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ASSOCIATION STUDIES ARTICLE

A meta-analysis of 120 246 individuals identifies 18 new loci for fibrinogen concentration

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Abstract

Genome-wide association studies have previously identified 23 genetic loci associated with circulating fibrinogen concentration. These studies used HapMap imputation and did not examine the X-chromosome. 1000 Genomes imputation provides better coverage of uncommon variants, and includes indels. We conducted a genome-wide association analysis of 34 studies imputed to the 1000 Genomes Project reference panel and including ~120 000 participants of European ancestry (95 806 participants with data on the X-chromosome). Approximately 10.7 million single-nucleotide polymorphisms and 1.2 million indels were examined. We identified 41 genome-wide significant fibrinogen loci; of which, 18 were newly identified. There were no genome-wide significant signals on the X-chromosome. The lead variants of five significant loci were indels. We further identified six additional independent signals, including three rare variants, at two previously characterized loci: *FGB* and *IRF1*. Together the 41 loci explain 3% of the variance in plasma fibrinogen concentration.

Fibrinogen is a coagulation factor crucial to clot formation, and an active regulator of the inflammatory response (1). It is a strong and established predictor of cardiovascular disease, autoimmune disorders and cancer (1–5). Circulating fibrinogen concentration has a moderate heritability of 34–46% (6–8). Previous genome-wide association studies (GWAS) have highlighted genetic loci involved in inflammatory pathways such as the acute-phase response and interleukin-1 and -6 signaling as main determinants of fibrinogen concentration (9–13).

The variance in fibrinogen concentration explained by genetic loci identified in these previous GWAS is less than 1/10th of its estimated heritability (11). It is, therefore, likely that a part of the heritability stems from genetic variants that are not well-tagged by the single-nucleotide polymorphisms (SNPs) found in HapMap, including further common, uncommon and rare SNPs, and other types of variants such as insertions or deletions (indels). Additionally, a part of the heritability could be explained by variants on the X-chromosome, which has not previously been interrogated.

To better interrogate the full range of genetic variants, including those with low minor allele frequency (MAF) that may have been poorly tagged by HapMap variants, we performed a meta-analysis of 34 GWAS imputed using 1000 Genomes Project reference panels (14), including the X-chromosome. We performed a joint/conditional analysis to identify additional independent signals within known and new loci associated with plasma fibrinogen concentration.

Results

Autosomal meta-analysis

Participant characteristics in each study are shown in Supplementary Material, Table S1, covariates adjusted for by each study are shown in Supplementary Material, Table S2 and genomic inflation factors are shown in Supplementary Material, Table S3. The meta-analysis of the autosomes included 9 492 263 SNPs and 841 128 indels, of which 4354 SNPs and 420 indels at 41 loci were genome-wide significant. Of these, 18 loci are new signals (Table 1), while 23 have been associated with fibrinogen concentration by previous GWAS (Table 2). Among genome-wide significant variants, 14 of 4354 were rare (MAF ≤ 0.01), and a further 477 were uncommon (0.01 < MAF ≤ 0.05). The lead variants of known locus *SNX13* and novel loci *ATXN2L*, *GYS2*, *GIMAP4* and *IFT122* were indels. Separate QQ plots of all autosomal variants, common variants, uncommon variants, rare variants, SNPs and indels are shown in Supplementary Material, Figure S1. A Manhattan plot of all autosomal variants is shown in Supplementary Material, Figure S2. Additionally, a Manhattan plot highlighting rare and uncommon variants is shown in Supplementary Material, Figure S3. Heterogeneity I^2 and *P*-values are shown in Supplementary Material, Table S4. Only rs7439150 at the fibrinogen gene cluster showed significant heterogeneity ($I^2 = 50.0$, $P = 0.0004$). Regional plots are shown in Supplementary Material, Figure S4, and forest plots are shown in Supplementary Material, Figure S5. Associations with rare variants were found at the two most robust fibrinogen loci: the fibrinogen gene cluster and the *IRF1* locus (lead variant annotated to *C5orf56*). Associations with uncommon variants were also found at these loci, as well as at *SPPL2A* and *HNF4A*. At one known locus (*SNX13*) and four new loci (*IFT122*, *GIMAP4*, *GYS2* and *ATXN2L*) the lead variant was an indel. At each of these loci there were also SNPs in linkage disequilibrium with the indel that reached genome-wide significance. *CD300LF* was the only previously identified locus that was not represented among our significant results. The previously reported lead variant in *CD300LF*, rs10512597 (*P*-value: 1.8×10^{-7}), had a smaller effect size [β: −0.006 ln(g/l)] than was previously reported [$\beta = -0.008$ ln(g/l)]. There was no strong evidence of heterogeneity ($I^2 = 22.7$, $P = 0.11$).

