A Genome-Wide Association Scan on the Levels of Markers of Inflammation in Sardinians Reveals Associations That Underpin Its Complex Regulation

Silvia Naitza1, Eleonora Porcu1, Maristella Steri1, Dennis D. Taub2, Antonella Mulas1, Xiang Xiao3, James Strait2, Mariano Dei1, Sandra Lai1, Fabio Busonero1, Andrea Maschio1, Gianluca Usala1, Magdalena Zoledziewska4, Carlo Sidore1,4,5, Ilenia Zara6, Maristella Pitzalis4, Alessia Loi1, Francesca Virdis1, Roberta Piras1, Francesca Deidda6, Michael B. Whalen6, Laura Crisponi1, Antonio Concas7, Carlo Podda7, Sergio Uzzau4,8, Paul Scheet3, Dan L. Longo2, Edward Lakatta2, Gonçalo R. Abecasis5, Antonio Cao1, David Schlessinger2, Manuela Uda1, Serena Sanna1,9, Francesco Cucca1,4,9

1 Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche, Cagliari, Italy, 2 Intramural Research Program, National Institute on Aging, Baltimore, Maryland, United States of America, 3 University of Texas, MD Anderson Cancer Center, Department of Epidemiology, Houston, Texas, United States of America, 4 Dipartimento di Scienze Biomediche, Università di Sassari, Sassari, Italy, 5 Center for Statistical Genetics, Department of Biostatistics, University of Michigan, Ann Arbor, Michigan, United States of America, 6 Center for Advanced Studies, Research, and Development in Sardinia (CRS4), AGCT Program, Parco Scientifico e Tecnologico della Sardegna, Pula, Italy, 7 High Performance Computing and Networking, CRS4, Parco Tecnologico della Sardegna, Pula, Italy, 8 Porto Conte Ricerche, Località Tramariglio, Alghero, Sassari, Italy

Abstract

Identifying the genes that influence levels of pro-inflammatory molecules can help to elucidate the mechanisms underlying this process. We first conducted a two-stage genome-wide association scan (GWAS) for the key inflammatory biomarkers Interleukin-6 (IL-6), the general measure of inflammation erythrocyte sedimentation rate (ESR), monocyte chemotactic protein-1 (MCP-1), and high-sensitivity C-reactive protein (hsCRP) in a large cohort of individuals from the founder population of Sardinia. By analysing 731,213 autosomal or X chromosome SNPs and an additional ~1.9 million imputed variants in 4,694 individuals, we identified several SNPs associated with the selected quantitative trait loci (QTLs) and replicated all the top signals in an independent sample of 1,392 individuals from the same population. Next, to increase power to detect and resolve associations, we further genotyped the whole cohort (6,145 individuals) for 293,875 variants included on the ImmunoChip and MetaboChip custom arrays. Overall, our combined approach led to the identification of 9 genome-wide significant novel independent signals—5 of which were identified only with the custom arrays—and provided confirmatory evidence for an additional 7. Novel signals include: for IL-6, in the ABO gene (rs657152, p = 2.13 × 10^-29); for ESR, at the HBB (rs4910472, p = 2.31 × 10^-11) and UC119B/SPPL3 (rs11829037, p = 8.91 × 10^-10) loci; for MCP-1, near its receptor CCR2 (rs17141006, p = 7.53 × 10^-15) and in CADM3 (rs3026968, p = 6.73 × 10^-13); for hsCRP, within the CRP gene (rs3093077, p = 5.73 × 10^-2), near DARC (rs3845624, p = 1.43 × 10^-10), UNC119B/SPPL3 (rs11829037, p = 1.50 × 10^-14), and ICOSLG/AIRE (rs113459440, p = 1.54 × 10^-08) loci. Confirmatory evidence was found for IL-6 in the IL-6R gene (rs4129267); for ESR at CR1 (rs12567990) and TMEM517 (rs10903129); for MCP-1 at DARC (rs12075); and for hsCRP at CRP (rs1205), HNF1A (rs225918), and APOC-I (rs4420638). Our results improve the current knowledge of genetic variants underlying inflammation and provide novel clues for the understanding of the molecular mechanisms regulating this complex process.

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* E-mail: francesco.cucca@inn.cnr.it

† These authors contributed equally to this work.

Introduction

Inflammation is a critical response to pathogens and injuries. Its control entails a coordinated cascade of biological events regulated by specific cells and molecular signals, in a complex process that is only partially understood. In this context, genetics can provide important clues, given that population studies indicate that about half of the inter-individual variability in biomarkers of inflammation is genetically determined and considering the achievements of GWA scans (GWAS) in complex trait analysis during the last few
Author Summary

Inflammation is a protective response of our organism to harmful stimuli—such as germs, damaged cells, or irritants—and to initiate the healing process. It has also been implicated, with both protective and predisposing effects, in a number of different diseases; but many important details of this complex phenomenon are still unknown. Identifying the genes that influence levels of pro-inflammatory molecules can help to elucidate the factors and mechanisms underlying inflammation and their consequence on health. Genome-wide association scans (GWAS) have proved successful in revealing robust associations in both common diseases and quantitative traits. Here, we thus performed a multistage GWAS in a large cohort of individuals from Sardinia to examine the role of common genetic variants on the key inflammatory biomarkers Interleukin-6, erythrocyte sedimentation rate, monocyte chemotactic protein-1, and high-sensitivity C-reactive protein. Our work identified new genetic determinants associated with the quantitative levels of these inflammatory biomarkers and confirmed known ones. Overall, the data highlight an intricate regulation of this complex biological phenomenon and reveal proteins and mechanisms that can now be followed up with adequate functional studies.

Results

We initially assessed 731,213 autosomal or X chromosome SNPs and imputed further ~1.9 million variants in 4,694 individuals (Step 1) and then replicated the top signals in an additional 1,392 individuals (Step 2). To increase the detection power and provide useful information for the fine mapping stage we have also evaluated the whole cohort of 6,145 individuals with the ImmunoChip (151,085 variants) [12] and the MetaboChip (142,790 variants, of which 9,920 overlapped with ImmunoChip) [13] (Step 3). Overall we detected variants significantly associated with each of the traits assessed. The salient results are reported below.

