Association of polymorphisms in the erythropoietin gene with diabetic retinopathy: a case-control study and systematic review with meta-analysis

Luís Fernando Castagnino Sesti
Universidade Luterana do Brasil (ULBRA)
Renan Cesar Sbruzzi
Universidade Luterana do Brasil (ULBRA)
Evelise Regina Polina
Universidade Luterana do Brasil (ULBRA)
Douglas dos Santos Soares
Hospital de Clínicas de Porto Alegre (HCPA)
Daisy Crispim
Hospital de Clínicas de Porto Alegre (HCPA)
Luís Henrique Canani
Universidade Federal do Rio Grande do Sul (UFRGS)
Kátia Gonçalves dos Santos (katia.santos@ulbra.br)
Universidade Luterana do Brasil (ULBRA)

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Abstract

Background: Diabetic retinopathy (DR) is characterized by ischemia, hypoxia, and angiogenesis. Erythropoietin (EPO), an angiogenic hormone, is upregulated in DR, and the association of *EPO* genetic variants with DR is still uncertain, as conflicting results have been reported. Therefore, we performed a case-control study followed by a meta-analysis to investigate whether the rs1617640, rs507392, and rs551238 polymorphisms in *EPO* gene are associated with DR.

Methods: The case-control study included 1042 Southern Brazilians with type 2 diabetes (488 without DR and 554 with DR). Eligible studies for the meta-analysis were searched from electronic databases up to June 1, 2021. Pooled odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for five genetic inheritance models.

Results: The minor alleles of the *EPO* polymorphisms had nearly the same frequency in all groups of patients (35%), and no association was detected with DR in the case-control study. The meta-analysis included 14 independent sets of cases and controls with 9117 subjects for the rs1617640 polymorphism and nine independent sets with more than 5000 subjects for the rs507392 and rs551238 polymorphisms. The G allele of the rs1617640 polymorphism was associated with DR under the dominant (OR = 0.82, 95% CI: 0.68-0.98), heterozygous additive (OR = 0.82, 95% CI: 0.69-0.97) and overdominant (OR = 0.88, 95% CI: 0.79-0.97) models. In the subgroup analyses, the G allele was also associated with DR among patients with type 1 diabetes or non-Asian ancestry. Regarding the rs507392 and rs551238 polymorphisms, no association was found between these variants and DR.

Conclusion: Our findings provide additional support to *EPO* as a susceptibility gene for DR, with the rs1617640 polymorphism deserving further investigation.

Background

Diabetic retinopathy (DR) is a chronic complication of diabetes mellitus and the leading cause of blindness in working-age adults. Clinically, it is classified as non-proliferative (NPDR) and proliferative (PDR). Non-proliferative DR is characterized by microaneurysms, exudates, venous beading, and intraretinal microvascular abnormalities, whereas PDR is characterized by neovascularization, which can result in intraocular bleeding, vision loss, and retinal detachment [1]. Chronic hyperglycemia augments the activation of biochemical pathways that promote the production of inflammatory cytokines, reactive oxygen species, and vasoactive substances. Collectively, these changes disrupt the neurovascular structures and alter normal retinal function by leading to the blood–retinal barrier breakdown, pericyte loss, neuronal death, and angiogenesis [2, 3].

Erythropoietin (EPO) is a pleiotropic hormone produced mainly by the adult kidney in response to hypoxia or anemia to increase the production of red blood cells [4, 5]. Erythropoietin and its receptors are also expressed in several other tissues in response to tissue injury [5, 6], including the retinal pigment epithelium, outer and inner nuclear layers, and ganglion cell layer of the retina [4], where they exert cytoprotective effects. Experimental studies have shown that EPO has antiapoptotic, anti-inflammatory, antioxidant, and angiogenic properties [4–6], thereby protecting against retinal damage by reducing the pericyte loss, formation of acellular capillaries, and degeneration of neuroretinal layers, amongst several other features of early DR. Despite the beneficial effects of EPO administration reported in small human clinical trials and several experimental models of ocular diseases [4, 5], patients with PDR have high levels of EPO in the vitreous fluid, aqueous humor [5], postmortem retinal tissue [7], plasma [7, 8], and serum [9].

