PROTECTIVE LOW FREQUENCY VARIANTS FOR PREECLAMPSIA IN THE *FLT1* GENE IN THE FINNISH POPULATION

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Abstract

Preeclampsia is a common pregnancy-specific vascular disorder characterized by new-onset hypertension and proteinuria during the second half of pregnancy. The etiology of the disease is

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Disclosures
None.

unknown but predisposition to preeclampsia is in part heritable. Preeclampsia is associated with an increased risk of cardiovascular disease later in life. We have sequenced 124 candidate genes implicated in preeclampsia to pinpoint genetic variants contributing to predisposition to or protection from preeclampsia. First, targeted exomic sequencing was carried out in 500 preeclamptic women and 190 controls from the Finnish Genetics of Preeclampsia Consortium (FINNPEC) cohort. Then 122 women with a history of preeclampsia and 1,905 parous women with no such history from the National FINRISK Study were included in the analyses. We tested 146 rare and low-frequency variants and found an excess (observed 13 versus expected 7.3) nominally associated with preeclampsia (p< 0.05). The most significantly associated sequence variants were protective variants rs35832528 (E982A; p=2.49E-4, OR=0.387) and rs141440705 (R54S; p=0.003, OR=0.442) in Fms Related Tyrosine Kinase 1 (*FLT1*). These variants are enriched in the Finnish population with minor allele frequencies 0.026 and 0.017, respectively. They may also be associated with a lower risk of heart failure in 11,257 FINRISK women. This study provides the first evidence of maternal protective genetic variants in preeclampsia.

Keywords

Targeted exomic sequencing; candidate gene; preeclampsia; *FLT1*; angiogenesis

Introduction

Preeclampsia (PE) is a common vascular disorder that affects 3% of pregnant women [1]. Worldwide, it annually accounts for approximately 50,000 maternal and 900,000 perinatal deaths [2, 3]. The clinical characteristics are diverse and the course of the disease is unpredictable. Both a PE mother and a child born from a PE pregnancy are at higher risk for later-life cardiovascular diseases and type 2 diabetes [4].

Angiogenesis is tightly involved in the pathophysiology of PE [5]. A high ratio of soluble fms related tyrosine kinase 1 (sFLT1) to placental growth factor (PlGF) is among the most promising biomarkers for predicting the onset of the disease [6]. Several susceptibility loci for PE have been identified in genome-wide linkage studies [7, 8]. However, linkage and candidate gene studies have been plagued with poor reproducibility.

The population of Finland is genetically unique in Europe [9]. The repeated bottleneck events that caused strong founder effects, and geographic isolation over centuries have resulted in the enrichment of variants that are rare or absent in other populations [10]. These features provide an opportunity to overcome many of the obstacles in studying rare enriched variants that contribute to the risk of complex diseases [11].

We designed a targeted gene sequencing protocol to screen the coding and splicing areas of genes of interest within angiogenic and vascular pathways and other putative candidate genes in a sizeable cohort of PE cases and controls from Finland. Here we explore the potential causal role of variation in candidate genes in PE.

Methods

Diagnostic criteria and patient cohorts

The study design is outlined in Figure 1. At Stage 1 we studied 500 non-obese (body mass index [BMI]<30 kg/m^2) women with PE pregnancies and 190 non-PE controls that were matched geographically, in age, and in BMI from The Finnish Genetics of PE Consortium (FINNPEC) cohort, a case-control cohort recruited from the five Finnish University Hospitals [12]. Nulliparous or multiparous women with a singleton pregnancy were eligible for the study. PE was defined as hypertension and proteinuria occurring after 20 week's gestation. Hypertension was defined as systolic blood pressure 140 mm Hg or diastolic blood pressure 90 mm Hg after 20 weeks of gestation. Proteinuria was defined as the urinary excretion of 0.3 g protein in a 24-h specimen, or 0.3 g/l, or two 1+ readings on dipstick in a random urine determination with no evidence of urinary tract infection. All diagnoses were independently verified by a research nurse and a physician. Seven PE and one control were excluded due to a failure in genotyping and further nine cases were excluded due to non-Finnish ethnicity and ovum donation pregnancy. The characteristics of the study participants are presented in S1. All women provided a written informed consent and the FINNPEC study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa.