Conditional analysis

Two loci (fibrinogen gene cluster and *IRF1*) harbored multiple jointly significant variants (Table 3). Forest plots of the additional variants discovered through conditional analysis are shown in Supplementary Material, Figure S6, and their heterogeneity I^2 and *P*-values are shown in Supplementary Material, Table S4. At the fibrinogen gene cluster, five variants were jointly significant: the lead variant rs7439150, an additional common variant rs76289367, and three rare variants, rs150768229, rs6054 and rs148685782. rs148685782 showed significant heterogeneity ($I^2 = 65.0$, *P*-value = 0.0004). At the *IRF1* locus three variants were jointly significant: the lead variant, rs2057655, and two

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Table 1. Association of the lead variants at 18 newly identified loci with natural log transformed plasma fibrinogen concentration (g/l)

Locus	Variant	Position	Closest gene	eQTL	NSYN variants	A1/A2	Frequency	β	P-values
2p25.3	rs7588285	3 648 186	*COLEC11*			C/G	0.20	0.0074	1.2×10^{-08}
3p25.3	rs62246343	9 543 642	*LHFPL4*			T/C	0.17	0.0071	2.2×10^{-08}
3q21.1	rs1976714	122 864 771	*PDIA5*			T/G	0.35	−0.0055	2.3×10^{-08}
3q21.3	3:129228166	129 228 166	*IFT122*	*RPL32P3*		D/R	0.10	0.009	1.0×10^{-08}
7p14.2	rs2710804	36 084 529	*EEPD1*			C/T	0.37	0.0055	2.9×10^{-09}
7q36.1	7:150289652	150 289 652	*GIMAP4*	*GIMAP4*		D/R	0.21	−0.0073	9.3×10^{-11}
8p23.1	rs7012814	9 173 358	*LOC157273*			A/G	0.47	0.0060	2.1×10^{-10}
9q22.2	rs3138493	92 219 260	*GADD45G*	*SEMA4D*		T/C	0.48	−0.0054	2.5×10^{-09}
10q23.31	rs2250644	91 008 879	*LIPA*			T/C	0.33	0.0054	2.2×10^{-08}
10q26.13	rs2420915	122 840 277	*MIR5694*	*WDR11*		A/G	0.09	−0.0094	5.2×10^{-09}
11p12	rs7934094	43 505 707	*TTC17*			G/T	0.22	−0.0083	2.5×10^{-13}
12p12.1	12:21703935	21 703 935	*GYS2*			R/D	0.37	0.0062	8.4×10^{-09}
12q24.12	rs7310615	111 865 049	*SH2B3*	*SH2B3*	*SH2B3*	C/G	0.50	−0.0069	1.5×10^{-13}
15q15.1	rs56702977	42 671 308	*CAPN3*	*ZFP106*		A/G	0.13	0.0080	2.1×10^{-09}
16p11.2	16:28845027	28 845 027	*ATXN2L*	*TUFM*		D/R	0.39	0.0061	7.7×10^{-10}
16q22.2	rs1035560	72 032 730	*PKD1L3*	*HP*		C/T	0.40	0.0064	2.6×10^{-12}
17q21.2	rs7224737	40 289 364	*RAB5C*	*STAT3*	*HSPB9*	A/G	0.24	0.0061	6.1×10^{-09}
19q13.33	rs73058052	50 099 422	*PRR12*	*IRF3*	*PRRG2*	T/C	0.16	0.0074	2.0×10^{-08}