The human *EPO* gene is located on chromosome 7q22.1, contains five exons and encodes a precursor protein of 193 amino acids (https://www.ncbi.nlm.nih.gov/gene/2056). The rs1617640 (G > T), rs507392 (C > T), and rs551238 (C > A) polymorphisms in the *EPO* gene were first investigated regarding their potential association with PDR and end-stage renal disease (ESRD) in European-Americans. In that study, the T allele of the rs1617640 polymorphism was associated with PDR and ESRD in three different cohorts and had a functional role in *EPO* expression [10]. Since then, the relationship between these genetic variants and DR has been evaluated in other populations [11–20], with half of the studies reporting positive associations with either allele [11, 16, 17, 19, 20] and the other half reporting no association [12–15, 18].

Here, we aimed to investigate whether the rs1617640, rs507392, and rs551238 polymorphisms in the *EPO* gene are associated with DR. To address this question, we performed a case-control study in Southern Brazilians with type 2 diabetes mellitus (T2DM) and conducted a systematic review followed by a meta-analysis of previous studies and ours.

Methods

This study was reported in accordance with the STrengthening the REporting of Genetic Association Studies (STREGA) [21] and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [22] statements.

Case-control study

Study population and clinical data collection. Our case-control study was carried out on a total of 1042 adult T2DM patients from Rio Grande do Sul, the southernmost Brazilian state. Most patients (n = 740) were enrolled between 1999 and 2010 as part of a multicenter study that aimed to investigate risk factors for chronic complications of diabetes. It mainly included the endocrinology outpatient clinics and the dialysis centers of four public tertiary care hospitals in the cities of Porto Alegre (Hospital de Clínicas de Porto Alegre – HCPA and Hospital Nossa Senhora da Conceição), Passo Fundo (Hospital São Vicente de Paulo) and Rio Grande (Hospital Universitário de Rio Grande). The remaining patients (n = 302) were enrolled between 2015 and 2017 in the endocrinology and the ophthalmology outpatient clinics of HCPA. This study was approved by the Human Research Ethics Committee of Universidade Luterana do Brasil – ULBRA (CAAE number: 55236216.2.0000.5349; consolidated review number: 1.553.469) and all patients gave their written informed consent prior to the data and blood collection.

Type 2 diabetes was diagnosed according to the criteria of the American Diabetes Association [23], and the inclusion criteria of this study were as follows: age at the diagnosis of diabetes ≥ 30 years, no need for daily insulin treatment within the first year of diagnosis, and no previous episodes of ketoacidosis. Patients underwent a complete clinical evaluation consisting of physical examination and routine biochemical exams, including the measurement of glycated hemoglobin (HbA1c), creatinine and lipid levels, which were determined according to standard methods as described elsewhere [24]. Glomerular filtration rate (eGFR) was estimated using the CKD-EPI equation [25] and diabetic kidney disease was defined according to the KDIGO 2012 classification, as previously described [26]. A structured questionnaire was used to collect demographic data and information regarding the clinical history, such as the age at the diagnosis of diabetes, history of cigarette smoking, and presence of comorbidities, which were obtained directly by interview with the patient or from medical records. Skin color/ethnicity was self-reported and dichotomized as white or non-white (pardo or black).

Diagnosis of DR was based on either ophthalmoscopy (for patients included in the study until 2010) or retinography (for patients included between 2015 and 2017) after mydriasis by staff retinal ophthalmologists in each institution. All eye examinations were performed before DNA isolation and genotyping procedures, and patients who had any eye condition that impaired the funduscopic examination, such as severe cataract, were excluded from the study. Retinopathy was defined according to the worst affected eye and was classified as absent (no fundus abnormalities), NPDR (microaneurysms, intraretinal hemorrhages, and/or venous beading and intraretinal microvascular abnormalities), or PDR (neovascularization and/or vitreous/preretinal hemorrhage) [27]. Patients with a prior history of panretinal photocoagulation were also included in the PDR group. Overall, of the 1042 T2DM patients included in this case-control study, 488 patients did not have DR, 317 had NPDR, and 237 had PDR.