At Stage 2 we included in the analyses whole exome sequencing from an additional 122 women with a history of PE and 1905 parous women with no such history from the national FINRISK study cohort (FINRISK license 8/2016)[13]. For identifying PE and eclampsia cases in the FINRISK study cohort, we used following Finnish ICD codes in the comprehensive National Hospital Discharge Register covering years 1992 to 2007: **ICD-10** (in use since 1996): O14.0, O14.1, O14.9, O15.0, O15.1, O15.2, O15.9, **ICD-9** (in use from 1987–1996): 6424–6426, 6427A and **ICD-8** (in use from 1968–1986) :637.03, 637.04, 637.09, 637.10, 637.99). Controls from FINRISK were all women who had given birth at least once and did not have any of these diagnoses recorded. In the final association analyses, we included genotypes of FINNPEC and FINRISK which combined totaled 609 cases and 2,092 controls. All polymorphic sites in the Stage 1 targeted sequencing were queried from the population cohort exomes. All of the National FINRISK Study methodology and ethical approvals are available online: https://www.thl.fi/en/web/thlfi-en/research-and-expertwork/population-studies/the-national-finrisk-study.

Preparation of Genomic DNA

Genomic DNA was extracted from whole blood using the NucleoSpin Blood XL DNA extraction kit (Macherey-Nagel GmbH & Co.) or the Chemagic Magnetic Separation Module I –machine (Chemagen) and subsequently stored at −20°C.

Targeted Sequencing and Capture Enrichment

Genes implicated in human biomarker studies were chosen as targeted candidate genes, as were their signalling partners and proteases that alter their function and levels. Specific single nucleotide polymorphisms (SNPs) and any neighbouring or associated gene that have been enriched in PE women based on meta-analysis, genomewide association studies

(GWAS) or functional studies were included. This list of existing candidate genes was based on review of literature in January 2013 and it included genes involved in angiogenesis, such as *FLT1*, and pregnancy, such as pregnancy-zone protein *PZP*, among other relevant functional pathways. The studied genes are listed in S2.

Libraries from genomic DNA were prepared in-house (Washington University School of Medicine) [14]. Enzymes were purchased from Enzymatics (Beverly, MA). Briefly, the ends of sheared genomic DNA fragments were repaired by treatment with T4 DNA Polymerase and T4 DNA Polynucleotide Kinase, which phosphorylates the 5′ hydroxyl group. An adenosine was then added to the 3′ position at each end of the DNA fragment with Taq Polymerase. Illumina adapters with an overhanging "T" were ligated onto the DNA fragment followed by bead-based size selection procedure to remove adapter-dimers and fragments below the desired size. A unique index sequence was added by PCR by targeting the two ligated universal adapters on each fragment end.

Sequence capture by hybridization was performed according to the manufacturer's recommendations with modifications (Roche SeqCap Hybridization and Wash Kit No. 05634261001). We utilized longer blocking oligos containing an additional 7 bp inosine segment for promiscuous pairing with different index sequences. Following hybridization, captured DNA was washed and eluted according to the manufacturer's instructions.

Sequencing and Analysis

Sequencing was performed on an Illumina HiSeq 2000 at the Washington University Genome Technology Access Center using 2×101 bp, 2×135 bp and 2×150 bp reads. We aligned sequencing to GRCh37 using bwa aln (v0.6.1-r104) and genotyped the samples using the Genome Analysis ToolKit (v2.5.2-gf57256b) Unified Genotyper [15–17].

Statistical Analyses

Quality control before meta-analysis included removal of singleton and monomorphic variants, removal of sites with >10% missing data in the targeted sequencing or a significant departure from Hardy-Weinberg equilibrium in controls (p<0.001). The combined dataset was analyzed using Fisher's exact test which served as our primary association test. The analysis was divided into two classes: low frequency and rare variants (minor allele frequency (MAF) <10%) or common variants (MAF>10%). After quality control, 622 variants (443 variants with MAF<10% and 179 variants with MAF>10%) were advanced to the combined primary analysis. Of these, 201 were in putatively functional categories (missense, nonsense, or splice region variants) and 421 were in likely benign categories (synonymous, intronic, intergenic).

The two SNPs with strongest associations were further compared to 672 predefined epidemiological diagnoses derived from national health care registries using imputed genotypes of 11,257 FINRISK women. Genotypes were imputed using a combined panel of Finnish whole genome sequences and 1000 Genomes phase I reference with a high imputation confidence (info metric > 0.97) [18, 19]. Associations were calculated using logistic regression (SNPTEST v2.5.2, EM algorithm) and then further confirmed with Fisher's exact test given the rarity of variants and several diagnoses. Due to the explorative nature of this

part of the analysis, no multiple testing corrections to estimates were performed. Heart failure was identified using codes I50, 4289X, and 42700 in Finnish **ICD-10**, **ICD-9**, and **ICD-8**, respectively.

