier. Joint genotyping was performed on the samples using the tool GATK Genotype GVCFs for the variants found by the HaplotypeCaller, but only for those with a minimum call confidence of 20. Samples' genotypes and varying positions were then filtered using GATK (SelectVariants and VariantFiltration modules), BCFtools software v1.9 (annotate, filter, norm and view modules) and PLINK whole genome association analysis toolset v1.9 [25,26]. Firstly, individual samples' genotypes were filtered when the read depth was lower than 10× and an unbalance putative allelic heterozygosity was observed (accepted allelic balance between 0.3 and 0.7). Then, varying positions were sequentially filtered when: (1) the variant was found in a repetitive region with more than eight repetitive units; (2) a SNP variant was in a range of 2 bps from an INDEL; and (3) it was not possible to confidently genotype at least 50% of the samples. Finally, only biallelic SNPs were considered.

NGS phase III quality control was performed with the PLINK software v1.7 [27], using an adaptation of the tutorial by McDonald and collaborators [28]. The threshold values applied were those recommended for CGAS [29]. The extreme criterion of the Hardy-Weinberg equilibrium (HWE) (p-value $< 10^{-3}$) was used to reject the null hypothesis of HWE in the control population. When, verified only in the control group, the deviation from the HWE assumptions was used to detect genotyping errors.

2.4. Genetic association

The basic allelic test, used to compare allele frequencies between the cases and control groups, and the general Portuguese population group, was performed with PLINK softwares v1.7 and v1.9 (www.cog-genomics.org/plink/1.9/, accessed August 1, 2022) [26,27].

Statistically significant was considered at p-value ≤ 0.05. To control for type I errors, the Bonferroni correction for multiple comparisons was performed [30].

The Ensembl Genome Browser [24] was used for comparing the genotypic and allelic frequencies of the associated SNPs to those in the subpopulations CEU (Utah residents with Northern and Western European ancestry) and IBS (Iberian populations in Spain) of the 1000 Genomes Project [31]. The frequencies in the cases and control groups were also compared to those observed for the general Portuguese population targeted by the present study.

For all the SNPs identified (associated SNPs and SNPs in LD), the characteristics of the SNPs were obtained from the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/, accessed July 15, 2022).

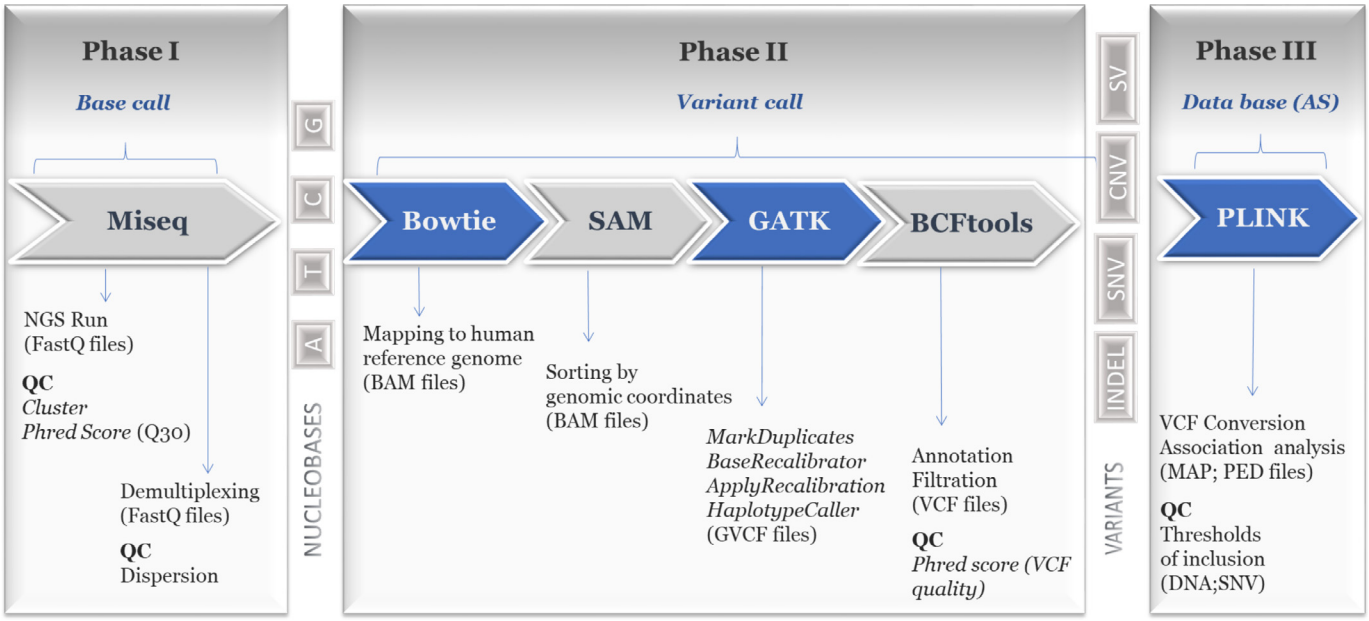


Fig. 1. NGS work flow for phases I, II and III, indicating the bioinformatics tools used in each step with the respective quality parameters. Legend: FASTQ- format based on ASCII characters; BAM- binary data storage file; GVCF- Genomic Variant Call Format; VCF- Variant Call Format; MAP- map of SNPs; PED- Pedigree Data; QC-Quality Control; * = in the reference genome.

The scientific literature was searched for citations and references of the variants in association with Q fever, other infectious diseases, or relevant traits.