DNA isolation and genotyping. Genomic DNA was isolated from peripheral white blood cells using a standard salting out procedure [28]. Genotyping of *EPO* polymorphisms was performed by real-time polymerase chain reaction (PCR) using specific primers and hydrolysis probes contained in validated commercial assays for allelic discrimination (TaqMan® Genotyping Assay, ID numbers C__8786860_10, C__27168915_10, and C__2868037_10 for rs1617640, rs507392, and rs551238 polymorphisms, respectively; Thermo Fisher Scientific, Waltham, USA). Amplification reactions were carried out in a total volume of 8 μL containing 2 μL genomic DNA (10 ng/μL), 4 μL TaqMan Genotyping Master Mix (2 X) (Thermo Fisher Scientific), and 0.4 μL genotyping assay (20 X). Plates were loaded into a real-time PCR thermal cycler (StepOnePlus Real-Time PCR System; Thermo Fisher Scientific) and heated under the standard conditions recommended by the manufacturer. The genotyping was done in the Laboratory of Human Molecular Genetics at ULBRA.

A sample of each genotype was used in all PCR runs as a positive control; the investigators who performed the genotyping were blinded to the patients' DR status (L.F.C.S. and R.C.S.) and the genotypes were read independently by two investigators (L.F.C.S. and E.R.P.). Genotyping success rate ranged from 97.8% (rs507392) to 99.1% (rs1617640), and 15% of the samples that were successfully genotyped for all three polymorphisms (152 out of 1010) were randomly selected to be re-genotyped to assess accuracy. One sample was discordant for the three polymorphisms, while another sample was discordant for only the rs551238 polymorphism. The discordant results were confirmed in a further PCR. The genotyping data generated in this study are available in a public repository (https://doi.org/10.6084/m9.figshare.16417161).

Statistical analysis. Continuous data are shown as the mean ± standard deviation or median (25th–75th percentiles), while categorical data are shown as absolute frequency (percentage), percentage, or relative frequency. After checking for the normal distribution using the Shapiro–Wilk test, continuous data were compared between groups by the Kruskal–Wallis test followed by the Dunn post hoc test for multiple comparisons where appropriate. Categorical data, including the genotype and allele frequencies, were compared between groups

using the chi-square test followed by Bonferroni correction for multiple pairwise comparisons, if appropriate. Allele frequencies were determined by gene counting, and deviations from Hardy–Weinberg equilibrium (HWE) were also verified by the chi-square test. Statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, USA) and WinPEPI version 11.50 [29] statistical software. Haplotype frequencies were estimated by a Bayesian method and compared between groups by a random permutation test (1000 replicates) implemented in the PHASE software version 2.1 [30, 31]. Linkage disequilibrium (LD) between the *EPO* polymorphisms was estimated and expressed in terms of D' and r^2 [32]. P values < 0.05 were considered statistically significant.

Sample size estimates were performed using the WinPEPI program. Calculations were based on the association effect sizes previously reported for *EPO* polymorphisms and DR [10, 11, 16, 17], considering a significance level of 5% and global frequencies of 0.33 (rs1617640 and rs507392) and 0.34 (rs551238) for the minor alleles, as described in the 1000 Genomes Project (https://www.ncbi.nlm.nih.gov/snp/). These estimates indicated that 1118 patients with T2DM (559 cases and 559 controls) would be needed to detect an odds ratio (OR) of as low as 1.30 (95% confidence interval [CI]): 1.02–1.65) for the association between the G allele of the rs1617640 polymorphism and DR, under the dominant model, as found in Chinese T2DM patients [17].