Data were analysed using PlinkSeq, Plink [20] and R. Kaviar[21] and VEP Build 37 was used in additional annotations [22]. Loss of function (LoF) analyses were conducted for each gene with associating variants *in silico* by the Loss of Function – tool (https://github.com/ensembl-variation/VEP_plugins/blob/master/LoFtool.pm). The following annotations were calculated: LoF score < 0.2 is probably damaging, LoF score 0.2–0.7 is possibly damaging, LoF score < 0.7 is benign. In addition to appropriate statistical probability tests, odds ratios (OR) with 95% confidence intervals (CI95) were calculated for all variants.

Results

Results of the association analyses are listed in Table 1. For these studies, genotypes from Stage 1 (FINNPEC) were combined with a population cohort in Stage 2 (FINRISK) for joint analysis. Forty-one variants demonstrated nominal association (p<0.05; 34 uncommon and 7 common). The tail of the p-value distribution of benign variants was as expected (0 results with p<0.001; expected   1 and 2 results with p<0.01; expected   4.2), suggesting that the overall study design and quality control were successful. Among the 201 putatively functional variants, one had p<0.001 (alpha=0.05) compared to the 0/421 observed among benign variants. The lack of inflated p-values points out that confounders such as stratification are not causing false positives.

Focusing on 146 putatively functional (missense or truncating) rare and low-frequency variants, we observed an excess (observed 13, expected 7.3) of nominally associated variants (p<0.05). The strongest associations observed were in *FLT1*, with two missense variants associated to PE: NM_002019.4:p.E982A (rs35832528; p-value=2.5E-4, odds ratio (OR)=0.387, withstands correction for multiple testing for the 146 rare and low-frequency coding variants), and NM_001159920.1:p.R54S (rs141440705; p-value=0.0027, OR=0.44). These variants are enriched in the Finnish population, with minor allele frequencies being 0.026 (1000 Genomes MAF=0.0014) and 0.017 (1000 Genomes MAF=0.0008), respectively (www.sisuproject.fi).

E982A and R54S were examined across registry-derived disease endpoints. Both of the assessed SNPs protected from heart failure (Fisher's exact p-value=0.007 for both, E982A: OR=0.368 95%CI=0.164–0.830, cases/controls=301/10956, minor allele (G) count in cases/controls=6/593; R54S: OR=0.340 95%CI=0.140–0.826) cases/controls=297/10960, minor allele (G) count in cases/controls=5/543).

Discussion

We discovered low frequency protective genetic variants in *FLT1* that contributed to lower PE risk. We also found associating genetic variants in known candidate genes using a targeted sequencing approach.. These *FLT1* variants may also be associated with lower risk of heart failure.

FLT1 codes for vascular endothelial growth factor receptor 1 (VEGFR1). It consists of seven immunoglobulin (Ig)-like domains in an extracellular ligand-binding region, a transmembrane segment and a cytoplasmic region containing a tyrosine kinase (TK) domain [23]. VEGFR1 is essential for survival by negatively regulating the levels of endogenous vascular endothelial growth factor (VEGF). Internalization and signaling of functional VEGF receptors will enhance angiogenic growth of blood vessels [24]. Sustaining angiogenesis is necessary for circulation and anti-angiogenic treatment causes cardiovascular morbidity [25]. There are multiple isoforms of VEGFR1 of which the soluble forms have been implicated in PE. The soluble forms of VEGFR1 only contain the extracellular parts of the protein encoded by the first 13 of 30 exons. In preeclampsia, an excess of VEGFR1 of placental origin has been recorded [26]. Furthermore, after healthy endothelium is restored, elevated levels of soluble VEGFR1 are observed in women with a history of PE [27].

