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Running title: *CES1* SNVs - gene expression and clopidogrel pharmacokinetics

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Abstract: Several single nucleotide variations (SNVs) affect carboxylesterase 1 (CES1) activity, but the effects of genetic variants on *CES1* gene expression have not been systematically investigated. Therefore, our aim was to investigate effects of genetic variants on *CES1* gene expression in two independent whole blood sample cohorts of 192 (discovery) and 88 (replication) healthy volunteers and in a liver sample cohort of 177 patients. Furthermore, we investigated possible effects of the found variants on clopidogrel pharmacokinetics (n=106) and pharmacodynamics (n=46) in healthy volunteers, who had ingested a single 300 mg or 600 mg dose of clopidogrel. Using massively parallel sequencing, we discovered two *CES1* SNVs, rs12443580 and rs8192935, to be strongly and independently associated with a 39% (P=4.0 x 10^{-13}) and 31% (P=2.5 x 10^{-8}) reduction in *CES1* whole blood expression per copy of the minor allele. These findings were replicated in the replication cohort. However, these SNVs did not affect *CES1* liver expression, or clopidogrel pharmacokinetics or pharmacodynamics. Conversely, the *CES1* c.428G>A missense SNV (rs71647871) impaired the hydrolysis of clopidogrel, increased exposure to clopidogrel active metabolite and enhanced its antiplatelet effects. In conclusion, the rs12443580 and rs8192935 variants reduce *CES1* expression in whole blood but not in the liver. These tissue-specific effects may result in substrate-dependent effects of the two SNVs on CES1-mediated drug metabolism.

Carboxylesterase 1 (CES1) catalyses the hydrolysis of a variety of ester-, thioester-, carbamate- and amide-containing xenobiotics and endogenous compounds to their respective free acids [1]. It is highly expressed in the liver and also observed in other tissues, such as blood, small intestine, lungs, heart and testes [1]. CES1 contributes to an estimated 80 to 95% of the total hydrolytic activity in the human liver.

CES1 plays an important role in the biotransformation of clopidogrel [2], which is widely used in the prevention and treatment of aetherothrombotic diseases. CES1 hydrolyses about 90% of the prodrug clopidogrel to an inactive carboxylic acid derivative. Therefore, ultimately only a small proportion of clopidogrel is converted to an active *cis* 5-thiol metabolite by cytochrome P450 (CYP) enzymes.

Several missense variants affect CES1 activity *in vitro.* Of these, the *CES1* c.428G>A (p.G143E, rs71647871) single nucleotide variation (SNV) markedly reduces the biotransformation of CES1 substrate drugs, such as clopidogrel, in humans [3, 4]. However, possible effects of genetic variants on *CES1* expression have not been systematically investigated. Therefore, we sequenced the *CES1* gene and its flanking regions in healthy volunteers and investigated the impact of the found variants on *CES1* whole blood expression. We replicated the findings in another whole blood sample cohort and further investigated the effects of the variants on *CES1* liver expression, as well as on clopidogrel pharmacokinetics and pharmacodynamics in humans.

MATERIALS AND METHODS

A written informed consent was obtained from all participants. The studies were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa or the Northern Savo Hospital District, and the Finnish Medicines Agency Fimea. A fasting whole blood RNA and DNA sample was

obtained from 212 (discovery cohort) and 106 (replication cohort) healthy volunteers during previous pharmacokinetic studies (Supplementary Table 1) [3, 5-8]. Eleven discovery cohort participants with a non-Caucasian background or excess relatedness were excluded from the analysis to minimise population stratification. Of the remaining participants, RNA expression data were obtained from 192 subjects in the discovery and 88 in the replication cohorts. A liver biopsy and a whole blood DNA sample were obtained from 201 patients undergoing laparoscopic gastric bypass operation at the Kuopio University Hospital (liver sample cohort), as part of the Kuopio Obesity Surgery Study [9, 10]. RNA expression data were obtained from 177 patients. The degree of liver steatosis was graded from 0 to 3 and that of lobular inflammation from 0 to 2. Seventy patients had type 2 diabetes, 29 had non-alcoholic fatty liver, 30 had non-alcoholic steatohepatitis and 54 used lipid-lowering medication. In addition, pharmacokinetic data on clopidogrel were obtained from all 106 healthy participants in the whole blood replication cohort. None of the healthy volunteers was a tobacco smoker or used any continuous medication.