eQTL indicates the gene with the strongest significant association between its expression levels in blood and the lead variant or its proxy. NSYN variants indicates genes containing non-synonymous variant correlated to the lead variant ($R^2 > 0.9$). A1 indicates the coded allele. A2 indicates the other allele. Frequency is the frequency of the coded allele. β indicates the β-coefficient adjusted for age, sex, population structure and study-specific covariates, such as center or case/control status. The β-coefficient can be interpreted as the ln(g/l) change in fibrinogen per 1 unit change in the dosage of the coded allele.

Table 2. Association of the lead variants at 23 known loci with natural log-transformed plasma fibrinogen concentration (g/l)

Locus	Variant	Position	Closest gene	eQTL	NSYN variants	A1/A2	Frequency	β	P-values
1p31.3	rs1892534	66 105 944	*LEPR*			T/C	0.38	−0.0073	4.3×10^{-15}
1q21.3	rs61812598	154 420 087	*IL6R*		*IL6R*	A/G	0.39	−0.0115	2.7×10^{-36}
1q44	rs10157379	247 605 599	*NLRP3*	*NLRP3*		C/T	0.38	−0.0103	6.3×10^{-29}
2q12	rs1558643	102 731 691	*IL1R1*			T/C	0.40	0.0058	3.1×10^{-10}
2q13	rs6734238	113 841 030	*IL1F10*	*IL1RN*		G/A	0.41	0.0106	6.7×10^{-30}
2q34	rs715	211 543 055	*CPS1*		*CPS1*	C/T	0.32	−0.0082	4.3×10^{-16}
2q37.3	rs59104589	242 237 902	*HDLBP*	*STK25*		T/C	0.34	−0.0083	8.2×10^{-19}
3q22.2	rs9840812	135 843 162	*PPP2R3A*	*PCCB*		C/T	0.23	0.0117	1.7×10^{-27}
4p16.3	rs59950280	3 452 345	*HGFAC*			A/G	0.34	0.0075	1.7×10^{-12}
4q31.3	rs7439150	155 481 541	*FGB*		*FBG*	A/G	0.20	0.0313	9.5×10^{-181}
5q31.1	rs2057655	131 807 624	*C5orf56*	*SLC22A4*		A/G	0.21	−0.0203	1.8×10^{-73}
7p21.1	7:17904452	17 904 452	*SNX13*			R/D	0.48	0.0067	1.3×10^{-13}
7p15.3	rs71520386	22 853 521	*TOMM7*			T/C	0.20	0.0066	5.1×10^{-09}
8q24.3	rs11780978	145 034 852	*PLEC*	*GRINA*		A/G	0.40	0.0059	5.5×10^{-10}
10q21.3	rs7916868	64 988 931	*JMJD1C*			A/T	0.49	0.0089	1.6×10^{-22}
11q12.2	rs11230201	59 996 994	*MS4A6A*	*MS4A6A*		G/C	0.41	−0.0057	4.5×10^{-10}
12q13.12	rs2731439	51 060 350	*DIP2B*	*DIP2B*		T/C	0.36	−0.0064	8.7×10^{-12}
14q24.1	rs367677	69 273 090	*ZFP36L1*			G/A	0.22	0.0077	1.8×10^{-12}
15q21.2	rs12913259	51 014 716	*SPPL2A*			T/C	0.30	−0.0068	2.3×10^{-12}
16q12.2	rs11859517	53 181 247	*CHD9*			T/C	0.29	−0.0074	8.9×10^{-14}
20q13.12	rs1800961	43 042 364	*HNF4A*		*HNF4A*	T/C	0.03	−0.0170	1.2×10^{-10}
21q22.2	rs9808651	40 466 468	*PSMG1*			A/G	0.27	−0.0095	2.5×10^{-20}
22q13.33	rs75347843	51 112 361	*SHANK3*	*ARSA*		A/G	0.19	0.0084	1.8×10^{-10}

eQTL indicates the gene with the strongest significant association between its expression levels in blood and the lead variant or its proxy. NSYN variants indicates genes containing non-synonymous variant correlated to the lead variant ($R^2 > 0.9$). A1 indicates the coded allele. A2 indicates the other allele. Frequency is the frequency of the coded allele. β indicates the β-coefficient adjusted for age, sex, population structure and study-specific covariates, such as center or case/control status. The β-coefficient can be interpreted as the ln(g/l) change in fibrinogen per 1 unit change in the dosage of the coded allele.