Step 1: Genome-Wide Association Scan

We identified several SNPs above the standard genome-wide significant threshold \((5 \times 10^{-8})\) in the SardiNIA discovery cohort (Table 1, Table S1 and Figure S1). The region surrounding each of these SNPs was studied in more detail as the respective traits were analyzed (see below).

IL-6

For IL-6, SNPs with \(p\)-values\(<5 \times 10^{-8}\) were all located in the ABO (\(a-1,3-N\)-acetylgalactosaminyitransferase) locus on chromosome 9q34.1-q34.2 (Table S1), encoding the Histo-blood group ABO system transferase, with the strongest signal at rs643434 in intron 1 of the gene \((p = 2.69 \times 10^{-21}, 0.69 \text{ pg/ml average increase per G allele}) (Table 1). This SNP is in strong linkage disequilibrium (LD) with another associated variant, rs687289, which tags the O allele of the ABO locus \((r^2 = 0.931 \text{ in HapMap CEU})\).

ESR

For ESR, we identified several associated SNPs on chromosome 1q32, all within the CR1 (complement component (3b/4b) receptor 1) gene, a member of the receptors of the complement activation family, recently shown associated with ESR (Table S1) [14]. The strongest signal was observed at rs12034598 in intron 22 of CR1, with a \(p\)-value of \(9.31 \times 10^{-11}\) (1.024 mm/h average increase per G allele) (Table 1). This SNP is in strong LD with other associated variants including rs2274567 \((r^2 = 1)\), a non-synonymous SNP in exon 22 that causes a His1208Arg substitution predicted as potentially damaging by PolyPhen and affecting expression levels of CR1 on the erythrocytes (Table S1) [15,16]. In the genomic region covered by CR1 several copy number variations (CNVs) have been identified. However, none of the 38 SNPs in this region with \(p\)-value\(<5 \times 10^{-8}\) (Table S1) tags the CNVs reported in a previous study [17,18]. In addition, CNVs analysis with PennCNV [19] in individuals genotyped with the Affymetrix 6.0 microarray did not show presence of CNVs in our samples (unpublished data). Still, we could not exclude the presence of population-specific CNVs or common CNVs not directly interrogated by the Affymetrix probes.

We also found a locus suggestively associated with ESR on chromosomes 11p15 in the \(\beta\)-globin locus control region (LCR), which coordinates the expression of the globin genes (Table S1). The top signal was at marker rs4910742 \((p = 6.34 \times 10^{-10})\) (Table 1), which is a surrogate for the \(B^{09/99}\) mutation carried by a large portion (11–13%) of the Sardinians and able to influence the levels of several blood indices, including number of RBCs [20], a parameter that has an inverse relationship with ESR. Accordingly, when we tested the association analysis including in the model \(\beta\)-Thalassemia (\(\beta\)-Thal) carrier status as a covariate, the association at rs4910742 disappeared \((p = 0.54 \text{ in SardiNIA})\).

MCP-1

The GWAS results for MCP-1 levels revealed strong association signals on chromosome 1q22-q23 (Table S1). The associated region encompassed ~500 kb and contained several genes, with the top signal detected in the DARC (Duffy blood group chemokine receptor) gene at marker rs12075 \((p = 1.68 \times 10^{-30}, 36.78 \text{ pg/ml average increase per A allele})\), as also shown in a recent meta-analysis (Table 1) [21]. The association curve encompasses several other genes, including the C4ADMA3 (cell adhesion molecule 3) locus upstream of DARC, as well as in the FCER1A (Fc fragment of IgE, high
Table 1. Top genome-wide association results for IL-6, ESR, MCP-1, and hsCRP.

| Trait | Gene | Marker | Allele Minor/Major N | RSQR | Freq | Effect(SE) | p-value N | Freq | Effect(SE) | p-value |
|-------|------|--------|---------------------|------|-------|-----------|----------|-------|-----------|----------|
| IL-6  | ABO  | rs643434 A/G | 4621 0.999 0.258 -0.245(0.026) | 2.69×10⁻⁷ | 23190 0.264 -0.160(0.039) | 4.07×10⁻⁸ | 6011 8.68×10⁻⁸ |
|       | ESR  | CR1    | rs12034598 A/G | 4689 GEN 0.408 -0.143(0.022) | 9.31×10⁻¹² | 1392 0.370 -0.128(0.035) | 2.19×10⁻⁶ | 6081 8.82×10⁻⁸ |
|       | HBB  | rs4910742 G/A | 4689 GEN 0.066 -0.229(0.042) | 6.34×10⁻⁶ | 1375 0.051 -0.199(0.076) | 8.62×10⁻⁹ | 6064 1.89×10⁻⁹ |
| MCP-1 | DARc | rs12075 A/G | 4624 0.709 0.490 0.303(0.026) | 1.68×10⁻⁶ | 1392 0.560 0.399(0.039) | 4.93×10⁻⁸ | 6016 4.33×10⁻⁸ |
|       | CADMs | rs3026968 T/C | 4153 GEN 0.120 0.239(0.033) | 7.63×10⁻¹² | 1226 0.120 0.151(0.062) | 1.47×10⁻¹⁰ | 5379 8.70×10⁻¹⁰ |
| hsCRP | CRP  | rs1341655 A/G | 4434 0.963 0.417 -0.195(0.024) | 2.82×10⁻¹⁰⁰ | 1069 0.371 -0.188(0.043) | 1.32×10⁻⁶ | 5503 1.98×10⁻⁶ |
|       | DARC | rs3845624 C/A | 3985 GEN 0.470 0.140(0.220) | 1.43×10⁻¹⁰² | 941 0.470 0.102(0.046) | 2.66×10⁻¹⁰² | 4926 1.65×10⁻¹¹ |

The table summarizes top genome-wide association signals for IL-6, ESR, MCP-1 and hsCRP phenotypes in the HapMap based GWAS (Step 1), as well as results in the replication independent cohort (Step 2) and in the combined data-sets. For each marker, frequency and effect estimates are given with respect to the minor allele. Imputation quality scores (RSQ) are reported for imputed SNPs. Novel signals are indicated in bold. The effect size is measured in standard deviation units, being estimated as the β coefficient of the regression model when using the normalized trait (e.g. an effect size of 1.0 implies each additional copy of the allele being evaluated increases trait values by 1.0 standard deviations).