Systematic review and meta-analysis

Search strategy and eligibility criteria. PubMed and Virtual Health Library (Biblioteca Virtual en Salud – BVS) databases were last searched on June 1, 2021, to retrieve all studies that investigated the association of at least one *EPO* gene polymorphism with DR (PDR, NPDR, or both combined), with no restriction on language. The following search terms were used: diabetes AND retinopathy AND (erythropoietin OR EPO) AND (polymorphism OR polymorphisms OR SNP OR SNPs OR variant OR variants OR mutation OR mutations OR rs1617640 OR rs507392 OR rs551238). In addition, we searched abstracts presented from 2018 to 2020 at scientific meetings of the following societies of diabetes, endocrinology, and ophthalmology: American Academy of Ophthalmology (AAO), American Diabetes Association, Endocrine Society (ENDO), European Association for the Study of Diabetes (EASD), International Society for Eye Research (ISER), and the Association for Research in Vision and Ophthalmology (ARVO).

Reference lists of the retrieved papers were also searched to identify additional studies. Studies presented in the form of a thesis or published in predatory journals were not included in our meta-analysis. Titles and abstracts of the retrieved articles were screened for eligibility, and then original studies on human subjects were reviewed in full. Authors of the papers that did not report the genotype and/or allele frequencies were contacted by e-mail. In case of no reply, the study was not included in the meta-analysis.

Data extraction and methodological quality assessment. Data from eligible studies were extracted independently by two of the authors (D.S.S. and K.G.S.) and disagreements were resolved by discussion and consensus. The following data were extracted and entered in an electronic spreadsheet: (i) characteristics of the study setting (name of the first author, year of publication, design type, period of inclusion, total sample size, number of cases and controls, degrees of DR, and diagnosis method of DR); (ii) characteristics of the study population (country and region, ethnicity, age, gender, type of diabetes, duration of diabetes, and presence of other diabetic complications); (iii) information regarding the polymorphisms (genotyping method, HWE in the control group, minor allele, and allele and genotype frequencies in cases and controls). Where available, haplotype frequencies and genotype/allele frequencies reported separately by the degree of DR were also registered for further analysis.

Genotype and allele data were extracted and carefully checked for accuracy. In the cases that genotype frequencies could not be clearly deduced from the paper or seemed to be incomplete, incorrect, or unclear, at least one of the authors was contacted regarding the data. Incomplete, incorrect, or unclear information regarding the DR definition was also checked with the authors of the eligible studies by e-mail. The methodological quality of each study included in our meta-analysis was assessed independently by two of the authors (D.S.S. and K.G.S.) using the Newcastle–Ottawa scoring Scale (NOS) for case–control studies [33].

Quantitative synthesis. The association between *EPO* polymorphisms and DR was evaluated by estimating pooled ORs and corresponding 95% CIs, using the minor allele for the rs1617640 (G), rs507392 (C), and rs551238 (C) polymorphisms as the exposure factor in the following five genetic inheritance models: (i) dominant (GG + TG vs. TT, CC + TC vs. TT, and CC + AC vs. AA); (ii) recessive (GG vs. TG + TT, CC vs. TC + TT, and CC vs. AC + AA); (iii) homozygous (GG vs. TT, CC vs. TT, and CC vs. AA) and heterozygous additive (TG vs. TT, TC vs. TT, and AC vs. AA); (iv) overdominant (TG vs. GG + TT, TC vs. CC + TT, and AC vs. CC + AA); and, (v) allele contrast (G vs. T, C vs. T, and C vs. A). The overdominant model is also referred to by some authors as codominant [34, 35]. Meta-analysis of haplotypes was also performed by comparing the GCC haplotype against the TTA haplotype.

Genotype frequencies were tested for HWE using the goodness-of-fit chi-square test and the overall analyses were repeated by excluding the studies in which the genotype frequencies deviated from HWE in the control group, as recommended elsewhere [34–36]. In addition, subgroup analyses were performed stratifying for the degree of DR (NPDR or PDR vs. no DR), type of diabetes (type 1 diabetes mellitus

[T1DM] or T2DM), and ethnicity (Asian or non-Asian), including only the studies that met HWE. Some of the studies included in the meta-analysis enrolled two or more independent sets of cases and controls [10, 11, present study]; therefore, these groups were analyzed as separate populations.