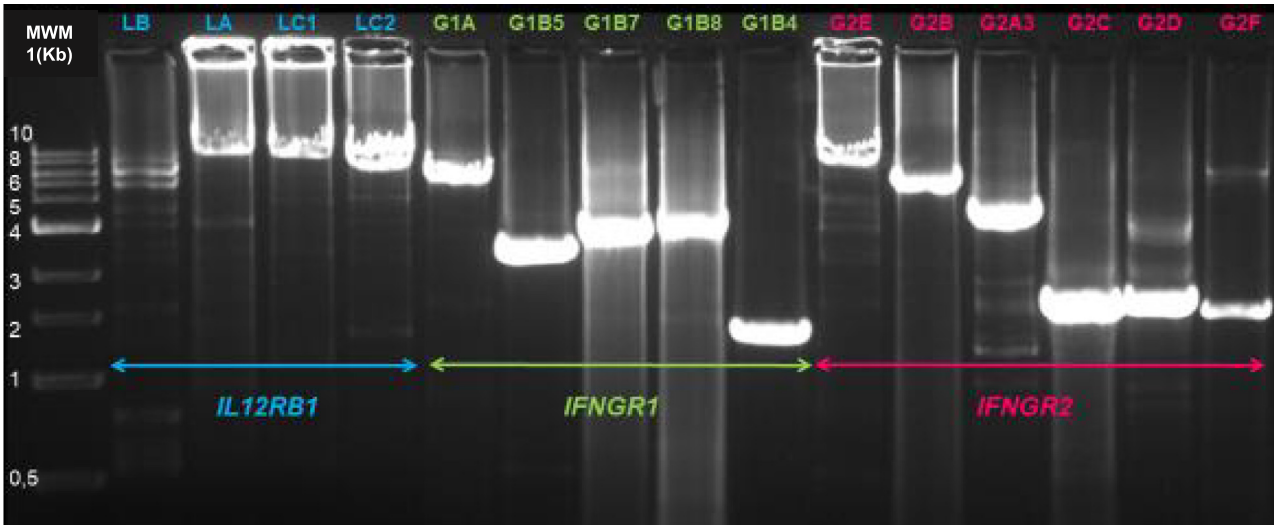
2.5. Validation of the results

Sanger sequencing was used to validate the NGS genotyping of SNPs associated with chronic Q fever withstanding the Bonferroni correction and to analyse these SNPs in an independent replication study on 32 Q fever patients of the same ethnic origin as in the original study (21 acute and 11 chronic forms). The two-tailed Fisher's exact test was used to evaluate statistical significance.

2.6. Preliminary studies for functional immunological analysis

Associated variants in putative regulatory regions were analyzed using the Genotype-Tissue Expression (GTEx) functional genomics database [32]. The SNPs in statistically significant association with chronic Q fever were investigated for linkage disequilibrium (LD) patterns in the subpopulation CEU using Haploview [33]. Haplotype blocks were estimated following the default procedure in Haploview via PLINK v1.7 using pairwise LD.

Preliminary expression studies were performed using three convenient samples from a group of three healthy individuals having the alternative allele haplotype relative to a group of three with the ancestral allele. Briefly, RNA was isolated and purified from peripheral blood using the NucleoSpin® RNA kit following the manufacturer's protocol (Macherey-Nagel, Thermo Fisher Scientific, Portugal). From total RNA 10μg was used for RT-qPCR using a one-step QuantiTect® SYBR® Green RT-PCR kit according to the manufacturers instructions (Qiagen, Werfen, Portugal). *IFNGR2* primers were designed using the primer design software PrimerBlast (https://www.ncbi.nlm.nih.gov/-tools/primer-blast/). For *IFNGR2* amplification the forward 5′-GAATC CAACAGGTCAAAGGCCC-3′ (spanning an exon/exon junction) and



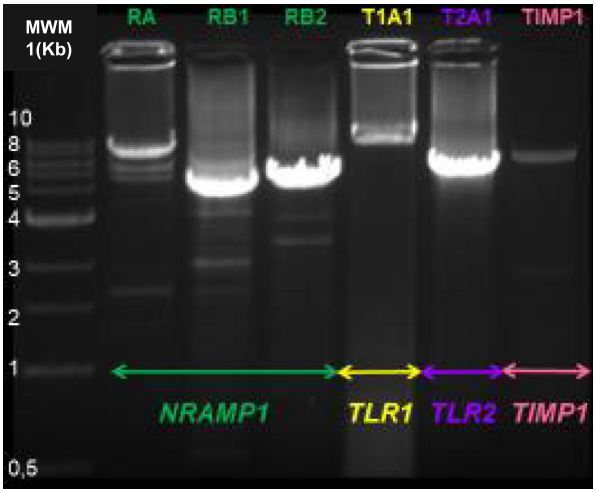


Fig. 2. Electrophoresis result of each of the amplified fragments of *IL12RB1*, *IFNGR1*, *IFNGR2*, *NRAMP1*, *TLR1*, *TLR2* and *TIMP1* genes. PCR products were visually quantified by electrophoreses using a 1 % agarose gel (Seakem® LE Agarose) in tris–borate EDTA buffer (TBE) and the expected bands identified using a 1 kb DNA ladder (New England BioLabs®, Werfen Portugal, Carnaxide, Portugal). The electrophoresis was run at 80 V for 90 min using a BIORAD® PowerPac300 power supply. Legend: MWM – Molecular Weight Marker, Quick-Load® 1 Kb DNA Ladder from New England BioLabs ® Inc.

reverse 5′-TTCCAAAGCAGTTGTGCCTGG-3′ primers were used. The mRNA expression profiles were normalized with respect to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using the forward 5′-AAGGTCGGAGTCAACGGATTT-3′ and reverse 5′-TGAAGGGGTCATTGATGGCA-3′ primers. The primers were used at a final concentration of 0.5 μM. The PCR reaction proceeded as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60°C for 1 min. For comparative purposes, the Ct data were analyzed using the ΔΔCt method. For each condition, three biological replicates were tested; and for every biological replicate, three technical replicates were performed.