Increased levels of sFLT1/sVEGFR1 have also been indicated in peripartum cardiomyopathy where high levels of sFLT1 correlate with the symptoms' severity [28]. Similarly, heart failure after myocardial infarction independent of pregnancy is reflected in extreme levels of sFLT1 [29]. Rhee *et al.* report extreme sFLT1 levels in at least the 95th percentile in a cases of heart failure during pregnancy [30]. Although further research is required to assess the effect of underlying risk factor profile in preeclamptic women's increased risk of heart failure in later life, it was recently shown in a meta-analysis of results from seven studies that 3.6-fold increase in risk of heart failure is associated with preeclampsia, particularly during the time period of 1–10 years after preeclampsia [30]. Preeclampsia increases the risk of peripartum cardiomyopathy and it has been suggested that sFLT1 may be toxic to the heart. Also the driver of the cardiac dysfunction in susceptible preeclamptics is likely mediated by antiangiogenic factors [31]. Our results indicate that *FLT1* variants that protect from preeclampsia may also protect from heart failure thereby adding to the growing body of evidence that imbalance of angiogenic factors may be the link between preeclampsia and cardiovascular morbidity in later life in susceptible individuals. Novel therapeutic options for these individuals may include blocking sFlt1 production, or neutralizing antibodies against angiogenic proteins [32, 33].

From the observed associating variants in *FLT1*, rs141440705 causing R54S is more likely of functional importance. This is because it results in a polar change (pos->neutral) within the Ig-like domain 1, a functional part of the protein [34]. Rs141440705 is located in the last nucleotide of an exon. Therefore, the variant may affect splicing as well as the coded amino acid sequence.

Epidemiological studies with many of the protective associations from severe cardiovascular diseases are strongest for R54S, supporting the suggestion of functional significance. The other variant resulting in the amino acid substitution E982A is not included in the s-FLT1 or the VEGFR1 isoform 2, which is the dominant isoform in the placenta. Even so, since VEGFR1 isoform 1 (canonical sequence) is expressed in vascular endothelium, E982A might have an important role in pregnancy in mediating decidual blood flow and in remodeling the spiral arteries.

We also found support for the role of several other candidate genes in PE. *Corin* has been previously indicated in PE with population-specific variants [35]. Stepanian *et al.* report very similar odds ratios of approximately 2.5 at two intronic SNPs, rs2271036 and rs2271037 to our finding at rs61759670 and rs61760500 [35]. Rs13406336 and rs4556933 in activin A receptor type 1C (*ACVR1C*) have been previously listed as a candidate SNPs, although no significant association to PE was established in a Norwegian population [36]. Interestingly, rs7830 features in numerous studies that pinpoint the association of nitric oxide synthase 3 (*NOS3*) to a spectrum of multifactorial diseases. Most relevantly, a haplotype including rs7830 was found to protect women from pregnancy hypertension and PE [37]. Rs1554286 in interleukin 10 (*IL-10*) has been shown to belong to an intronic haplotype, which strongly predisposes women of Bahraini Arab population to idiopathic recurrent miscarriages [38]. Our findings support the hypothesis that common immunological etiology may be shared between recurrent miscarriages and PE [39, 40].

Perspectives

To our knowledge, this study provides the first evidence that maternal *FLT1* sequence variants associate with lower PE susceptibility. Further research is required to pinpoint the mechanism of protection from PE and heart failure and the effect of *FLT1* variants on gene transcription. Genetic associations may open new avenues of drug development once the functional consequences of our findings are further deciphered. Genetic protection from PE due to *FLT1* variants may also protect these women from heart failure in later life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and significance

1. What Is New?

- We have established a genetic association between PE mothers and the *FLT1* gene.

- The same variants may also protect Finnish women from heart failure in later life

2. What Is Relevant?

- Preeclampsia is a common hypertensive disorder of the pregnancy.

- Identification of protective variants in *FLT1*, a known candidate gene, opens an opportunity for drug development to target the women most at risk of the disease.