The table includes information on the imputation quality scores (RSQ) and the effect sizes (SE) for the SNPs with the strongest association signal at each locus as well as a combined analysis. Follow-up analysis of the top SNP rs643434 in ABO showed replication of this signal in SardiNIA stage 2 (p = 4.07×10⁻⁸), Table 1), supporting a role for this gene in regulating the levels of IL-6.

For ESR, replication was observed for both the top marker in the known CR1 gene (rs12034598, p = 2.19×10⁻⁸ for its genotyped proxy rs630877 with r² = 1), and the SNP in the β-globin LCR, (rs4910742, p = 8.62×10⁻⁵ for its genotyped proxy rs10506047 with r² = 0.661) (see Table 1). As observed in the Step 1, when we repeated the association analysis for the latter SNP, including in the model β-Thal carrier status as a covariate, the association disappeared (p = 0.34).

The top SNP in the chemokine receptor gene DARC known to be associated with MCP-1 levels was also strongly replicated (rs12075, p = 4.93×10⁻⁸). The relatively lower association signal showed by rs12075 in the SardiNIA discovery cohort (Step 1) compared to the follow-up cohort SardiNIA stage 2 is most likely due to the fact that it was imputed with a modest imputation score in the initial GWAS, whereas it was directly genotyped in the replication cohort. The association signal at rs3026968 in CADMs was also confirmed in SardiNIA stage 2 (p = 0.0147, Table 1), whereas the association at rs9405112 in the HLA-DRA9 region was not (p = 0.59). Neither the signal in the CDH4 gene, or that at rs3918357 in the CCR2 gene were followed up; however, the latter supports a previously reported suggestive association in the CCR2/CCL5 cytokine receptor gene cluster (rs12495098, r² = 1 with rs3918357) [21].

Finally for hsCRP, the top SNP associated in the known CRP gene were followed up; however, the latter supports a previously reported suggestive association in the CCR2/CCL5 cytokine receptor gene cluster (rs12495098, r² = 1 with rs3918357) [21].

Step 2: Follow-Up of Initial Findings

To corroborate our initial findings, we examined with TaqMan genotyping technology the 4 top associated SNPs (p<5×10⁻⁶), as well as 3 additional SNPs including the 2 independent signals in the CADMs and DARC loci (rs3026968 and rs3845624), and one suggestive SNP with p<10⁻⁶ near HLA-DRA9 (rs9268858), in a group of 1,392 Sardinians enrolled in the same SardiNIA study but unrelated to the individuals analyzed in Step 1 GWAS. This independent cohort has been previously described as SardiNIA stage 2 [27]. Table 1 provides a summary of the follow-up results for the SNPs with the strongest association signal at each locus as well as a combined analysis.

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Step 3: Gene-Specific Scan using Custom Genotyping Arrays

To refine the contribution of the detected loci and increase the power to detect novel signals, we performed an additional association scan by testing 293,875 variants assessed in the whole SardiNIA cohort (6,145 individuals, including the discovery and...
follow-up cohorts from Steps 1 and 2) by genotyping with the ImmunoChip [12] and the MetaboChip [13], two Illumina custom arrays designed to follow up regions of prior interest in immune- and metabolic-related traits and diseases, respectively, as detailed in the Methods section. With this approach, we not only validated with an independent genotyping method, and refined all the association results at the previously described loci (see Figure S1, Table 1), but also identified novel signals for all traits (Figure 1, Table 2). A detailed view of the associated regions is illustrated in Figure 2 and Figure 3, and results discussed below. The effect of the associated variants on trait variability per genotype is represented in Figure S2 and Figure S3.

**IL-6**

For IL-6 levels, besides corroborating the association at the ABO gene (strongest hit rs657152, $p = 2.13 \times 10^{-39}$), we also observed a signal at rs4129267 ($p = 2.36 \times 10^{-20}$, with an average increase of 0.220 pg/ml per T allele), in the IL-6R (IL-6 receptor) gene (Table 2, Figure 2A and 2B, and Figure S2). This SNP is a proxy of the functional SNP rs8192284 ($r^2 = 0.982$ in HapMap CEU) affecting cleavage of IL-6 soluble receptor (IL-6 sR), which was previously found associated with both IL-6 sR and IL-6 levels by admixture mapping and candidate gene analysis in African and European Americans [28]. SNP rs4129267 was genotyped in the Step 1 GWAS but observed with a lower p-value ($p = 2.45 \times 10^{-14}$).

Conditional analysis did not reveal any independent signals at such loci. The top variants at ABO (rs657152) and IL-6R (rs4129267) explain 2.2% of the total phenotype variation.

**ESR**

The scan for ESR, in addition to strong confirmatory signals in CR1 (strongest hit rs12567990, $p = 8.26 \times 10^{-15}$), detected a novel associated SNP, rs10903129, in TMEM57 (Transmembrane protein 57) ($p = 3.91 \times 10^{-08}$, with an average increase of 0.581 mm/h per G allele) (Table 2, Figure 2C and 2D, and Figure S2). SNP rs10903129 was analysed in the Step 1 GWAS, but its p-value did not reach genome-wide significance ($p = 9.30 \times 10^{-05}$) and thus it was not considered for follow-up in Step 2. Although this gene encodes a largely uncharacterized protein, polymorphisms in the region have been previously reported associated with lipid levels, CHD and more recently with ESR [14,29]. We also detected a novel signal on chromosome 12q24.31 near the UNC119B (Unc-119 homolog B) and SPPL3 (Signal peptide peptidase-like 3) genes at a low frequency SNP, rs11829037, with a large effect ($p = 8.91 \times 10^{-10}$, MAF = 0.009, average increase of 4.657 mm/h per the minor T allele) (Figure 2E and Table 2). It was...