3. Results

3.1. Genotyping

PCR target amplification for NGS of the genes *IL12RB1*, *IFNGR1*, *IFNGR2*, *NRAMPI*, *TLR1*, *TLR2* and *TIMP1* is shown in Fig. 2. An example of the five groups of five size compatible PCR products, after confirmed standardization of amplicon concentration prior to the final pooling of all 20 PCR products per sample is shown in Fig. 3.

3.2. NGS quality control parameters

NGS phase I quality control results showed that the percentage of reads attributed to each sample ranged between 0.6–1.6% for 95% (89/94) of the samples and that 89.8% of the total sequenced bases had ≥Q30. At the end of NGS phase II, after annotation and filtration, 607 SNPs from 94 DNA samples passed quality control. After NGS phase III quality control and exploratory data analysis, 405 SNPs and 82 DNA samples were available for association testing (Fig. 4). This included 38 acute and 15 chronic, samples, and 29 general Portuguese population samples.

3.3. Association testing

The allelic model in PLINK identified 29 SNPs, in the *IL12RB1 and IFNGR2* genes, showing statistically significant association with chronic Q fever. Of these, rs78407108 (NC_000021.9:g.33426675G > A), rs17879956 (NC_000021.9:g.33438631C > T), rs7277167 (NC_000021.9:g.33401471C > T), and rs9974603 (NC_000021.9:g.33403994C > A) in the *IFNGR2* gene, withstood the Bonferroni correction for multiple comparisons (adjusted $\alpha = 0.000154321$) (Table 2). Sanger sequencing confirmed the NGS genotyping in all the study samples. The alternative alleles were associated with the chronic Q fever phenotype.

Table 3 shows the allelic and the genotypic frequencies of the four *IFNGR2* variants. For the population study, these are given for the subpopulation with northern and western European ancestry (CEU) and the Iberian populations in Spain (IBS), as well as for the Portuguese population targeted by the present study. For the association study, the results for the chronic and acute Q fever groups are also displayed. SNP rs78407108 is an *IFNGR2* intron variant, rs17879956, a *TMEM50B* intron variant, rs7277167, an *IFNGR2* 2kb upstream variant, and rs9974603, an *IFNGR2* intron variant. In the chronic Q fever patients, the frequencies of the alternative alleles of rs78407108 (20%), rs17879956 (20%), rs7277167 (20%), and rs9974603 (23%) are well above those frequencies observed in the general Portuguese population (12%, 16%, 14% and 14% respectively), and the acute Q fever group (0%, 0%, 0% and 1%, respectively) (Table 3). In the population of our study, the associated variants were in LD (Fig. 5). They are referenced in the GTEx database as possible eQTLs where the alter-

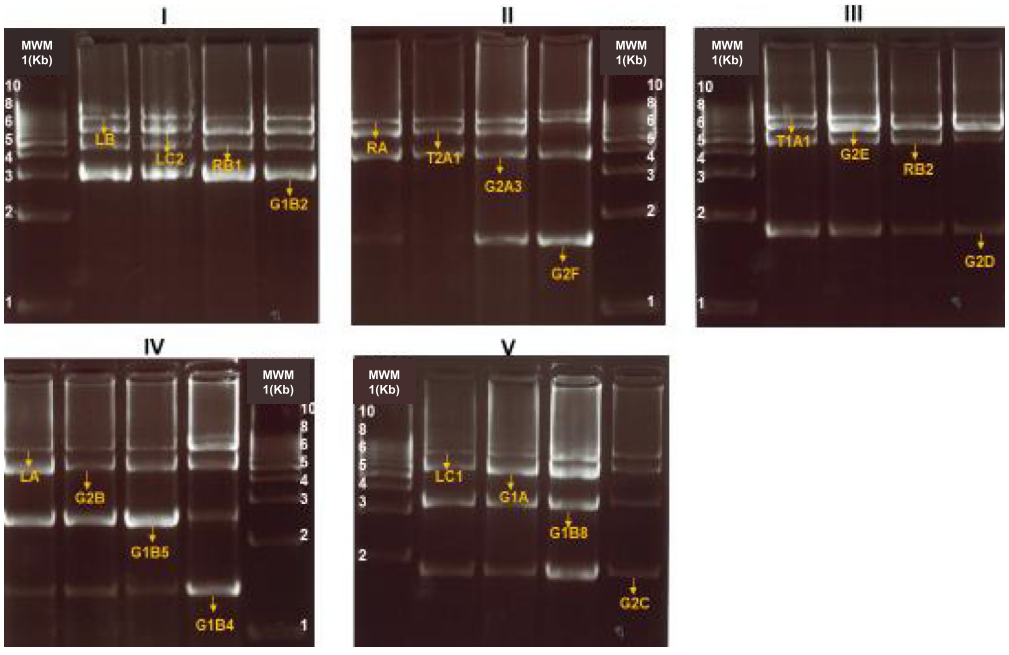


Fig. 3. Electrophoresis result of 5 groupings of 4 PCR amplified fragments each. Legend: I – Gel relative to the amplification of the LB (13559 bp), LC2 (7576 bp), RB1 (4851 bp) and G1B7 (3206 bp) fragments; II – Gel relative to the amplification of RA (8823 bp), T2A1 (6185 bp), G2A3 (3790 bp) and G2F (1636 bp) fragments; III – Gel relative to the amplification of the T1A1 (12110 bp), G2E (8034 bp), RB2 (5217 bp) and G2D (1793 bp) fragments; IV– Gel relative to the amplification of LA (10760 bp), G2B (5587 bp), G1B5 (2626 bp) and G1B4 (1415 bp) fragments; V – Gel relative to the amplification of LC1 (9748 bp), G1A (6069 bp) and G1B8 (3330 bp) and G2C (1821 bp) fragments; MWM – Molecular Weight Marker, Quick-Load® 1 Kb DNA Ladder from New England BioLabs ® Inc.