3. Summary of the conclusions of the study

- Genetic variants within the maternal *Flt1* protect Finnish women from preeclampsia.
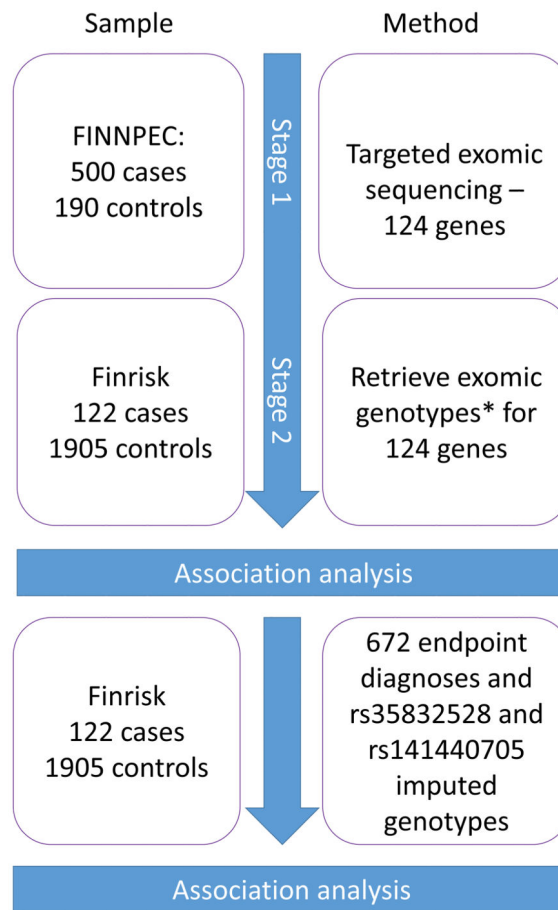
Figure 1.
Methods and samples of study design.

*Genotyping performed by Agilent 1.1 refseq (60 cases and 4 controls), Illumina coding v1 (162 cases and 10 controls) and Nimblegen SeqCap EZ VCRome (1682 cases and 109 controls) platforms.

Table 1

The observed variants with significant associations to preeclampsia. Counts of minor alleles are given for the combined data; 615 cases and 2,094 controls, and per FINNPEC data set (in parentheses); 487 of 500 women with PE and 187 of 190 control women passed quality control.

RSID number	Gene name	P-value*	OR (95% confidence interval)	MAF in total sample	Count with minor allele $n_{cases}/n_{controls}$ FINNPEC minor allele count in parentheses (cases/controls)	HWE	Consequence (distance from exon, base pairs)	LoFtool†
rs35832528	FLT1	2.49E-4	0.387 (0.205 – 0.678)	0.010	14/122 (7/6)	1	missense variant, E982A	0.463
rs141440705	FLT1	0.003	0.442 (0.233 – 0.779)	0.010	14/106 (8/5)	1	missense variant, R54S	0.463
rs61758484	CORIN	0.003	2.658 (1.320 – 5.261)	0.013	17/22 (16/1)	1	non coding transcript exon variant, E10K	0.201
rs34106916	ANGPTL1	0.006	3.186 (1.325 – 7.597)	0.008	12/13 (8/3)	1	synonymous variant, Q103Q	0.658
rs61759670	CORIN	0.010	2.082 (1.147 – 3.693)	0.016	21/35 (18/4)	1	missense variant, Y907T	0.201
rs80338240	JAG1	0.010	0.256 (0.051 – 0.806)	0.004	3/40 (2/4)	1	intron variant (−11)	0.006
rs147998709	GPR98	0.011	13.757 (1.360 – 675.490)	0.003	4/1 (3/0)	0.004	intron variant (+24)	0.977
rs2290843	ADAM12	0.011	0.726 (0.561 – 0.932)	0.071	83/383 (63/33)	1	synonymous variant, T326T	0.320
rs61760500	CORIN	0.011	2.550 (1.179 – 5.382)	0.010	14/19 (11/3)	1.001	intron variant (+13)	0.201
rs147942437	ADAM28	0.012	5.181 (1.226 – 24.998)	0.004	6/4 (5/0)	1.002	missense variant, L449P	0.994
rs13406336	ACVR1	0.013	2.423 (1.129 – 5.062)	0.011	14/20 (13/2)	1.003	missense variant, A15G	0.138
rs142436579	ADAM28	0.014	0.366 (0.129 – 0.852)	0.005	6/56 (4/3)	1.004	missense variant, R219S	0.994
rs140437272	INHBE	0.014	0.477 (0.236 – 0.881)	0.013	12/85 (11/6)	1.005	missense variant, P27L	0.786
rs201756397	FLT4	0.014	Inf (1.283 – Inf)	0.001	3/0 (2/0)	1.006	synonymous variant, E926E	0.023
rs80069610	GPR98	0.016	2.499 (1.122 – 5.411)	0.010	13/18 (11/2)	1.007	synonymous variant, V1101V	0.977
rs139608664	INHA	0.018	5.716 (1.110 – 36.859)	0.003	5/3 (4/0)	1.008	synonymous variant, S225R	0.046
rs4556933	ACVR1C	0.019	0.847 (0.735 – 0.976)	0.323	1157/381 (314/120)	0.113	synonymous variant, F38F	0.076
rs41302834	GPR98	0.022	3.451 (1.031 – 11.557)	0.007	7/7 (7/2)	1.009	missense variant, D1944N	0.977
rs3736061	FLT4	0.023	0.803 (0.665 – 0.973)	0.135	485/175 (143/39)	0.868	synonymous variant, L252L	0.023
rs3741849	PZP	0.039	1.309 (1.004 – 1.696)	0.068	88/235 (71/21)	1	synonymous variant, K563K, splice region variant	0.988
rs34307240	LCT	0.026	2.201 (1.038 – 4.515)	0.010	14/22 (12/2)	1.011	missense variant, D106E	0.571
rs36032184	INHA	0.027	1.906 (1.058 – 3.346)	0.017	21/38 (20/3)	1.012	synonymous variant, G109G	0.046
rs2228048	TGFBR2	0.029	0.672 (0.459 – 0.963)	0.030	38/191 (27/13)	0.450	synonymous variant, N354N	0.060
rs138819536	INHBA-AS1	0.032	4.311 (0.926 – 21.758)	0.005	5/4 (4/1)	1	missense variant, R229Q	0.043
rs1466360	ADAM12	0.033	1.152 (1.010 – 1.315)	0.414	1852/504 (404/152)	0.691	intron variant (+30)	0.320