uncommon variants, rs12777 and 5:131786964. Of the secondary signals, rs12777 is in strong linkage disequilibrium with a previously associated SNP, rs1242111 ($R^2 = 0.8$), while 5:131786964 is a new independent signal ($R^2 = 0.0$). The uncommon variants near *SPPL2A* were not significant in the conditional analysis. The uncommon lead variant rs141272690 was only marginally significant in the primary analysis ($P = 1.89 \times 10^{-8}$), so that even a small correlation with the lead common variant rs12913259 ($R^2 = 0.02$) raised the P-value above the threshold in the conditional analysis.

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Table 3. Joint/conditional association of eight variants at two loci with natural log transformed plasma fibrinogen concentration (g/l)

Locus	Variant	Position	Closest gene	Annotation	A1/A2	Frequency	β	P-values	Joint β	Joint P-values
4q31.3	rs7439150	155 481 541	*FGB*	Intergenic	A/G	0.205	0.0313	9.5×10^{-181}	0.0259	1.9×10^{-92}
4q31.3	rs150768229	155 488 301	*FGB*	Intronic	C/A	0.009	−0.0458	6.4×10^{-12}	−0.0385	9.3×10^{-09}
4q31.3	rs6054	155 489 608	*FGB*	NSYN	T/C	0.005	−0.1228	2.4×10^{-53}	−0.1222	4.9×10^{-52}
4q31.3	rs148685782	155 533 035	*FGG*	NSYN	C/G	0.005	−0.2239	1.2×10^{-87}	−0.2179	4.0×10^{-82}
4q31.3	rs76289367	155 546 159	*FGG*	Intergenic	G/T	0.148	0.0263	2.0×10^{-76}	0.0109	1.6×10^{-11}
5q31.1	rs12777	131 671 662	*SLC22A4*	SYN	G/C	0.044	0.0240	9.3×10^{-27}	0.0207	6.9×10^{-21}
5q31.1	5:131786964	131 786 964	*C5orf56*	ncRNA	I/R	0.015	−0.0543	2.5×10^{-14}	−0.0428	2.0×10^{-09}
5q31.1	rs2057655	131 807 624	*C5orf56*	ncRNA	A/G	0.207	−0.0203	1.8×10^{-73}	−0.0188	1.9×10^{-64}

A1 indicates the coded allele. A2 indicates the other allele. Frequency is the frequency of the coded allele. NSYN indicates a non-synonymous exonic variant. SYN indicates a synonymous exonic variant. β indicates the β-coefficient adjusted for age, sex, population structure and study-specific covariates, such as center or case/control status. Joint β indicates the β-coefficient of the jointly significant variants, adjusted for the above and for each other. All β coefficients can be interpreted as the ln(g/l) change in fibrinogen per 1 unit change in the dosage of the coded allele.

X-chromosome meta-analysis

The meta-analysis of the X-chromosome included 251 747 SNPs and 26 448 indels. There were no genome-wide significant variants detected on the X-chromosome. This was true in both sex-specific meta-analyses, and in the combined meta-analyses, irrespective of whether the sex-specific results were combined using inverse-variance weighted meta-analysis or sample size based meta-analyses. QQ plots and Manhattan plots for the X-chromosome are shown in Supplementary Material, Figures S7 and S8.