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**Figure 1. Manhattan plot and QQ plot of association findings.** The figure summarizes the association results obtained on the ImmunoChip and MetaboChip markers (Step 3). The blue dotted line marks the Bonferroni threshold significance levels ($1.7 \times 10^{-7}$), and SNPs in loci exceeding this threshold are highlighted in green. The bottom panel represents the QQ plot, where the red line corresponds to all test statistics, and the blue line to results after excluding statistics at top markers (highlighted in green in the Manhattan Plot). The gray area corresponds to the 90% confidence region from a null distribution of P values (generated from 100 simulations).

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genotyped by the MetaboChip, but the association at this locus was supported by SNPs genotyped with both arrays, and by more common SNPs (MAF up to 0.05 for the 18 SNPs with p-value<10^{-6}). It is missing and not well tagged in the HapMap data set, which provides an explanation as to why it was not discovered in the initial scan (step 1). Finally, we confirmed the association at rs4910742 (p = 2.31×10^{-11}) in the HBB locus (Figure 2F). Conditional analysis did not reveal any independent signals at such loci. The top variants at CR1 (rs12567990), HBB (rs4910742), TMEM57 (rs10903129) and UNC119B/SPPL3 (rs11829037) explain 2.3% of the total trait variation.

MCP-1

For MCP-1, the association with the coding SNP in DARC was corroborated with a striking p-value (rs12075, p = 7.43×10^{-102}) (Figure 3A and Table 2). In addition, SNP rs17141006, 10 kb upstream of its receptor CCR2, correlated with the previous borderline signal (r^2 = 0.997), reached genome-wide significance (rs17141006, p = 7.53×10^{-105}; average increase of 5.35 mg/L per T allele), and the 8th HBB gene (rs4420638, 0.094 (rs3845624) explain 9.8% of the phenotypic variation.

**Table 2.** Top association signals for IL-6, ESR, MCP-1, and hsCRP in the ImmunoChip and MetaboChip data-sets.

| Trait | Gene | Marker | Allele Minor/ Major | N   | Freq  | Effect(SE) | p-value | Array | r^2 with GWAS (SNP) |
|-------|------|--------|---------------------|-----|-------|------------|---------|-------|-------------------|
| IL-6  | ABO  | rs657152| T/G                 | 5915| 0.269 | -0.219 (0.019) | 2.13×10^{-20} | L | 1 (rs643434) |
|       | IL6R | rs4129267| T/C                 | 5915| 0.260 | 0.109 (0.020) | 2.36×10^{-08} | L | - |
| ESR   | CR1  | rs12567990| C/T                | 6021| 0.408 | -0.152 (0.020) | 8.26×10^{-15} | L | 0.945 (rs12034598) |
|       | HBB  | rs4910742| G/A                | 6021| 0.075 | -0.208 (0.031) | 2.31×10^{-11} | L | (same SNP) |
|       | TMEM57| rs10903129| G/A              | 6021| 0.339 | -0.093 (0.017) | 3.91×10^{-08} | L | - |
|       | UNC119B/SPPL3| rs11829037| T/C       | 6106| 0.009 | 0.523 (0.085) | 8.91×10^{-10} | M | - |
| MCP-1 | DARC | rs12075| G/A               | 6010| 0.445 | -0.405 (0.019) | 7.43×10^{-102} | M | (same SNP) |
|       | CCR2 | rs17141006| C/T            | 5924| 0.101 | 0.253 (0.035) | 7.53×10^{-13} | L | 0.997 (rs3918357) |
| hsCRP | CRP  | rs1205  | T/C              | 5705| 0.383 | -0.209 (0.018) | 8.20×10^{-30} | L | 0.961 (rs1341665) |
|       | CRP  | rs3090377| G/T               | 5705| 0.118 | 0.173 (0.027) | 5.73×10^{-21} | L | 0.043 (rs3845624) |
|       | UNC119B/SPPL3| rs11829037| T/C       | 5791| 0.009 | 0.713 (0.093) | 1.50×10^{-14} | M | - |
|       | HNF1Ac | rs2259816| A/C            | 5703| 0.335 | -0.114 (0.019) | 5.41×10^{-06} | L | - |
|       | APOC-I| rs4420638| G/A           | 5657| 0.094 | -0.200 (0.031) | 7.13×10^{-11} | L | - |
|       | ISOCG/AIRE| rs113459440| T/C       | 5704| 0.003 | 0.819 (0.145) | 1.54×10^{-08} | L | - |

The table summarizes top association signals for IL-6, ESR, MCP-1 and hsCRP phenotypes in the ImmunoChip and MetaboChip data-sets (Step 3). For each marker, frequency and effect estimates are given with respect to the minor allele. We also reported the r^2 with the SNP detected in the GWAS scan (Step 1). Novel signals are indicated in bold.

- The effect size is measured in standard deviation units, being estimated as the β coefficient of the regression model when using the normalized trait (e.g. an effect size of 1.0 implies each additional copy of the allele being evaluated increases trait values by 1.0 standard deviations).
- Allele frequencies SNP at rs12075, p = 7.43×10^{-102} M (same SNP).
- The table reports the p-value on the primary analysis. On the conditional analysis, the p-value for independent SNPs were: rs12378220, 9.43×10^{-06}; rs3093077, 9.02×10^{-11}; rs2259816, 7.58×10^{-10}.