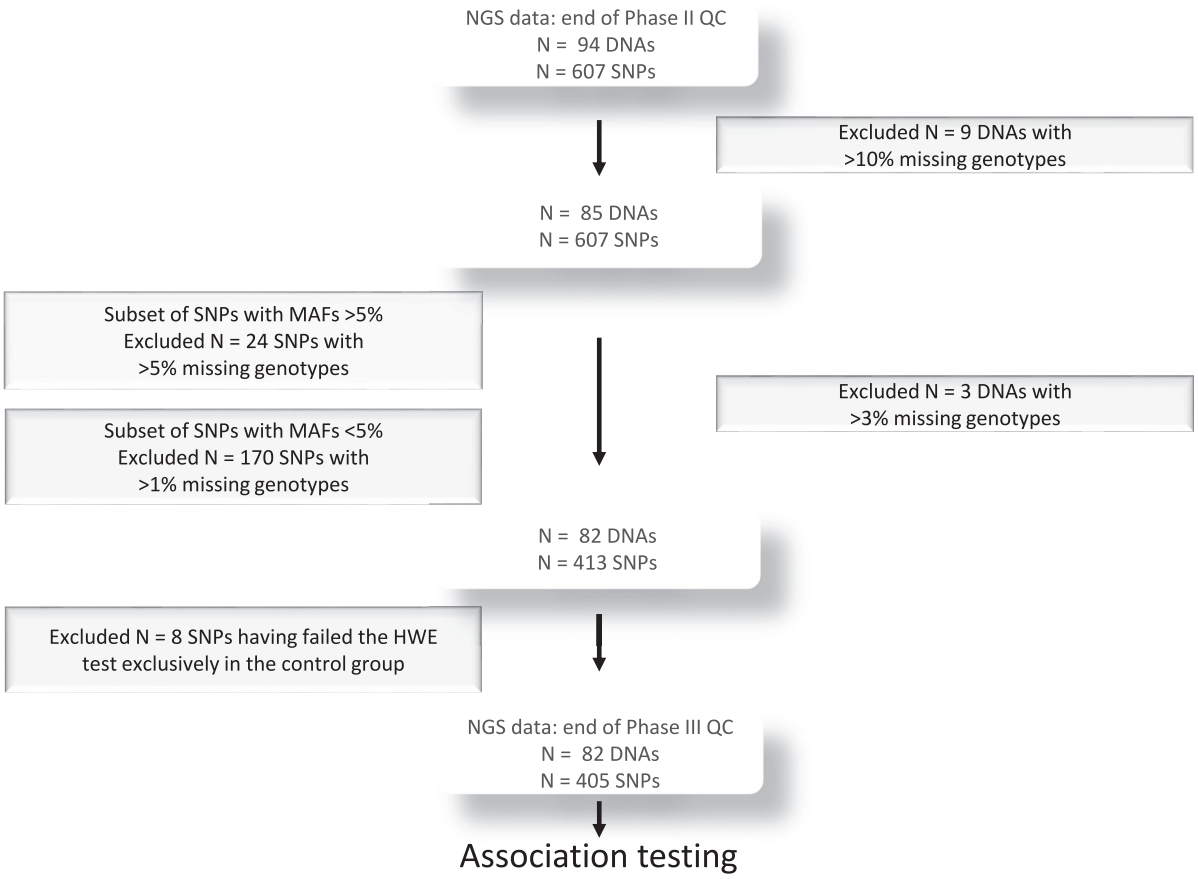


Fig. 4. Phase III Quality Control (QC) of genotyping data. Adapted methodology from the communication by McDonald and collaborators [28]. Definitions: N = number. Targetted next generation sequencing (NGS) was performed on 94 sample, including 60 samples from unrelated Q fever patients (17 chronic and 43 acute forms) and 34 convenient samples from the general Portuguese population. After NGS quality control, the number of samples available for the case-control genetic association study totaled 53 confirmed Q fever cases (15 chronic and 38 acute forms) and 29 samples from the general Portuguese population.

native alleles appear associated with an increase in *IFNGR2* expression in whole blood as well as with a decrease in *TMEM50B* expression in whole blood and in the heart [32].

3.4. LD analysis

Haploview [33], was used to investigated the entire sequenced region of the *IFNGR2* gene, between positions 21:33390000–33450000, for patterns of LD according to HapMap for the CEU population, more representative of the Portuguese population, than the IBS subpopulation (Supplementary Table 2). Variants in LD with the four associated SNPs were identified as variants in the *IFNGR2* and/or *TMEM50B* genes. None were reported in the ClinVar database, however, five were cited in the scientific literature: rs9808753 (NC_000021.9:g.33415005A > G), rs17880053 (NC_000021.9:g.33401192dup), rs8126756 (NC_000021.9:g.33403138T > C/T > G), rs8134145 (NC_000021.9:g.33403128A > C/A > G) and rs9978223 (NC_000021.9:g.33398052G > A/G > C). In the GTEx database, the alternative alleles of rs9808753, rs17880053 and rs9978223 appeared to be associated with higher expression of *IFNGR2* and lower expression of *TMEM50B* in the heart.

3.5. Haplotype analysis

Twelve haplotype blocks were estimated from our data, totaling 63 haplotypes (Supplementary Table 3). A large 64.17 kb block of 23 variants located at 21:33414268–33478437 (H12), showed a statistically significant association with chronic Q fever (p-value < 0.05), withstanding the Bonferroni correction (adjusted $\alpha = 0.000675676$) (Supplementary Tables 4 and 5). Frequency analysis of chronic versus acute Q fever showed this haplotype to be present only in the chronic Q fever group (20% of the cases). It was detected in 7% of the samples from the general Portuguese population (Supplementary Table 6).