Genomic DNA was extracted using Maxwell 16 LEV Blood DNA Kit (Promega, Madison, WI, USA; discovery and replication cohorts) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany; liver sample cohort). For the sequencing library preparation, 3 μg of DNA was processed according to the NEBNext DNA Sample Prep protocol (New England BioLaps, Ipswich, MA, USA). The *CES1* gene ± 20 kb (chr16: 55,816,763–55,887,075; genome build GRCh37) was enriched in discovery cohort samples using the NimbleGen SeqCap EZ Choice capture protocol (Roche Sequencing, Pleasanton, CA, USA) and sequenced on the HiSeq2000 platform with 100 bp paired-end reads (Illumina, San Diego, CA, USA). Quality control, short read alignment and variant calling and annotation were carried out using an in-house developed pipeline, as described previously [11]. The sequencing and bioinformatics pipelines were carried out at the Institute for Molecular Medicine Finland (Helsinki, Finland). Mean coverage depth was 24.9X. Coverage depth ≥ 10X, Hardy-Weinberg equilibrium $P<9.92 \times 10^{-5}$

(Bonferroni correction) and proportion missing ≤ 0.25 were employed as quality thresholds for including genotype data in the statistical analysis. No data were obtained from exon 12 to 12 kb downstream of *CES1* due to insufficient coverage. The *CES1* rs12443580 (c.52+579A>G) and rs8192935 (c.257+885T>C) SNVs were genotyped with custom TaqMan assays on a QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Call identity with sequencing data was 99.4% for rs12443580 and 100% for rs8192935. The replication cohort samples were also genotyped for the *CES1* c.428G>A and *CYP2C19* c.681G>A (**2*, rs4244285), c.636G>A (**3*, rs4986893) and c.-806C>T (**17*, rs12248560) SNVs.

RNA was extracted using the Maxwell 16 LEV simplyRNA Blood Kit (Promega; discovery and replication cohorts) or miRNeasy Mini Kit (Qiagen; liver sample cohort), and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit or SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed on custom OpenArray® plates containing *CES1* (Hs00275607_m1) and reference gene assays.

In clopidogrel pharmacokinetic studies, the participants ingested a 300 mg (*n*=49) or 600 mg (*n*=57) dose of clopidogrel (Plavix, Sanofi Pharma Bristol-Myers Squibb SNC, Paris, France) after an overnight fast [3, 5-8]. The plasma concentrations of clopidogrel, clopidogrel active *cis* 5-thiol metabolite and clopidogrel carboxylic acid were measured as described previously [3]. The peak plasma concentration (C_{max}), time to C_{max} (t_{max}), elimination half-life and area under the plasma concentration-time curve from 0 to infinity ($AUC_{0-\infty}$) were calculated using concentrations for up to 4 hr (*n*=12) and 12 hr (*n*=94) with standard noncompartmental methods using Phoenix® WinNonlin®, version 6.3 (Certara, Princeton, NJ, USA). The platelet inhibitory effect of clopidogrel was measured

with a VerifyNow® P2Y12 Test for up to 12 hr after a 600 mg dose of clopidogrel in a subgroup of 46 participants (Accumetrics, San Diego, CA, USA).