Functional annotation

Genome-wide significant associations with other traits were found for 28 out of the 41 loci, of which 10 were associated with cholesterol levels, 7 were associated with C-reactive protein, and 5 were associated with platelet count (Supplementary Material, Table S5). Out of the 41 lead variants, 20 were associated with blood expression levels of one or more neighboring genes (Supplementary Material, Table S6). Notably, rs1035559 at 16q22.2 was exclusively associated with *HP* expression levels ($P = 9.8 \times 10^{-198}$), and rs7224737 at 17q21.2 was exclusively associated with *STAT3* expression levels ($P = 5.4 \times 10^{-12}$). Out of the 41 lead variants, 36 were available in HaploReg V2. Detailed annotation of these variants as well as 457 correlated SNPs is shown in Supplementary Material, Table S7. Eight of these SNPs are predicted to influence the binding of miRNAs to transcripts of their host gene. Further information about these SNPs and their effect on miRNA binding is shown in Supplementary Material, Table S8. Of these eight SNPs, two were lead variants. First, the fibrinogen decreasing minor allele of lead variant rs715 in the 3′-UTRof *CPS1* is predicted to create a miRNA-binding site for miR-3154. Secondly, the fibrinogen increasing minor allele of lead variant rs6224634 in the 3′-UTR of *LHFPL4* is predicted to disrupt the binding site of miR-6761-3p. In both cases, predicted successful miRNA-target gene binding is associated with lower fibrinogen concentration.

Variance explained

In the Women's Genome Health Study, the lead variant at the fibrinogen gene cluster explained 0.8% of the variance, and all five jointly significant variants together explained 1.6% of the variance. At 5q31.1 the lead variant explained 0.2% of the variance, while all three jointly significant variants together explained 0.3% of the variance. The 47 independently significant variants at 41 loci explained 3.0% of the variance in circulating fibrinogen concentration. The variance explained by the 23 previously identified loci was 2.6%.

Discussion

We identified 18 new autosomal loci associated with circulating fibrinogen concentration in individuals of European ancestry, increasing the variance explained from 2.6 to 3.0%. The small increase in the variance explained relative to the large number of new loci is suggestive of a highly polygenic genetic architecture. At two loci (fibrinogen gene cluster and *IRF1* locus) rare or uncommon variants were jointly significant alongside common lead variants. In five cases, the lead variant at an associated locus was an indel. There were no significant associations on the X-chromosome: this may be result of issues specific to the X-chromosome rather than the absence of relevant signals. The most important issue is that the X-chromosome is generally poorly covered by genotyping arrays (15).

Four of the 18 new loci implicate inflammatory pathways not previously linked to fibrinogen. First, the septin gene family is represented at two significant loci: *SEPT7* at 7p14.2 and *SEPT2* at 2q37.3. Proteins from the septin gene family form cage-like structures around bacteria to facilitate autophagy (16). The link between these processes and fibrinogen concentration is unclear. Secondly, our results also implicate genes from the GIMAP family, which are structurally similar to septins (17). The signal at 7q36.1 appears to be driven by one or more genes from a cluster of eight GIMAP genes, and the lead variant is associated with blood expression levels of four of these. Through their involvement in lymphocyte maturation, these genes influence lymphocyte counts and diversity, and thereby also the inflammatory response (18). Finally, the lead variant at 16q22.2 is strongly associated with blood expression levels of the neighboring *HP* ($P \leq 9.8 \times 10^{-198}$), the gene encoding haptoglobin. Like fibrinogen, haptoglobin is an acute-phase reactant. The association of rs1035560 with fibrinogen suggests that besides sharing upstream regulators, haptoglobin itself may be involved in the regulation of circulating fibrinogen.

Six of the new loci appear to be closely related to STAT3, a transcription factor working downstream of IL-6 that upregulates the expression of fibrinogen and other acute-phase proteins (19). At 17q21.2, the lead variant rs7224737 (175 kb from *STAT3*) was associated with *STAT3* blood expression levels ($P = 5.4 \times 10^{-12}$). At 9q22.2, the lead variant rs3138493 lies upstream of *GADD45G*. This gene is expressed in the liver, where it has been shown to inhibit the Tyr705 phosphorylation of STAT3 (20). As Tyr705 phosphorylation of STAT3 allows it to dimerize and move into the