3.6. Replication study

The replication study did not evidence statistically significant associations. However, the same tendencies were observed with the presence of the alternative allele of the four SNPs, rs78407108, rs17879956, rs7277167, and rs9974603, being more common in the chronic Q fever group. Whereas in the NGS studies the frequencies of the alternative allele varied from 25 to 29% in the chronic Q fever patients compared to 0–3% in the acute, in the replication these frequencies were 10–22% in the chronic disease group as compared to 5–8%, in the acute. However, if the Q fever cases, original and replication studies, were pooled (26 chronic and 59 acute) the presence of the alternative allele was significantly associated with chronic Q fever. The association withstood the Bonferroni criteria for statistical significance only for rs1789956, odds ratio 13.8095 (95% CI 2.9057–65.6316; p = 0.000142550).

3.7. Preliminary studies for functional immunological analysis

For the haplotype rs7277167_T (C > A,T); rs9974603_A (C > A,G); rs78407108_A (G > A,T); rs17879956_T (C > T), *IFNGR2* expression of the alternative allele (genotype TAAT) relative to the ancestral allele

Table 2
Statistically significant results for allelic association with chronic Q fever.

SNP	Location	Gene	Minor allele	MAF chronic	MAF acute	Major allele	$\chi 2$	P	OR
rs78407108	21:33426675	*IFNGR2*	A	0,20	0,00	G	16,11	0,0000597***	NA
rs17879956	21:33438631	*IFNGR2*	T	0,20	0,00	C	16,11	0,0000597***	NA
rs7277167	21:33401471	*IFNGR2*	T	0,20	0,00	C	15,71	0,0000740***	NA
rs9974603	21:33403994	*IFNGR2*	A	0,23	0,01	C	14,53	0,0001382***	22,22
rs9808753	21:33415005	*IFNGR2*	G	0,20	0,01	A	12,17	0,0004844***	18,75
rs412133	19:18094574	*IL12RB1*	T	0,43	0,18	C	7,032	0,0080070**	3,39
rs374326	19:18090456	*IL12RB1*	C	0,43	0,20	T	6,161	0,0130600*	3,11
rs418892	19:18094975	*IL12RB1*	T	0,43	0,20	C	6,161	0,0130600*	3,11
rs419540	19:18095263	*IL12RB1*	T	0,43	0,20	C	6,161	0,0130600*	3,11
rs845380	19:18070759	*IL12RB1*	A	0,43	0,20	G	5,771	0,0162900*	3,01
rs117612494	19:18059422	*IL12RB1*	T	0,07	0,00	C	5,164	0,0230600*	NA
rs184963792	21:33412855	*IFNGR2*	G	0,07	0,00	A	5,164	0,0230600*	NA
rs121913199	21:33421400	*IFNGR2*	A	0,07	0,00	G	5,164	0,0230600*	NA
rs185459305	21:33427424	*IFNGR2*	T	0,07	0,00	C	5,164	0,0230600*	NA
rs2045386	19:18071456	*IL12RB1*	A	0,18	0,39	G	4,282	0,0385100*	0,33
rs17879591	19:18060585	*IL12RB1*	T	0,30	0,13	C	4,147	0,0417000*	2,83
rs17878594	19:18062703	*IL12RB1*	T	0,30	0,13	C	4,147	0,0417000*	2,83
rs17879124	19:18062748	*IL12RB1*	A	0,30	0,13	G	4,147	0,0417000*	2,83
rs17878265	19:18066763	*IL12RB1*	A	0,30	0,13	G	4,147	0,0417000*	2,83
rs12150884	19:18069163	*IL12RB1*	A	0,07	0,24	G	4,069	0,0436700*	0,23
rs2305739	19:18069384	*IL12RB1*	A	0,07	0,24	G	4,069	0,0436700*	0,23
rs2305740	19:18069426	*IL12RB1*	G	0,07	0,24	A	4,069	0,0436700*	0,23
rs2305741	19:18069455	*IL12RB1*	A	0,07	0,24	G	4,069	0,0436700*	0,23
rs453427	19:18072680	*IL12RB1*	T	0,07	0,24	G	4,069	0,0436700*	0,23
rs447009	19:18074382	*IL12RB1*	A	0,07	0,24	C	4,069	0,0436700*	0,23
rs404068	19:18092159	*IL12RB1*	G	0,07	0,24	A	4,069	0,0436700*	0,23
rs433821	19:18093031	*IL12RB1*	G	0,07	0,24	A	4,069	0,0436700*	0,23
rs845332	19:18093283	*IL12RB1*	G	0,07	0,24	C	4,069	0,0436700*	0,23
rs438421	19:18065276	*IL12RB1*	A	0,37	0,18	G	3,973	0,0462300.	2,56

Definitions: MAF chronic = Minor allele frequency in the chronic Q fever cases; MAF acute = Minor allele frequency in the acute Q fever cases; $\chi 2$ = Basic allelic test chi-square; P = asymptotic p-value for the chi-square test; OR = odds ratio; NA = Not applicable; significance codes: 0 '***' 0,001 '**' 0,01 '*' 0,05 '.' 0,1 ' '.

(genotype CCGC) in healthy individuals showed an average fold difference of 1.6 ± 1.6. This result was thus inconclusive as to the existence of a fold increase in the expression of *IFNGR2* in the samples with the alternative allele.

4. Discussion

In humans, *C. burnetii* causes acute disabling disease (acute Q fever) that can evolve into the persistent potentially fatal forms (chronic Q fever), including endocarditis in the majority of cases.

Genotype to phenotype association studies are pivotal in the identification of markers for early diagnosis to channel prophylactic medical interventions, care and surveillance. However, only a limited number of genetic association studies for Q fever have been performed [15,34–37].

The success of the genetic model in tuberculosis led us to reflect on some similarities in the pathophysiology of infection by its etiological agent, *M. tuberculosis*, and that of infection by *C. burnetii*. The most characteristic of their common features is the formation of protective granuloma [5,38,39]. This approach provided some guidance in the choice of candidate genes for Q fever CGAS.