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For IL-6, ESR, and MCP-1, the association signals were detected in the previously described CRP gene (rs1205, p = 8.20×10^{-30}) and in the 3’ of APOC-I (Apolipoprotein C-I) gene (rs4420638, p = 7.12×10^{-11}) (Figure 3C and 3D and Table 2), a well known determinant of serum hsCRP that did not reach statistical significance (p = 2.85×10^{-14}) in our initial scan (Stage 1) [25]. A novel and previously unknown signal with a large phenotypic impact was identified at a rare variant, rs113459440 (p = 1.54×10^{-08}, MAF = 0.003, average increase of 5.35 mg/L per T allele), near ICOSLG (Inducible T-cell co-stimulator ligand) and the AIRE (Autoimmune regulator) genes (Figure 3E and Figure S3). Common variants at the first gene have been associated by GWAS with the risk of Ulcerative colitis, Celiac disease, Chron’s disease and ankylosin spondiliitis in Europeans [30–32], whereas at the latter gene with Rheumatoid arthritis in Japanese [33]. The AIRE gene is also responsible for Autoimmune polyendocrinopathy syndrome, type I (APECED), an autosomal recessive autoimmune disease relatively common in Sardinia (OMIM # 607358). Association was supported by other SNPs genotyped with the ImmunoChip (7 SNPs with p-value<10^{-6}, with MAF up to 0.007). In addition, we observed that the same low frequency SNP at UNC119B/SPPL3 associated with ESR levels was also associated with hsCRP (rs11829037, p = 1.50×10^{-14}, average increase of 3.68 mg/L per T allele) (Figure 3F, Table 2)
and Figure S3). Similarly to ESR, association is likely to be genuine, supported by SNPs genotyped with both arrays and several common SNPs (MAF up to 0.38 for the 22 SNPs with p-value $< 10^{-6}$). Notably, signals at SNPs within SSLP3 were previously detected associated with CRP levels in an isolated founder population from the Pacific Island of Kosrae, although the p-values did not reach the genome-wide threshold [34]. None of those SNPs was correlated to the top SNP associated in our study; however the four SNPs which were genotyped (rs10437838, rs6489780, rs1039302, rs10431387) showed consistent direction of allele effects (increasing value for the minor allele) as in Lowe et al., albeit with weak evidence ($0.04 < p < 0.09$). This further indicates that association at this locus cannot be spurious. Conditional analysis revealed the presence of two independent signals. The first was within the CRP gene, at SNP rs3093077 ($p = 9.02 	imes 10^{-11}$ after conditioning for rs1205, with average increase of 0.724 mg/L per G allele) (Figure 3C, Table 2 and Figure S3). This marker is independent from the signal observed 461 Kb downstream in the Step 1 GWAS scan, rs3845624, near the DARC gene ($r^2 = 0.043$).

Figure 2. Zoom views of the association results in the loci associated with IL-6 and ESR. Each panel shows the association curve around the strongest SNP, which is highlighted with a purple dot. The SNPs are coloured according to their linkage disequilibrium ($r^2$) with the top variant in the 1000 Genomes European data set, with symbols that reflect genomic annotation as indicated in the legend. Arrows highlight independent signals, if any, described in the manuscript; while light blue lines indicate the recombination rate, according to the right-hand Y axis. Genomic positions are as in build 37. Gene transcripts are annotated in the lower box. Plots were drawn using the standalone LocusZoom version [65]. doi:10.1371/journal.pgen.1002480.g002
Indeed, when accounting for rs1205 and rs3093077 in the HapMap-based GWAS data set, the association signal at rs3845624 was still significant. The second independent signal was at rs2259816 ($p = 7.58 \times 10^{-10}$ after conditioning for rs11829037, with average increase of 0.381 mg/L per C allele), in an intron of the \textit{HNF1A} (Hepatic nuclear factor-1a) gene about 300 Kb downstream from the \textit{UNC119B/SPPL3} locus (Figure 3F, Table 2, and Figure S3). This marker is a perfect proxy of rs1169310, a variant reported by a previous study [24]. The best signal at this locus on our initial GWAS (Step 1) was at a linked SNP, rs7953249 ($r^2 = 0.5$), which did not reach genome-wide significance level ($p = 7 \times 10^{-06}$). Overall the top variants at \textit{CRP} (rs1205), \textit{APOC-I} (rs4420638), \textit{ICOSLG/AIRE} (rs113459440), \textit{UNC119B/SPPL3} (rs11829037), and the independent variants at \textit{CRP} (rs3093077), \textit{DARC} (rs3845624) and \textit{HNF1A} (rs2259816) explain 5.6% of the phenotypic variation of this trait.

Figure 3. Zoom views of the association results in the loci associated with MCP-1 and hsCRP. Each panel shows the association curve around the strongest SNP, which is highlighted with a purple dot. The SNPs are coloured according to their linkage disequilibrium ($r^2$) with the top variant in the 1000 Genomes European data set, with symbols that reflect genomic annotation as indicated in the legend. Arrows highlight independent signals, if any, described in the manuscript; while light blue lines indicate the recombination rate, according to the right-hand Y axis. Genomic positions are as in build 37. Gene transcripts are annotated in the lower box. Plots were drawn using the standalone LocusZoom version [65].

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Discussion

Our results, besides confirming previous associations, highlight new determinants for variation at the major inflammatory biomarkers IL-6, ESR, MCP-1 and hsCRP.

Specifically, we found a novel highly significant association between IL-6 expression levels and the ABO locus, with our top associated marker tagging the O allele. This association is of special interest, given the numerous biological effects of this cytokine as well as the associations previously reported of the O allele with both inflammatory traits and diseases [35–38]. In contrast to individuals with A and B alleles, individuals with the O blood group do not produce either the A or B antigens because of a single-base deletion in the gene sequence, whose product catalyzes the transfer of carbohydrates to the H antigen, forming the antigenic structure of the ABO blood group. Our data show that individuals carrying two copies of the G allele at our top SNP, corresponding to blood type O carriers, display highly increased IL-6 circulating levels compared to non-O carriers (average increase of 1.38 pg/ml for homozygotes of the G allele, compared to opposite homozygotes). This is consistent with the observation that blood type O individuals show an enhanced inflammatory response to Helicobacter pylori, with a significantly higher release of IL-6 [39]. The detected association at the ABO locus with the IL-6 phenotype may also provide a mechanistic clue for previous associations of the O blood group with various diseases with an inflammatory component such as cancer and heart disease, although determining the workings of this puzzle will likely also require specific functional studies.