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nucleus, it is essential for the upregulation of STAT3 targets like the fibrinogen genes. At 10q26.13, the lead variant rs2420915 is an intergenic SNP close to *FGFR2*. Over-expression of *FGFR2*, or the related *FGFR1* is required for the Tyr705 phosphorylation of STAT3 (20). At 19q13.33, the lead variant rs73058052 is associated with blood expression levels of *IRF3*. After activation in response to viral infection, *IRF3* enables the expression of type I interferons *INFA* and *INFB*, leading to the upregulation of *STAT3* (21,22). Furthermore, our results point toward two SH2B adaptor proteins implicated in STAT3 signaling. At 12q24.12, the lead variant rs7310615 was associated with blood expression levels of *SH2B3*. Using immortalized B lymphoblastoid cell lines, a loss of the SH2B3 protein was accompanied by increased STAT3 phosphorylation (23). At 16p11.2, the lead variant 16:28845027 lies close to *SH2B1*. The β variant of SH2B1 appears to form a complex with STAT3, allowing STAT3 to cross through the membrane into the nucleus as an alternative to STAT3 dimerization (24). Collectively, these findings suggest that a wide range of disturbances to STAT3 may affect circulating fibrinogen concentration.

In addition to STAT3, our results highlight HNF4A, another transcription factor known to regulate fibrinogen gene expression. The association between lead variant rs1800961 and circulating fibrinogen has been previously been described by Wassel *et al.* and Hufman *et al.* (12,25). rs1800961 is a non-synonymous coding variant that has been shown to decrease *HNF4A* expression *in vitro* (26).

The majority of rare and uncommon variants associated with fibrinogen concentration were found at loci with common variant signals. Only the signal at *HNF4A* was led by an uncommon variant, and no signals were led by rare variants. Conditional analysis suggests that there are two secondary signals at the *IRF1* locus led by uncommon variants, and three secondary signals near the fibrinogen gene cluster led by rare variants. The uncommon variants that were significant near *SPPL2A* were not significant in the conditional analysis, but the linkage disequilibrium with the lead common variant was very low. Our results suggest that common and rare variant signals are often independent of each other, and do not support the hypothesis that associations with common variants are synthetic associations merely reflecting linkage disequilibrium with rare variants (27,28).

Absolute effect sizes of significant variants ranged from 0.005 to 0.033 ln(g/l) among common variants, 0.013 to 0.087 ln(g/l) among uncommon variants and 0.036 to 0.254 ln(g/l) among rare variants. Despite their small effect size, common variants have helped discover biologically relevant fibrinogen loci. Therefore, the complete lack of overlap between the effect sizes of significant common and rare variants suggests that further rare variants with smaller effect sizes are likely to exist at important and possibly unknown fibrinogen loci. While the rare variants with large effects we found were limited to the two most important fibrinogen loci, rare variants with moderate effects may be more widespread.

When considering not only the primary signal at the fibrinogen gene cluster, but also the four additional signals the variance explained by the locus doubles from 0.8 to 1.6%. Two of these additional signals are driven by rare non-synonymous exonic variants (rs6054 and rs148685782) with very large effect sizes [$\beta = -0.12$ and $\beta = -0.21$ ln(g/l), respectively]. The association between rs6054 and fibrinogen has been described earlier in a candidate gene study (12), and rs148685782 (also known as γAla82Gly) has previously been reported as a causal variant for mild congenital hypofibrinogenaemia (29–31). Furthermore, in a previous study, we examined exome-wide genotypes using exome arrays and identified independent associations of both rs6054 and rs148685782 with fibrinogen (25). In the present study, however, two further variants, rs140473879 and rs149234484, are in strong linkage disequilibrium with rs148685782 and tag this signal. These variants are intergenic, but each changes several regulatory motifs. Thus, the identification of rs148685782 as a causal variant is not conclusive.