The CGAS was performed using NGS to genotype four candidate genes having been implicated in life-threatening mycobacterial diseases (*IL12RB1, IFNGR1, IFNGR2, NRAMP1*) [40–44], and three previously investigated for association with severe Q fever (*TLR1, TLR2, TIMP1*) [34,36,45]. Statistically significant associations with chronic Q fever were observed for *IFNGR2* and *IL12RB1*, whereas results withstanding the Bonferroni correction were only observed for four variants in the genomic locus containing *IFNGR2*. This targeted locus also contained the *TMEM50B* gene. These four variants were referenced in the GTEx database as eQTLs. In GTEx the alternative alleles appear associated with higher expressions of *IFNGR2* in whole blood and lower expression of the *TMEM50* in the heart. The variants were in LD in the CEU subpopulation. The observed enrichment of the alternative alleles in chronic Q fever compared to the general population further supported the association with clinical severity. Furthermore, a higher MAF observed in the Portuguese population than that reported for the CEU and IBS subpopulations could represent an increased vulnerability in this population.

Moreover, we identified a large 64.17 kb haplotype of 23 variants, located at 21: 33414268–33478437 in the *IFNGR2* and *TMEM50B* genomic region, with significant statistical association with Q fever, withstanding the Bonferroni correction. This haplotype is relatively frequent in the Portuguese general population (7%), announcing a potentially high risk of severe cases in a possible outbreak.

A replication study using an independent sample of the same ethnic origin did not evidence a statistically significant association. Nevertheless, when this sample was pooled with the original study population, statistically significant associations of the alternative allele with chronic Q fever were observed. Failure to observe a statistically significant association with the replicate study could be due to the small size of the sample. However, another issue could be the cross-sectional nature of this study and the inherent risk it poses for a misclassification of patients. Q fever patients were diagnosed at the INSA, but no follow-up was carried out to monitor an eventual evolution of acute Q fever to the chronic form. Chronic Q fever usually develops within 2 years after the primary infection; however, decade-long intervals between acute and chronic infection have been reported [46].

LD investigations in the CEU subpopulation identified several variants, reported in the international literature, that are in LD with the four SNPs associated with chronic Q fever; rs9808753 has been reported possibly related to immune system imbalance [47–52], and rs8126756 and rs8134145 associated with resistance to tuberculosis in the Vietnamese population in a haplotype context [53]. In the GTEx database, the alternative alleles of three of these variants, rs9808753, rs17880053 and rs9978223, appeared to associate with increased

Table 3
Allelic and genotypic frequencies of the *IFNGR2* variants significantly associated with chronic Q fever,[a] in different populations from the 1000 Genomes Project and in the general Portuguese population.[b]

Variant	Consequence[c]	Genotype	Populations from the 1000 Genomes Project (2015)				Study population			
			World (N = 2504)	European (N = 503)	CEU (N = 99)	IBS (N = 107)	Total (N = 82)	Chronic Qf (N = 15)	Acute Qf (N = 38)	GPP (N = 29)
rs78407108 NC_000021.9: g.33426675G>A	*IFNGR2* Intron Variant	GG	0,6890 (1725/2504)	0,8052 (405/503)	0,8283 (82/99)	0,8785 (94/107)	0,8415 (69/82)	0,6000 (9/15)	1,0000 (38/38)	0,7586 (22/29)
		GA	0,2396 (600/2504)	0,1769(89/503)	0,1718 (17/99)	0,1028 (11/107)	0,1585 (13/82)	0,4000 (6/15)	0,0000 (0/38)	0,2414 (7/29)
		AA	0,0715 (179/2504)	0,0179 (9/503)	0,0000 (0/99)	0,0187 (2/107)	0,0000 (0/82)	0,0000 (0/15)	0,0000 (0/38)	0,0000 (0/29)
		Allele A	0,1913 (958/5008)	0,106 (107/1006)	0,0859 (17/198)	0,0700 (15/214)	0,07927 (13/164)	0,2000 (6/30)	0,0000 (0/76)	0,1207 (7/58)
		Allele G	0,8087 (4050/5008)	0,8936 (899/1006)	0,9141 (181/198)	0,9299 (199/214)	0,92073 (151/164)	0,8000 (24/30)	1,0000 (76/76)	0,8793 (51/58)
rs17879956 NC_000021.9: g.33438631C>T	*TMEM50B* Intron Variant	CC	0,5735 (1436/2504)	0,7972 (401/503)	0,8081 (80/99)	0,8785 (94/107)	0,8171 (67/82)	0,6000 (9/15)	1,0000 (38/38)	0,6897 (20/29)
		CT	0,3415 (855/2504)	0,1809 (91/503)	0,1919 (19/99)	0,1028 (11/107)	0,1829 (15/82)	0,4000 (6/15)	0,0000 (0/38)	0,3103 (9/29)
		TT	0,0851 (213/2504)	0,0219 (11/503)	0,0000 (0/99)	0,0187 (2/107)	0,0000 (0/82)	0,0000 (0/15)	0,0000 (0/38)	0,0000 (0/29)
		Allele T	0,256 (1281/5008)	0,1123 (113/1006)	0,0960 (19/198)	0,0701 (15/214)	0,0915 (15/164)	0,2000 (6/30)	0,0000 (0/76)	0,1552 (9/58)
		Allele C	0,7442 (3727/5008)	0,8877 (893/1006)	0,9040 (179/198)	0,9299 (199/214)	0,9085 (149/164)	0,8000 (24/30)	1,0000 (76/76)	0,8448 (49/58)
rs7277167 NC_000021.9: g.33401471C>T	*IFNGR2* 2KB Upstream Variant	CC	0,6270 (1570/2504)	0,8032 (404/503)	0,8283 (82/99)	0,8785 (94/107)	0,8272 (67/81)	0,6000 (9/15)	1,0000 (37/37)	0,7241 (21/29)
		CT	0,2955 (740/2504)	0,1809 (91/503)	0,1717 (17/99)	0,1028 (11/107)	0,1728 (14/81)	0,4000 (6/15)	0,0000 (0/37)	0,2759 (8/29)
		TT	0,0775 (194/2504)	0,0159 (8/503)	0,0000 (0/99)	0,0187 (2/107)	0,0000 (0/81)	0,0000 (0/15)	0,0000 (0/37)	0,0000 (0/29)
		Allele T	0,2252 (1128/5008)	0,1064 (107/1006)	0,0859 (17/198)	0,0701 (15/214)	0,0864 (14/162)	0,2000 (6/30)	0,0000 (0/74)	0,1379 (8/58)
		Allele C	0,7748 (3880/5008)	0,8936 (899/1006)	0,9141 (181/198)	0,9299 (199/214)	0,9136 (148/162)	0,8000 (24/30)	1,0000 (74/74)	0,8621 (50/58)
rs9974603 NC_000021.9: g.33403994C>A	*IFNGR2* Intron Variant	CC	0,4884 (1223/2504)	0,7455 (375/503)	0,7374 (73/99)	0,8318 (89/107)	0,8148 (66/81)	0,6000 (9/15)	0,9730 (36/37)	0,7241 (21/29)
		CA	0,3750 (939/2504)	0,2247 (113/503)	0,2424 (24/99)	0,1495 (16/107)	0,1728 (14/81)	0,3333(5/15)	0,0270 (1/37)	0,2759 (8/29)
		AA	0,1366 (342/2504)	0,0298 (15/503)	0,0202 (2/99)	0,0187 (2/107)	0,0123 (1/81)	0,0667 (1/15)	0,0000 (0/37)	0,0000 (0/29)
		Allele A	0,3241 (1623/5008)	0,1421 (143/1006)	0,1414 (28/198)	0,0935 (20/214)	0,0988(16/162)	0,2333 (7/30)	0,0135 (1/74)	0,1379 (8/58)
		Allele C	0,6759 (3385/5008)	0,8579 (863/1006)	0,8586 (170/198)	0,9065 (194/214)	0,9012 (146/162)	0,7667 (23/30)	0,9865 (73/74)	0,8621 (50/58)