Heterogeneity among studies was evaluated using the I^2, τ^2, and Q metrics, and all the individual and pooled ORs were estimated using both fixed- and random-effects models. In the presence of moderate/high heterogeneity, as defined by $I^2 \geq 50\%$ and $P < 0.10$ in the Q-test, the random-effects model was considered more suitable than the fixed-effects model for interpretating our meta-analysis. Otherwise, the fixed-effects model was considered as the appropriate model [34, 35]. Following the recommendations of Sterne et al. [37], small-study effects were examined by visual inspection of funnel plots and formal statistical testing for the rs1617640 polymorphism, as this was the only polymorphism for which at least 10 studies were included in the meta-analysis. Rücker's test, based on arcsine transformation of the effect measure, was used to test for funnel plot asymmetry because it is indicated for meta-analyses with binary outcomes and performs reasonably well in the presence of substantial between-study heterogeneity, as defined by $\tau^2 > 0.1$ [37, 38]. Statistical analyses were performed using the 'meta' package version 4.14-0 [39] in R version 4.0.2 [40].

Results

Case-control study

Characteristics of study population. Subjects with T2DM included in our case-control study were predominantly elderly (60.3 ± 9.5 years), female (53.3%), and white (89.0%). Subjects with DR were more often male and daily insulin users, had a longer duration of diabetes, and lower body mass index than those without this complication. In addition, patients with PDR were older and had worse renal function than those without DR (Supplementary Table 1).

Relationship between EPO polymorphisms and DR. Genotype frequencies were in agreement with those predicted by the Hardy–Weinberg equation for the three *EPO* polymorphisms in all T2DM groups. As the genotype and allele frequencies did not differ according to the period of inclusion in the study (Supplementary Table 2) and were quite similar in white and non-white subjects (Supplementary Table 3), all T2DM patients were analyzed together in relation to DR. As shown in Table 1, the genotype frequencies were similar between subjects with PDR, NPDR, and without DR, and the minor alleles had a frequency of approximately 0.35 in these three groups.

Table 1
Genotype and allele frequencies of *EPO* polymorphisms in Brazilians with type 2 diabetes

SNPs	Genotypes and alleles	All subjects	Without DR	NPDR	PDR	*P*
	Genotype	*n* = 1033	*n* = 483	*n* = 316	*n* = 234	
	TT	438 (42.4)	208 (43.1)	138 (43.7)	92 (39.3)	0.519
	TG	480 (46.5)	221 (45.7)	149 (47.1)	110 (47.0)	
rs1617640	GG	115 (11.1)	54 (11.2)	29 (9.2)	32 (13.7)	
	Allele					
	T	0.66	0.66	0.67	0.63	0.299
	G	0.34	0.34	0.33	0.37	
	Genotype	*n* = 1019	*n* = 473	*n* = 314	*n* = 232	
	TT	426 (41.8)	198 (41.9)	137 (43.6)	91 (39.2)	0.538
	TC	477 (46.8)	220 (46.5)	148 (47.2)	109 (47.0)	
rs507392	CC	116 (11.4)	55 (11.6)	29 (9.2)	32 (13.8)	
	Allele					
	T	0.65	0.65	0.67	0.63	0.306
	C	0.35	0.35	0.33	0.37	
	Genotype	*n* = 1028	*n* = 481	*n* = 315	*n* = 232	
	AA	427 (41.5)	200 (41.6)	138 (43.8)	89 (38.4)	0.628
	AC	477 (46.4)	223 (46.3)	145 (46.0)	110 (47.4)	
rs551238	CC	124 (12.1)	58 (12.1)	32 (10.2)	33 (14.2)	
	Allele					
	A	0.65	0.65	0.67	0.62	0.266
	C	0.35	0.35	0.33	0.38	