Our study also revealed novel associations with ESR levels at the HBB and the UNC119B/SPPL3 loci. Although the impact of the associated variants at HBB in ESR values is somewhat expected in Sardinia because of the high frequency of carriers of β-Thalassemia (see Results), our work indicates a direct link supported by a genetic association. The link of UNC119B/SPPL3 with ESR is currently less clear. Interestingly, we also found that the same UNC119B/SPPL3 variant was associated with hsCRP levels, a finding supported by a recent study showing suggestive evidence at SNPs within the SPPL3 gene with CRP levels variation [34]. As expected by the strong correlation of CRP blood circulating levels and ESR (i.e., high blood levels of acute phase proteins increase ESR), the same allele of the associated SNP at UNC119B/SPPL3 increases both CRP and ESR, further supporting that the association at this locus is genuine.

Our results highlighted a novel association at the MCP-1 receptor CCR2, with a clear involvement with MCP-1 levels, previously only suggestively associated with this trait [21]. Confirming previous findings of Schabel and colleagues [21], we also found robust evidence of association between MCP-1 and SNPs in the DARC gene, an unusual transmembrane chemokine receptor, which binds the two main families of inflammatory chemokines, CXC and CC (i.e., MCP-1). The top signal (rs12075) is a non-synonymous SNP located in exon 3 of the gene, that generates a Gly42Asp amino acid change in the DARC protein. The predicted impact of the mutation, as well as the strength of the association signal compared to all nearby variants, suggests that it represents a causal variant, as previously hypothesized [21]. However and intriguingly, our results indicate that the association in the region is complex, with one novel genome-wide significant independent signal at the upstream gene CADM3. In addition, we also observed that SNPs near the DARC gene are associated with variation in CRP levels. Although the biological implications of these SNPs on DARC function are at present unclear, this is consistent with the observation that MCP-1 production by endothelial cells rises in response to CRP [40]. The DARC associations with CRP and MCP-1 were genetically independent of each other, supporting the notion of a complex correlation between hsCRP and MCP-1, and suggesting a multi-layered control of expression of the inflammation response in the DARC region.

Finally, in spite of the small sample size compared with the large meta-analyses conducted so far [25], our study identified several new variants associated with hsCRP levels, including an independent signal at CRP, the signal at the previously discussed UNC119B/SPPL3 locus and an unexpected signal at ICOCG/AIRE. Although the SNP associated with hsCRP at the UNC119B/SPPL3 locus is independent and not correlated (r2<0.1) with the known signal at HNF1A located about 300 kb downstream [24], at present we cannot exclude that this SNP may act as an eQTL or more generally in the regulation of HNF1A expression.

Interestingly, the majority of the association signals (and specifically at IL6R, TAF1E57, UNC119B/SPPL3, CCR2, APOC-1, HNF1A, the CRP independent signal, and ICOCG/AIRE), were observed, at least at the genome-wide level of significance, only after genotyping the MetaboChip and ImmunoChip custom arrays, which were typed in our cohort primarily to assess other phenotypes. All these signals, apart from that at ICOCG/AIRE, had supporting evidence for the involvement in the specific trait variation from previous reports, indicating that the associations are not spurious. The strongest variants were either not genotyped with the commercial arrays used in our initial scan, missing or poorly tagged in the HapMap-based reference panel we used for imputation, or only partially genotyped (given our genotyping strategy), resulting in inadequate power for being detected at the required significance level in the GWAS scan. This suggests that cost effective custom arrays could improve our understanding of the genetics underlying trait variation even for a phenotype, such as inflammation, for which the array was not specifically designed.

Understanding the effects of the protein products of all the discovered loci in inflammation is an important goal, which may also likely have clinical implications. For instance, whereas CRP and ESR are the most widely used non-specific diagnostic markers of inflammation, the factors and fine mechanisms regulating their levels and interfering with them are only partially understood.

Overall, our results contribute to improve the current knowledge of the regulation of the inflammatory response. While inflammation is canonically thought of as involving leukocyte migration and infiltration, the fact that several of the variants identified are better noted in erythrocyte function may suggest a more active role for the red cell in this process, beyond its obligate role in ESR. Notably, four of the associated loci (ABO, HBB, DARC and GCR1) have been implicated in resistance to malaria, a disease endemic in Sardinia until a few decades ago [41–45]. This raises the possibility that the genetic selection imposed by malaria may have contributed to shaping levels of inflammation, at least for these specific inflammatory biomarkers, in this population [46]. Still, a link between these specific genes and variants with malaria remains speculative and needs to be further assessed with adequate biological and genetic analyses; for instance, they could be tested and cross-compared with future statistically well powered GWAS on Malaria and other infectious diseases.

A related potential detrimental consequence of inflammation is that polymorphisms which have been selected because they promote pro-inflammatory responses may increase the risk for diseases with an inflammatory component [47,48], particularly those that show a high frequency in Sardinia, such as Multiple Sclerosis (MS) and Type 1 diabetes (T1D) [49,50]. However, we could not find any evidence of association of the top SNPs
associated with pro-inflammatory markers in a sample-set of 2,280 MS cases, 1,377 T1D cases and 1,922 unrelated controls, all from Sardinia [51], with a power of 60% and 33% to detect variants with an odds ratio of 1.4 and MAF of 0.1 at a significance level of 1\times10^{-6}, indicating that larger sample sizes are required to identify association at variants with smaller effects or of lower frequency (data not shown). Similarly, these variants were not found associated to other autoimmune diseases in larger data-sets (1T1Dbase, http://1t1dbase.org; and the GWAS Catalog, http://www.genome.gov/gwastudies/).

Another possibility is that these pro-inflammatory variants play a positive role in protection against serious diseases. For instance, the ABO O allele is also associated with a reduced risk of myocardial infarction and pancreatic and skin cancer [52–54]. Our results suggest that an increase in the circulating levels of IL-6 can indeed contribute to these associations involving the O group.

In conclusion, our work highlights important aspects of the complex and multilayered regulation of inflammation and may provide a route to understanding possible attendant effects on a number of serious diseases.

Methods

Ethics Statement

All individuals studied and all analyses on their samples were done according to the Declaration of Helsinki and informed consents were approved by the local ethics committee for the Istituto di Ricerca Genetica e Biomedica-CNR (IRGB-CNR; Cagliari, Italy) and by MedStar Research Institute, responsible for intramural research at the National Institutes of Aging, Baltimore, Maryland, United States.