Strengths of this study include the use of a large ethnically homogenous sample, and coverage of previously unexamined uncommon and rare variants, indels and variants on the X-chromosome. At the same time, the lack of ethnic heterogeneity may also be a limitation, as including different ethnicities can help narrow down the association signal to a smaller region (32). This study has other limitations that should be acknowledged. To most effectively use the available data, we used all 34 studies in the discovery sample (33). The results have thus not been replicated. Nevertheless, the consistent association of these loci across the 34 studies and the strict Bonferroni correction enforcing a 5% false discovery rate ensure that essentially all of the loci represent true associations. A second limitation is that an approximation based on meta-analysis summary data was used to identify additional independently associated variants at the identified loci rather than a stepwise conditional analysis using individual-level data. Different methods were used to measure plasma fibrinogen across the studies: ethylenediaminetetraacetic acid (EDTA) or citrate plasma samples were used, and a variety of assays were used (34). While the association between fibrinogen and cardiovascular disease has previously been shown to be independent of assay type, the genetic etiology of fibrinogen may differ across assay types (35). However, to minimize the impact on our results, studies that used multiple assays to measure fibrinogen performed their analyses stratified by the assay.

Finally, our ability to attribute these signals to causal genes remains limited. For each locus we reported the gene closest to the lead variant, but proximity alone is not strong evidence that a gene is the underlying causal gene. Thus, we also reported the genes whose expression levels in blood were most strongly associated with the lead variant, and we reported genes with non-synonymous exonic variants in high linkage disequilibrium with the lead variant. Based on blood expression levels, some signals were characterized by a single promising candidate causal gene, but other signals were associated with either no candidate causal genes, or more than one. Furthermore, genetic variants can have effects on the expression of multiple genes across different tissues, and these effects can be tissue specific.

We identified 41 loci that collectively explain 3% of the variance in plasma fibrinogen concentration. Of these loci, 18 had not been identified previously through GWAS. The new loci emphasize the importance of STAT3 to fibrinogen regulation, and highlight several new potential pathways that should be experimentally confirmed. The use of 1000 Genomes Project imputation increased our ability to assess the role of uncommon variants, resulting in an in depth characterization of the two most important fibrinogen loci.

Materials and Methods

Study sample

This meta-analysis was conducted within the framework of the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) consortium (36). The study sample consists of 34 studies with 120 246 individuals of European ancestry. Twelve studies

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with 25 453 participants were not included in the previous fibrinogen GWAS (11). Fibrinogen concentration was measured in citrated or EDTA plasma samples using a variety of methods including the Clauss method, immunonephelometric methods, immunoturbidimetric methods and prothrombin time-derived methods as described in Supplementary Material, Table S1 and the Supplementary Material, Methods, which further describe the studies. All studies were approved by appropriate research ethics committees and all respondents signed informed consent prior to participation.

Genotyping and imputation

Genotyping, pre-imputation quality control, imputation and analysis methods are presented in Supplementary Material, Table S2. All studies imputed variant dosages using reference panels from the 1000 Genomes Project using markov chain haplotyping (MACH) or IMPUTE (14,37–39). The Phase I version 3 reference panel was used by all studies except two, which used the Phase I version 2 reference panel. Before meta-analysis, we excluded variants with MACH imputation quality<0.3 or IMPUTE imputation quality<0.4 and variants with effective minor allele count (minor allele count × imputation quality)<10. These filters were applied at the level of individual studies. Because we wanted to focus only on those variants that passed these filters in a large proportion of the studies, we additionally excluded variants with a total sample size of less than half of the maximum sample size at the meta-analysis level.

Autosomal association analysis

Plasma fibrinogen concentration was converted to g/l and natural log-transformed. All studies adjusted for age and sex. When necessary, analyses were also adjusted for study-specific covariates, such as center or case/control status. In family studies, linear mixed models were used to account for the family structure. Analyses were adjusted for principal components to account for the population structure and cryptic relatedness. These adjustments are shown in Supplementary Material, Table S2. To account for remaining stratification, we applied a genomic control correction to the results of each of the studies before meta-analysis. We used an inverse-variance model with fixed-effects implemented in METAL to meta-analyze association results (40). Heterogeneity was assessed using I^2 and corresponding P-values.