Definitions: World – World population from the 1000 Genomes Project (2015 version); European − European population from the 1000 Genomes Project (2015 version) that includes the subpopulations CEU (Utah residents with Northern and Western European ancestry) and IBS (Iberian populations in Spain), amongst others; N = number; Qf = Chronic Q fever group of patients; acute Qf = Acute Q fever group of patients; GPP = General Portuguese population this study.

[a] Variants with statistically significant association that resisted the Bonferroni criterion (adjusted $\alpha = 0.00016339869$).

[b] General Portuguese population from this study.

[c] Functional Consequence (dbSNP, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi).

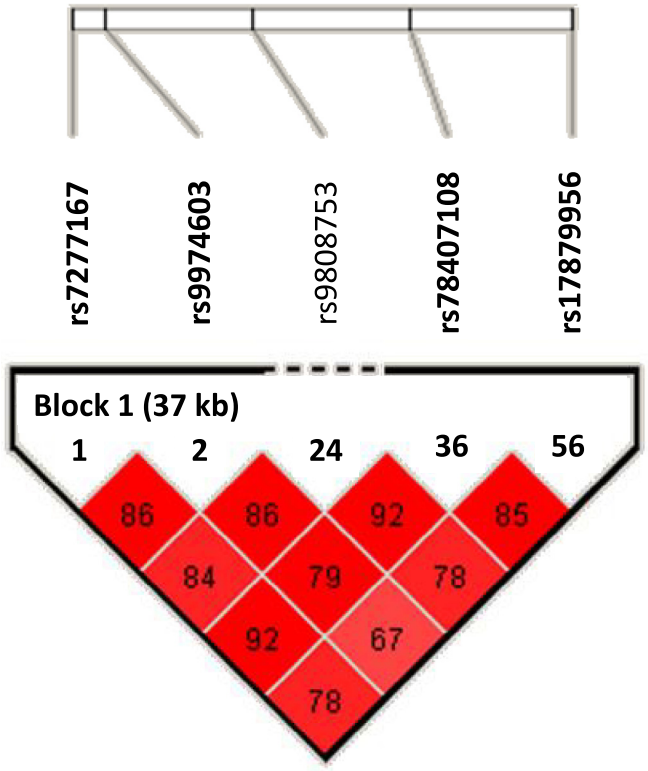


Fig. 5. LD patterns in the genomic region around the SNPs rs78407108 G > A, rs17879956 C > T, rs7277167 C > T, and rs9974603 C > A in the IFNGR2 gene, associated with chronic Q fever, having withstood the Bonferroni correction for multiple comparisons. Patterns are according to HapMap, visualized using the Haploview software [33], in the population with ancestry from northern and western Europe (CEU). The display uses the standard color scheme and the LD-values shown are the R-squared.

IFNGR2 expression in whole blood and decreased *TMEM50B* expression in the heart.

Interestingly, SNP rs9978223 has been identified as showing a significant association with acute myocardial infarction in the Indian population [54]. Although little documented in the literature, there are references that have correlated myocardial infarction with infective endocarditis [55–57]. As endocarditis is the main consequence of chronic Q fever, the hypothesis raised about the indirect implication of the rs9978223 variant with this phenotype deserves further investigation.