Sample Description

We recruited and phenotyped 6,148 individuals, males and females, ages 14–102 y, from a cluster of four towns in the Lanusei Valley of Sardinia [11]. During physical examination, a blood sample was collected from each individual and divided into two aliquots. One aliquot was used for DNA extraction and the other to characterize several blood phenotypes, including evaluation of serum levels of hsCRP, IL-6, MCP-1, and values of ESR. Descriptive statistics of the study cohort are shown in Table S2. Serum levels of hsCRP were measured by the high sensitivity Vermont assay (University of Vermont, Burlington), an enzyme-linked immunosorbent assay calibrated with WHO Reference Material [55]. The lower detection limit of this assay is 0.007 mg/l, with an inter-assay coefficient of variation of 5.14%. Serum levels of IL-6 and MCP-1 were measured by Quantikine High Sensitive Human Immunoassays (R&D Systems, Inc.), according to manufacturer’s instructions. This method employs solid-phase ELISA techniques. For IL-6, the lower detection limit is 0.039 pg/ml. The intra-assay coefficient of variations (CVs) were 6.9% to 7.8% over the range 0.43–5.53 pg/ml. For MCP-1, the lower detection limit is 5.0 pg/ml. The intra-assay coefficient of variations (CVs) were 4.7% to 7.8% over the range 76.7–1121 pg/ml. ESR was measured using sedimentation measurement tubes buffered with 3.8% sodium citrate (Venoject-Terumo). After mixing of 2.4 ml of blood with the additive, tubes were left in a vertical position in the specific support with graduation markings for 30 minutes to allow sedimentation of the erythrocytes by gravity. The erythrocyte sedimentation rate is calculated in Westergreen units (mm/h) determining the length at the plasma/erythrocyte cell interface level within the sedimentation tube. Samples affected by multiple sclerosis (MS) and type 1 diabetes (T1D) used for the side case-control analysis briefly reported in the Discussion were recruited from all the island as previously described [51]. Only 20 of these samples overlapped with those in the SardiNIA cohort.

GWAS Genotyping and Statistical Analysis

During the study, we genotyped 4,694 individuals selected from the whole sample to represent the largest available families, regardless of their phenotypic values. Specifically, 1,412 were genotyped with the 500 K Affymetrix Mapping Array set, 3,329 with the 10 K Mapping Array set, with 436 individuals genotyped with both arrays. We also recently typed 1,997 individuals with the Affymetrix 6.0 chip, of which 1,004 and 66 were also typed with the 10 K and 300 K chips respectively. This genotyping strategy allowed us to examine the majority of our cohort in a cost-effective manner since genotypes for the SNPs that passed quality control checks could be propagated through the pedigree using imputation. Measurements of inflammatory biomarkers were available for 4,137, 4,292, 4,295 and 3,596 individuals for hsCRP, IL-6, MCP-1 and ESR, respectively, among the 4,694 genotyped. A total of 731,209 autosomal SNPs passed stringent quality control checks. Quality checks for the 10 K and 500 K chips were described previously [56]. For the Affymetrix 6.0 chip, similar criteria were used, as detailed in Table S3. In addition, we also removed SNPs in common between the other chips that showed an high level of discordance or that generated too many discrepancies when comparing genotypes across 11 duplicates. After performing quality control checks and merging genotypes from the three gene chip platforms, we used the quality controlled 731,209 autosomal markers to estimate genotypes for all polymorphic SNPs in the CEU HapMap population (release 22) [57], in the individuals genotyped with the 500 K Array and the 6.0 Affymetrix chip separately using the MaCH software [58]. Taking advantage of the relatedness among individuals in the SardiNIA sample, we carried out a second round of computational analysis to impute genotypes at all SNPs in the individuals who were genotyped only with the Affymetrix Mapping 10 K Array, being mostly offspring and siblings of the individuals genotyped at high density. At this second round of imputation, we focused on the SNPs for which the imputation procedure predicted $\hat{\rho}^2>0.30$ between true and imputed genotypes and for which the inferred genotype did not generate an excess of Mendelian errors. We then used a modified version of the Lander-Green algorithm, as previously described [3,56] to estimate IBD sharing at the location of the SNPs being tested and identify stretches of haplotype shared with close relatives who were genotyped at higher density and probabilistically infer missing genotypes. The within-family imputation procedure and the association test are implemented in Merlin software [59,60]. Due to computational constraints, we divided large pedigrees into sub-units with “bit-complexity” of 21 or less (typically, 25–30 individuals) before analysis.

For association, we evaluated the additive effect of genotyped and imputed SNPs on inflammatory biomarker levels using a family-based association test implemented in Merlin (−fastassoc option) [59,60]. This test accounts for relatedness under the assumption that the samples analyzed are from an ethnically homogeneous population [59,60], and this is suggested by demographic records indicating that 89% of the participants were born in the same 31 Km² area and for 95% of the volunteers, both parents and all grandparents were born in Sardinia [11]. At each SNP, levels of each biomarker of inflammation were regressed onto allele counts in a regression model that included gender, age, and age-squared as covariates. We also used a second model, which included body-mass index (BMI) and smoking status as additional covariates, as these have been previously implicated as
being associated with inflammatory biomarker levels [24]. Here we report the results from the second model, where the inclusion of the additional covariates improved the variance explained by the model (from 1.3% to 3.7% for CRP, from 1.8% to 19.5% for ESR, from 6.8% to 7.6% for IL-6, and from 3% to 3.3% for MCP-1). Genomic control parameters show negligible inflation (1.049, 1.039, 1.031 and 1.115, respectively for hsCRP, MCP-1, IL-6 and ESR); nevertheless the corresponding correction factors were applied to the GWAS results to completely avoid spurious associations.

### Follow-Up Sample

The SardiNIA stage 2 cohort was used to follow-up initial findings [27]. Genotyping of specific SNPs was performed in Sardinian individuals selected for replication efforts using TaqMan single SNP genotyping assays (Applied Biosystems). In particular, we genotyped and analysed 1,392 individuals from the SardiNIA stage 2 cohort, who were unrelated (kinship coefficient = 0) to the individuals analysed in the GWAS.