Statistical analysis was performed using JMP Genomics 7.0 (SAS Institute Inc., Cary, NC, USA) and IBM SPSS 22.0 (Armonk, NY, USA). Possible effects of demographic covariates (age, sex and body weight for the discovery cohort; age, sex, body mass index, type 2 diabetes, degree of lobular inflammation and hepatic steatosis and use of lipid-lowering medication for the liver sample cohort) on *CES1* expression were investigated using a forward stepwise linear regression analysis. The effects of genetic variants on *CES1* whole blood and liver expression were then investigated using linear regression analysis adjusting for demographic covariates as necessary. Differences in the pharmacokinetic (except t_{max}) and pharmacodynamic variables were investigated using a forward stepwise linear regression analysis, with demographic covariates, and *CES1* and *CYP2C19* genotypes as independent variables. The $AUC_{0-\infty}$ and C_{max} values were adjusted for 70-kg body weight. Except for the t_{max}, the pharmacokinetic variables were logarithmically transformed. The t_{max} values were compared using Kruskal-Wallis 1-way analysis of variance. Differences were considered statistically significant when *P* was below 0.05. The discovery cohort *P* values were adjusted for multiple testing with the Benjamini-Hochberg false discovery rate (FDR) method. Consistency of genotype distribution with the Hardy-Weinberg equilibrium was tested with an exact test [12].

RESULTS

The *CES1* mRNA expression varied 82-fold among the discovery cohort and 42-fold among the replication cohort whole blood samples, and 10-fold among the liver samples. Age, sex or body weight were not associated with *CES1* expression in whole blood. Similarly, age, sex, body mass index, type 2 diabetes, degree of lobular inflammation and hepatic steatosis or use of lipid-lowering

medication were not associated with *CES1* expression in the liver. A total of 227 sequence variants with minor allele frequencies (MAFs) of at least 1% were discovered within ± 20 kb of the *CES1* gene among the 192 healthy participants. Of these, 97 were associated with *CES1* whole blood expression with FDR-adjusted *P* values below 0.05 (Supplementary Table 2). In a forward stepwise linear regression analysis, two intronic SNVs associated independently with *CES1* expression (Fig. 1, Table 1); the rs12443580 (MAF 35%) SNV was associated with a 39% reduction (90% confidence interval (CI), 32-45%; P=4.0 x 10^{-13}) and the rs8192935 (MAF 31%) SNV with an additional 31% reduction (90% CI, 24-38%; P=2.5 x 10^{-8}) per variant allele. In a replication cohort, the rs12443580 SNV was associated with a 28% reduction (90% CI, 15-38%; P=0.0015) and the rs8192935 SNV with a 25% reduction (90% CI, 10-37%; P=0.0091) of *CES1* expression per variant allele. However, these SNVs showed no significant association with *CES1* expression in the liver samples or the pharmacokinetics or pharmacodynamics of clopidogrel. The *CES1* c.428G>A missense SNV was associated with 58% lower clopidogrel carboxylic acid to clopidogrel $AUC_{0-\infty}$ ratio per variant allele (P=0.0004). This ratio was 74% higher following 600 mg dose than 300 mg dose of clopidogrel (90% CI, 35-124%; P=0.0005) and 1.9% lower per 1 kg body weight (90% CI, 0.9-2.8%; P=0.001). The $AUC_{0-\infty}$ of clopidogrel was 163% (90% CI, 76-293%; P=0.0001) and that of the active metabolite 60% (90% CI, 22-110%; P=0.005) higher per *CES1* c.428G>A variant allele. There was a tendency for a 13% lower $AUC_{0-\infty}$ of the active metabolite per *CYP2C19* c.681G>A variant allele (**2*, rs4244285) (P=0.058). The average percentage inhibition of $P2Y_{12}$-mediated platelet aggregation by clopidogrel was 21 percentage points higher per *CES1* c.428G>A variant allele (P=0.009) and 12 percentage points lower per *CYP2C19* c.681G>A variant allele (P=0.01).

DISCUSSION

Although CES1 has a recognised role in drug metabolism and its activity shows marked interindividual variability, few studies have investigated the effects of genetic variants on *CES1* expression in humans. We discovered two intronic *CES1* SNVs to be strongly and independently associated with its expression in two whole blood sample cohorts. However, these variants did not affect *CES1* liver expression, or the pharmacokinetics or pharmacodynamics of clopidogrel.