RSID number	Gene name	P-value[*]	OR (95% confidence interval)	MAF in total sample	Count with minor allele $n_{cases}/n_{controls}$ FINNPEC minor allele count in parentheses (cases/controls)	HWE	Consequence (distance from exon, base pairs)	LoFtool[†]
rs56133834	TEK	0.034	3.450 (0.921 – 12.934)	0.004	6/6 (6/0)	1	synonymous variant, E986E	0.046
rs140593977	TREX1	0.034	3.448 (0.920 – 12.928)	0.004	6/6 (6/0)	1	downstream gene variant (−44)	0.824
rs1466361	ADAM12	0.034	1.152 (1.010 – 1.316)	0.414	1810/501 (404/152)	0.691	intron variant (−46)	0.320
rs148671842	EHD3	0.035	2.372 (0.992 – 5.459)	0.007	11/16 (8/2)	1	synonymous variant, E188E	0.139
rs115734907	KDR	0.035	0.695 (0.483 – 0.979)	0.029	42/205 (28/11)	1	intron variant (+12)	0.196
rs1554286	IL10	0.037	1.203 (1.009 – 1.438)	0.153	761/191 (145/60)	0.231	intron variant (+18)	0.538
rs2453040	NOTCH2	0.037	0.818 (0.676 – 0.992)	0.141	482/172 (144/45)	0.423	intron variant (−45)	0.016
rs116951780	LCT	0.038	10.333 (0.829 – 541.218)	0.001	3/1 (2/0)	1	synonymous variant, NMD transcript variant, A921A	0.571
rs138894008	TEK	0.038	10.333 (0.829 – 541.218)	0.002	3/1 (3/0)	1	missense variant, R479H	0.046
rs148588802	FLT1	0.038	10.328 (0.829 – 540.960)	0.002	3/1 (3/0)	1	intron variant (+40)	0.463
rs200071734	FLT4	0.040	10.114 (0.811 – 529.805)	0.002	3/1 (3/0)	1	missense variant, V157M	0.023
rs150123876	ANGPT4	0.042	0.348 (0.090 – 0.968)	0.007	4/39 (3/7)	1	missense variant, R25H	0.773
rs368518386	FLT4	0.046	5.581 (0.799 – 61.819)	0.003	4/2 (4/0)	1	intron variant (−43)	0.023
rs3736062	FLT4	0.046	1.547 (0.988 – 2.374)	0.024	33/74 (26/6)	1	synonymous variant, Y531Y	0.023
rs7830	NOS3	0.049	0.877 (0.769 – 1.002)	0.434	1635/525 (411/172)	0.346	intron variant (+11)	0.817
rs61763183	FLT1	0.050	1.653 (0.967 – 2.754)	0.020	24/50 (20/7)	1	downstream gene variant (+73)	0.463

[*] Non-adjusted p-values.

[†] LoF tool - loss of function tool

MAF-minor allele frequency

HWE-Hardy-Weinberg Equilibrium