As proposed by Huang *et al.* (41), variants with $P < 2.5 \times 10^{-8}$ were considered genome-wide significant (based on a Bonferroni correction for 2 000 000 tests). Significant variants were assigned to loci in the order of ascending P-value. A variant was assigned to a new locus when there were no significant variants within 500 kb of it belonging to a previously defined locus. Variants were annotated to genes using ANNOVAR version 2013Mar07 (42).

X-chromosome association analysis

Of the 120 246 participants, 95 806 had imputed data on the X-chromosome. Dosages of variants on the X-chromosome were coded as [0,2] in men and [0,1,2] in women. This way one allele in men has the same value as two alleles in women. Thus, we assume full inactivation of one of the two X-chromosomes in women. Variants in the pseudo-autosomal region were excluded. Analyses of the X-chromosome were stratified by sex in each study, and the studies then were meta-analyzed separately for men and women using an inverse-variance model with fixed effects (40). We then combined the sex-specific meta-analysis results for variants on the X-chromosome using both an inverse-variance weighted model with fixed effects and a sample-size weighted model based on P-values and effect direction. The sample-size weighted model does not take the effect size into account, and thus may work better when there are different effects in men and women (43,44), as can happen when there is incomplete inactivation in women.

Conditional analysis

Some loci may harbor multiple independent variants that affect fibrinogen (11,45). To putatively identify these jointly significant variants, we used an approximate method for conditional and joint analysis using meta-analysis summary statistics implemented in genome-wide complex trait analysis (46,47). The method consists of a genome-wide stepwise selection procedure selecting variants according to their conditional P-values and, after the model has been optimized, the estimation of the joint effects of the selected variants. This method depends on a reference panel to estimate linkage disequilibrium patterns between variants. We used best-guess imputation for variants with imputation quality >0.3 in 5733 unrelated individuals from the Rotterdam Study as the reference panel (48). A description of the Rotterdam Study is given in the Supplementary Methods.

Functional annotation

For each locus, we searched the National Human Genome Research Institute GWAS catalog for genome-wide significant associations with other traits within 100 kb of the lead variant (49). We used the Blood eQTL browser, a publicly available database, to examine whether any lead variants, or their most correlated HapMap proxy (with $R^2 > 0.8$), were associated with expression levels of nearby genes in blood. Results from the blood eQTL browser are based on non-transformed peripheral blood samples from 5311 individuals with replication in 2775 individuals (50). For each lead SNP and its highly correlated neighbors (with $R^2 > 0.9$), we used HaploReg V2 to determine the level of conservation, association with gene expression in a range of tissues including the liver, and any overlap with ENCODE transcription factor binding sites, and DNAse-hypersensitive, promoter and enhancer regions in various cell types (51,52). Furthermore, we determined the overlap of these SNPs with microRNAs and microRNA binding sites (see Supplementary Methods) (53–55).

Variance explained

In the Women's Genome Health Study, the largest contributor to the meta-analysis, we computed a weighted genetic risk score based on the lead variants at each genome-wide significant locus, as well as any jointly significant variants identified in the conditional analysis (56). A description of the Women's Genome Health Study is given in the Supplementary Methods. Beta coefficients from the genome-wide association meta-analysis including all studies were used as weights, except in loci with multiple jointly significant variants. For variants at these loci, joint β-coefficients were obtained from the conditional analysis. The genetic risk score was computed as the sum of the weighted variants dosages. The variance in fibrinogen concentration explained was estimated using a linear regression model. Additionally, for any loci with jointly significant variants, we compared the variance explained by the lead variant to the variance explained by the jointly

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significant variants. We were not able to directly compare our estimate of the variance explained to previous estimates, as these had been computed in different populations and were adjusted for age and sex. Thus, we re-calculated the variance explained without adjustment for age and sex. For this, we used HapMap-imputed dosages of the independently associated SNPs reported by Sabater-Lleal *et al.* (11). Since the variance explained is estimated on the basis of imperfectly imputed dosages, we expect our estimates to be slightly lower than if they were based on measured genotypes.

Supplementary Material

Supplementary Material is available at *HMG* online.

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