The effects of two intronic *CES1* rs12443580 and rs8192935 SNVs on its whole blood expression may result from direct effects of these SNVs on *CES1* transcription or linkage disequilibrium (LD) with other variants affecting *CES1* expression. A total of 10 SNVs were in strong LD (r^2>0.30) with the rs12443580 SNV and 30 with the rs8192935 SNV (Supplementary Table 3). Of these, 8 and 0, respectively, were located upstream of the *CES1* gene. *CES1* expression is regulated by specificity protein 1, CCAAT/enhancer-binding protein, nuclear factor-erythroid 2 related factor and hepatocyte nuclear factor 4α transcription factors. However, none of the upstream variants in LD with rs12443580 SNV is located at known transcription factor binding sites. The lack of effect of these SNVs on *CES1* liver expression, despite strong effects on whole blood expression, suggests tissue-specific transcriptional regulation of *CES1*. This could be due to tissue differences in promoter methylation or transcription factor expression. Therefore, our findings highlight the potential downside of carrying out gene expression association studies in whole blood only, as regulatory variants affecting mRNA expression in whole blood might not affect gene expression in the liver. It should also be kept in mind that genetic variants affecting mRNA expression do not necessarily affect protein expression.

The finding that rs12443580 and rs8192935 variants had no effect on *CES1* liver expression or clopidogrel pharmacokinetics supports the hypothesis that the CES1-mediated hydrolysis of clopidogrel takes place mainly in the liver. Dabigatran etexilate, in contrast, is metabolised to active dabigatran by carboxylesterases probably already in the intestinal wall [13]. In a genome-wide association study, the rs8192935 SNV and a linked intronic rs2244613 SNV were associated with reduced plasma dabigatran concentrations [13]. Additionally, in a recent study, a haplotype containing the rs2244613 SNV was associated with increased capecitabine toxicity [14]. Due to the strong LD between these SNVs (r^2=0.47 in our study and r^2=0.45 in the dabigatran study) [13], it is unlikely that rs2244613 SNV would affect *CES1* liver expression. These data suggest substrate-dependent effects of the rs8192935 SNV on drug metabolism, which could be explained by tissue-specific effects on *CES1* expression.

In the present study, we employed an oligonucleotide-based target enrichment procedure, allowing for up to five close matches in the genome, and mapped the reads to the *CES1* gene and its flanking regions of human genome build GRCh37. A non-functional *CES1P1* pseudogene has sequence similarities with *CES1*, especially through exons 12-14 [14-16], which may explain the poor coverage in this region in our study. It should also be recognized that a fusion gene, known as *CES1P1VAR* (MAF 14% in Caucasians) is identical to the *CES1* gene except for sequence differences in 5´-UTR, exon 1 and intron 1 [16-18]. *CES1P1VAR* is not included in the human genome build and this may have caused uncertainties in sequence capture and some allele calls. However, especially the top associated SNVs were well in the Hardy-Weinberg equilibrium and successfully genotyped with another method. Moreover, the associations were replicated in an independent cohort. These data support the specificity of our findings.

Confirming the results of two previous studies [3, 4], the *CES1* c.428G>A SNV significantly impaired the hydrolysis of clopidogrel, increasing the exposure to the active metabolite and enhancing its antiplatelet effects. In addition to the *CES1* c.428G>A SNV, *CYP2C19* variants have a recognised effect on clopidogrel pharmacokinetics and pharmacodynamics [19, 20]. In the present study, the *CYP2C19* c.681G>A loss-of-function allele was associated with reduced antiplatelet effects, but only a non-significant trend existed towards reduced exposure to the active metabolite. As this study included participants from a previously published genotype panel study focusing on the *CES1* c.428G>A variant [3], the over-representation of this variant may have overshadowed the impact of the *CYP2C19* c.681G>A variant allele. In any case, the effect of the *CES1* c.428G>A SNV on clopidogrel antiplatelet effects is opposite to, and even stronger than that of the *CYP2C19* c.681G>A allele.