IFN-γ is the central effector of cell-mediated immunity. IFN-γ plays an important role in the defense against *C. burnetii* [5,58]. IFN-γ signals through binding of its receptor (IFNGR), a heterodimeric complex composed of a ligand binding alpha subunit (IFNGR1) and a signal transducing beta subunit (IFNGR2), both belonging to the class II cytokine receptor family. The IFNGR2 chain is generally the limiting factor in IFN-γ responsiveness, as it seems to be associated with the cellular response [59], whereas the IFNGR1 chain is required for ligand binding and signaling and is usually in surplus [60]. Moreover, the surface expression of *IFNGR2* determines whether a cell stimulated by IFN-γ undergoes proliferation or apoptosis [59,61]. Variants in interferon receptor genes have been associated with chronic disease [40–42,62].

Previously, it had been assumed that the chronicity of Q fever was due to unresponsive T lymphocytes and impaired production of IFN-γ in response to *C. burnetii* [63–65]. However, more recent studies have evidenced the high production of antigen-specific IFN-γ in chronic Q fever [15,66,67]. Schoffelen and collaborators demonstrated that IFN-γ production by *C. burnetii*-stimulated PBMCs from chronic Q fever patients was significantly higher than in healthy controls and that many IFN-γ response genes were also strongly upregulated [15]. The authors concluded that the IFN-γ signalling pathway was intact in chronic Q fever, being higher than in healthy individuals. These findings appear to contradict commonly held assumptions that IFN-γ production is characteristic of the protective immune response.

The apparent contradictory role of IFN-γ has also been observed in human Chagas' disease, where in chronically infected patients, higher levels of IFN-γ and IFN-γ-inducible genes are seen in myocardial tissues of patients suffering from Chagas' cardiomyopathy in comparison with asymptomatic (indeterminate) patients [68]. Moreover, a direct link of IFN-γ signaling to cardiac disease has been reported in a number of clinical studies. Improved cardiac function was observed to be concomitant with a reduction of serum IFN-γ levels [69]. Higher serum IFN-γ levels as compared to healthy controls have been reported in patients suffering from chronic heart failure [70].

In their study, Fenollar and collaborators encountered preexisting valvular disease more often in Q fever patients with endocarditis (p-value $< 10^{-7}$) [6]. The lack of cardiac vegetation and inflammation in these patients suggests a mechanism distinct from the colonization of cardiac valves found in usual infectious endocarditis, suggesting that factors related to the cardiac valve context, including the immune context, are involved in *C. burnetii* endocarditis [5,71–73]. In addition, *C. burnetii* endocarditis is more frequently observed in patients with bicuspid aortic valves [74], a frequent congenital heart disorder with a large heritable component [75,76]. The genetic component of the majority of these cases remains however, unexplained.

Although, our results do not point to any clear explanation of the role of the *IFNGR2* gene and its variants in chronic Q fever, the existence of immunogenetic factors related to the cardiac valve context is suggested. Preexisting valvular disease was the major risk factors present in our chronic Q fever patient group. This factor is known to enhance the risk of endocarditis in patients with *C. burnetii* infection. Given the selection of our group of cases and controls (1) chronic Q fever patients with cardiovascular predisposition to disease chronicity, namely in the form of valvular disease; (2) acute Q fever patients for the most part presenting no apparent cardiovascular risk factor, respectively, we hypothesize that the statistical association observed in our study evidenced a predisposing trait leading up to the subsequent development of the chronic form of the disease. The persistence of *C. burnetti* in favorable niches would allow for a temporal difference in the role of IFN-γ signaling possibly localized at the cardiac level of the infection. The phenotype definition used in our study may have introduced bias in the results given the fact that the case and control groups were not as identical as possible in relation to the disease phenotype as recommended for case control CGAS [77]. Nevertheless, it may have helped evidence variants and alleles more prone to detect an association with cardiovascular risk in chronic Q fever.

A major critique to our study was the small sample size. Due to this constraint, a strategy was selected in the quality control of NGS data applying a more stringent criterion of missing genotypes for rare variants (removal of samples with >1% of genotypes absent) than for the more common variants (removal of samples with >5% of genotypes absent) [28]. An additional weakness in our study was the lack of functional studies. The fact that RNA from patient groups was not available to us, did not allow us to investigate IFN-γ production in chronic Q fever patients. Our preliminary studies were only possible with healthy individuals, and failed to conclude if there was a fold increase in the expression of *IFNGR2* in the samples with the alternative allele relative to the ancestral allele haplotype.

Given the direct bearing of IFNGR2 on IFN-γ signaling, the possible involvement of the associated SNPs and haplotype identified in this study with a higher expression of the *IFNGR2* gene could be in line with observations suggesting that IFN-γ production from chronic patients is significantly higher than in healthy controls. Appropriate

models are needed for further investigations on IFN-γ signaling in Q fever, such as *ex vivo* and *in vivo* models coupled with IFN-γ and/ or infection challenges.

Although, the implication of the IFN-γ signaling, through the association of *IFNGR2* variants with chronic Q fever valvular disease and its evolution to endocarditis has been discussed, further investigations of the immunological processes are required to clarify the role of *IFNGR2* in association with chronic Q fever.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CRediT authorship contribution statement

Susana David: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Liliana Castro:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Elsa Duarte:** Writing – review & editing, Methodology. **Ulisses Gaspar:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Maria Rosário da Costa Rodrigues:** Writing – original draft, Methodology. **Maria Vanessa Cueto-Rojo:** Writing – original draft, Methodology. **Joana Mendonça:** Methodology, Data curation. **José Ferrão:** Writing – original draft, Investigation. **Miguel Machado:** Writing – original draft, Methodology, Formal analysis, Data curation. **José Poças:** Writing – original draft, Investigation. **João Lavinha:** Resources, Funding acquisition, Conceptualization. **Luís Vieira:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Ana Sofia Santos:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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U Gaspar's contribution was in the context of the preparation for his Master's degree in Biomedical Sciences, Universidade da Beira interior, Covilhã, Portugal.

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Appendix A. Supplementary data

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