### ImmunoChip and MetaboChip: Genotyping and Statistical Analysis

We successfully genotyped 6,145 samples using the MetaboChip and ImmunoChip arrays (Illumina). The MetaboChip was designed in collaboration with several international consortia [3,61,62] with the aim to fine map association loci detected through GWAS for a variety of traits. Part of the design included a set of wild-card SNPs chosen by individual research groups; the SardiNIA study promoted several SNPs associated with a wide range of traits, including rs12075. The ImmunoChip is also a consortium based array, designed to fine map loci associated to 12 immunologically related human diseases, or immune-mediated disease loci, as well as a set of wild-card SNPs. The SardiNIA study had not role in the design of this array, and a full detailed description is provided elsewhere [12]. All samples had a genotyping call rate >98%, and SNP genotypes were carefully assessed through several quality control checks. In particular, we removed markers with call rate <98%, with strong deviation from HWE (p<10^{-6}), that were monomorphic or leading to an excess of Mendelian errors (defined as >1% of the families). A detailed breakdown of markers excluded by each filter criteria is provided on Table S4. Since the majority of the variants included on these custom arrays are of low frequency compared to the GWAS data set (average MAF = 0.176, compared to 0.219 observed in GWAS), the impact of hidden population structure and imprecise modelling of relatedness due to pedigree splitting (task we performed on the GWAS data set due to computational constraints) could be problematic. Analysis was thus carried out using EMMAX, a variant component model that overcomes such issues by using a genomic-based kinship matrix [63]. To calculate the kinship matrix, we used all SNPs that passed quality control checks but excluding those with MAF between 0 and 1%. Association analysis was subsequently performed testing all QCed SNPs (Table S4), in spite of their minor allele frequency. Observed genomic lambda were 1.01, 0.962, 1.00 and 1.01, respectively for IL6, VES, MCP1, and hCRP (as a note, genomic lambda using Merlin on the same data set were 1.41, 1, 1.26 and 1.14, respectively). To declare an association significant, we used a Bonferroni threshold of 0.05/293,875 = 1.7×10^{-5}.

### Conditional Analysis

We performed conditional analysis at each locus by adding the top associated SNP to the already included covariates, and testing for association the remaining SNPs at the locus. A marker was declared independent only if the p-value observed in the conditional analysis reached genome-wide significance threshold (5×10^{-8} in the Step1 GWAS, and 1.7×10^{-5} in the custom-array based dataset) [64].

### Supporting Information

#### Figure S1: Manhattan plot and QQ plot of association findings in step 1 GWAS. The figure summarizes the genome-wide association scan results combined across the data-sets by inverse variance weighting. The blue dotted line marks the threshold for genome-wide significance (5×10^{-8}) [64]. SNPs in loci exceeding this threshold are highlighted in green. The bottom panel represents the QQ plot, where the red line corresponds to all test statistics, and the blue line to results after excluding statistics at top markers (highlighted in green in the Manhattan Plot). The gray area corresponds to the 90% confidence region from a null distribution of P values (generated from 100 simulations).

#### Figure S2: Boxplots for levels of IL-6, ESR, and MCP-1 for each genotype at the top associated SNPs. Standard boxplots are drawn with min, 0.25 quantile; median, 0.75 quantile; and 0.75 quantile + 1.5*IQR for the levels of unadjusted biomarkers IL-6 (pg/ml), ESR (mm/h), and MCP-1 (pg/ml) in the original units. For each boxplot the name of the biomarker and the associated SNP are indicated, as well as the number of individuals per genotype.

#### Figure S3: Boxplots for levels of hsCRP for each genotype at the top associated SNPs. Standard boxplots are drawn with min, 0.25 quantile; median, 0.75 quantile; and 0.75 quantile + 1.5*IQR for the levels of unadjusted hs-CRP (mg/ml) biomarker in the original units. For each boxplot the name of the biomarker and the associated SNP are indicated, as well as the number of individuals per genotype.

#### Table S1: Association results for SNPs with p-value<10^{-5} for inflammatory biomarkers in the step 1 GWAS. The table summarizes the association results at SNPs with p-value<10^{-5} for each inflammatory biomarkers. The effect size is measured in standard deviations (e.g. an effect size of 1.0 implies each additional copy of the allele being evaluated increases trait values by 1.0 standard deviations) and it refers to allele 1. The r^2 between each imputed genotype and the true underlying genotype is provided (RSQ) and serves as a quality-control metric. The percentage of the variance explained by each markers is also reported (H^2), and the column “L/G” indicates whether the SNP has been imputed or genotyped. Physical positions are given according to build 36. SNPs above genome-wide threshold (p<5×10^{-8}) are indicated in bold. EA, effect allele; OA, other allele.

#### Table S2: Descriptive statistics for the SardiNIA cohort. The table shows the basic clinical characteristics of the SardiNIA samples.
Table S3  Quality Control data for Affymetrix 6.0 chips and imputation. (DOCX)

Table S4  Quality Controls for the MetaboChip and ImmunoChip data-sets. The table shows the breakdown of each criteria applied and the relative number of markers removed. The same marker could have failed one or more criteria. a excess was defined as >1% of the families. b SNPs on chromosomes X and Y were discarded for the analysis with EMMAX. (DOCX)

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Author Contributions

Conceived and designed the experiments: S Naitza, S Uzzau, DL Longo, E Lakatta, A Concas, GR Abecasis, D Schlessinger, M Udà, S Sanna, F Cucca. Performed the experiments: DD Taub, A Mulas, M Dei, S Lai, F Busonero, A Maschio, G Usala, M Zoledziewska. Analyzed the data: E Porcu, M Steri, X Xiao, J Striado, C Sidore, I Zara, P Sitzalis, S Sanna. Contributed reagents/materials/analysis tools: M Pizalis, A Loi, F Virdis, R Piras, F Deidda, I Crisponi, A Concas, C Podda. Wrote the paper: S Naitza, MB Whalen, D Schlessinger, S Sanna, F Cucca.

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