In conclusion, the *CES1* rs12443580 and rs8192935 variants have a major effect on *CES1* expression in whole blood, but not in the liver, suggesting tissue-specific effects of these SNVs on *CES1* expression. While these SNVs have no effect on clopidogrel pharmacokinetics, the *CES1* c.428G>A SNV markedly reduces clopidogrel hydrolysis.

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FIGURE LEGENDS

Figure 1

The association of *CES1* genetic variants with its whole blood expression (a and b). Horizontal axis shows SNV location and vertical axis shows FDR-adjusted -$\log_{10}P$ value for each SNV tested by univariate linear regression analysis (a) or multivariate linear regression analysis adjusting for rs12443580 SNV (b). The horizontal dashed lines represent *P* value thresholds of 0.05. Box plots of the effects of rs12443580 and rs8192935 SNVs on *CES1* whole blood (c and d) and liver (e) expression. Box plots of the effects of *CES1* c.428G>A SNV (rs71647871) on dose-adjusted clopidogrel carboxylic acid to clopidogrel $AUC_{0-\infty}$ ratio (f), dose- and weight-adjusted active metabolite $AUC_{0-\infty}$ (g) and the effects of *CES1* c.428G>A and *CYP2C19* c.681G>A (**2*, rs4244285) SNVs on platelet inhibitory effects of 600 mg oral dose of clopidogrel (h). Individual data points are given

as circles for men and triangles for women (f-h). The horizontal lines inside the boxes represent the median, the box edges show the lower and upper quartiles and the whiskers show the 10th and 90th percentiles.

$AUC_{0-\infty}$, the area under the plasma concentration-time curve from 0 hr to infinity; CES1, carboxylesterase 1; CYP, cytochrome P450; FDR, false discovery rate; SNV, single nucleotide variation.

Table 1 Effects of *CES1* rs12443580 and rs8192935 SNVs on *CES1* whole blood and liver expression and effects of *CES1* c.428G>A and *CYP2C19* c.681G>A SNVs on clopidogrel pharmacokinetics and pharmacodynamics.

Parameter	Effect (per variant allele)	90% CI	*P* value
CES1 whole blood expression (discovery) (*n*=192)			
rs12443580	-38.8%	-44.9%, -32.1%	4.0×10^{-13}
rs8192935	-31.4%	-38.3%, -23.6%	2.5×10^{-8}
CES1 whole blood expression (replication) (*n*=88)			
rs12443580	-27.5%	-38.4%, -14.7%	0.0015
rs8192935	-24.8%	-37.1%, -10.2%	0.0091
CES1 liver expression (*n*=177)			
rs12443580	1.2%	-6.0%, 8.9%	0.798
rs8192935	3.3%	-5.0%, 12.3%	0.519
Clopidogrel carboxylic acid to clopidogrel $AUC_{0-\infty}$ ratio (*n*=106)			
CES1 c.428G>A SNV	-58.1%	-71.6%, -38.0%	0.00037

Clopidogrel $AUC_{0-\infty}$			
CES1 c.428G>A SNV	163.0%	76.0%, 293.1%	0.00012
Active metabolite $AUC_{0-\infty}$			
CES1 c.428G>A SNV	59.6%	21.5%, 109.8%	0.0054
Average platelet inhibition (0-12h) (*n*=46)			
CES1 c.428G>A SNV	21.4%	11.3%, 31.4%	0.00090
CYP2C19 c.681G>A SNV	-12.4%	-20.1%, -4.6%	0.010

$AUC_{0-\infty}$, the area under the plasma concentration-time curve from 0 hr to infinity; CES1, carboxylesterase 1; CI, confidence interval; CYP, cytochrome P450; SNV single nucleotide variation.

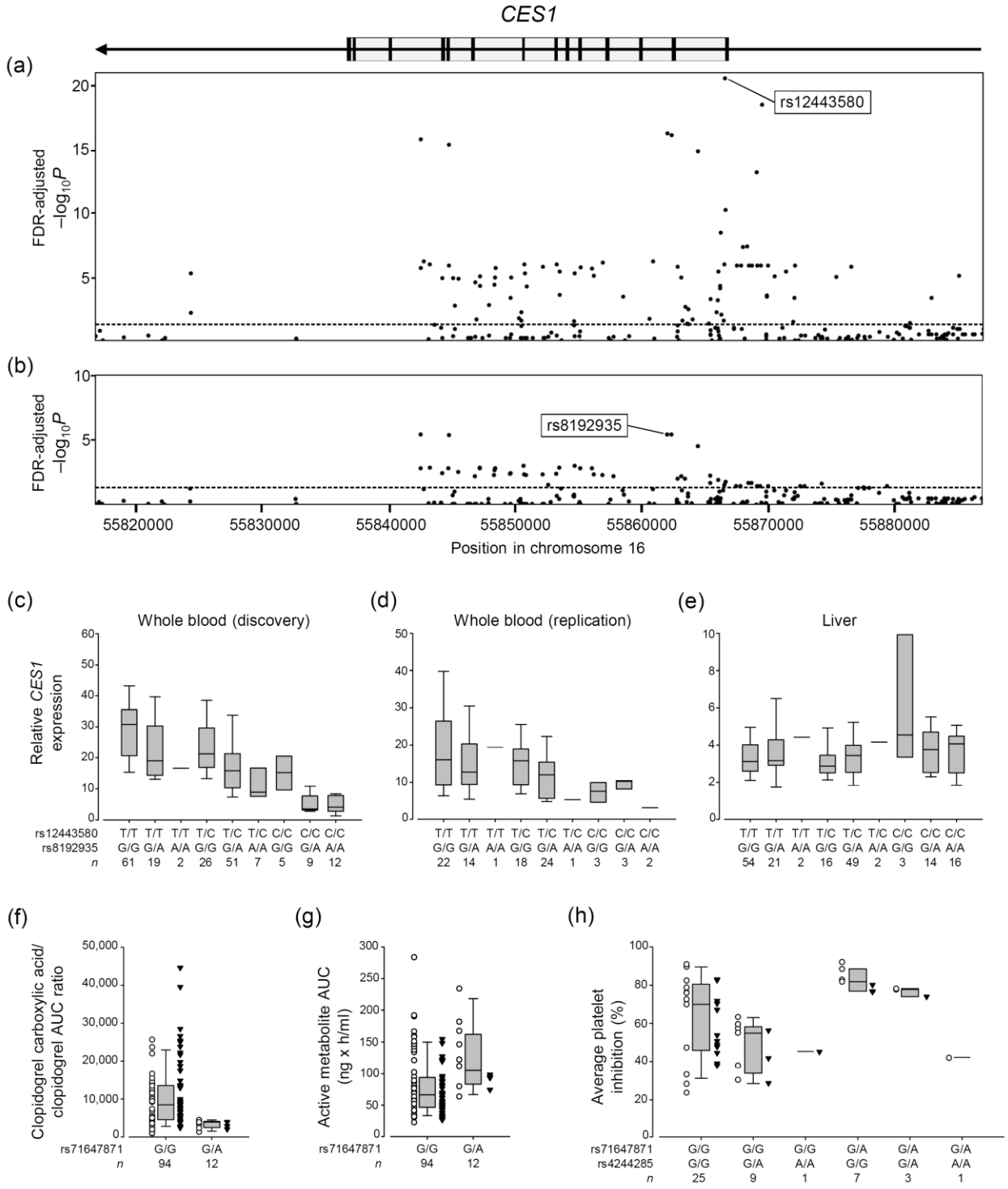

Figure 1