Data are shown as absolute frequency (and percentage) or relative frequency. SNPs: single nucleotide polymorphisms, DR: diabetic retinopathy, NPDR: non-proliferative DR, PDR: proliferative DR

Among the 1010 T2DM patients successfully genotyped for the three *EPO* polymorphisms, 413 (41%) were homozygous for the major alleles, 454 (45%) were triple heterozygotes, and 110 (11%) were homozygous for the minor alleles, while the remaining 33 patients (3%) had other genotype combinations. In fact, the three polymorphisms were in strong LD (D' = 0.96 and r^2 = 0.90, for rs1617640 vs. rs507392; D' = 0.95 and r^2 = 0.88, for rs507392 vs. rs551238; and D' = 0.98 and r^2 = 0.93, for rs1617640 vs. rs551238). Two haplotypes accounted for > 97% of the chromosomes in our population, whereas the other six possible haplotypes had estimated individual frequencies varying from 0.01–1.1%. Haplotype frequencies were not significantly different between patients with PDR, NPDR, and without DR (Supplementary Table 4).

Meta-analysis

Study characteristics. Nineteen non-duplicate articles were initially retrieved from PubMed and Virtual Health Library (BVS) databases, and another five studies were identified by checking the reference lists of the retrieved articles (Fig. 1). No studies were identified from the abstracts of the scientific meetings. After reviewing the titles and abstracts, 13 studies were excluded because they did not evaluate the association of *EPO* polymorphisms with DR, they did not report original data (reviews and meta-analysis), the full-text was not available, or they had not been published in peer-reviewed journals. Among the 11 full-texts reviewed, two of them were excluded because they did not report the genotype and/or the allele frequencies and the contacted author did not reply to our e-mail asking for these data [13, 15]. In addition to our case-control study, nine articles fulfilled the eligibility criteria and were included in the meta-analysis, giving 14 independent sets in total (Fig. 1) with 9117 subjects analyzed for the rs1617640 polymorphism [10–12, 14, 16–20]. Nine independent sets from seven

studies, with more than 5000 subjects, were analyzed for the rs507392 and rs551238 polymorphisms [11, 14, 16–19, present study] (Table 2).

Table 2
Characteristics of the studies included in the meta-analysis

First author and reference	Year	Country	Type of diabetes	*n* (cases/controls)	Cases		Controls		HWE
					Genotypes	Alleles	Genotypes	Alleles	
rs1617640				9117 (4462/4655)	TT/TG/GG	T/G	TT/TG/GG	T/G	
Tong (1) [10]	2008	USA	T2DM	613 (374/239)	150/172/52	472/276	66/127/46	259/219	Yes
Tong (2) [10]	2008	USA	T1DM	1439 (865/574)	335/419/111	1089/641	148/307/119	603/545	Yes
Tong (3) [10]	2008	USA	T1DM	520 (379/141)	139/180/60	458/300	35/78/28	148/134	Yes
Abhary (1) [11]	2010	Australia	T2DM	333 (170/163)	65/78/27	208/132	64/88/11	216/110	No
Abhary (2) [11]	2010	Australia	T1DM	167 (102/65)	40/44/18	124/80	24/30/11	78/52	Yes
Balasubbu [12]	2010	India	T2DM	702 (344/358)	31/163/150	225/463	30/171/157	231/485	Yes
Yang [14]	2014	China	T2DM	491 (211/280)	146/55/10	347/75	182/82/16	446/114	Yes
Song [16]	2015	China	T2DM	782 (444/338)	293/138/13	724/164	225/98/15	548/128	Yes
Fan [17]	2016	China	T2DM	1193 (397/796)	208/161/28	577/217	468/302/26	1238/354	No
Montesanto [18]	2018	Italy	T2DM	433 (107/326)	51/42/14	144/70	140/145/41	425/227	Yes
Kaur [19]	2021	India	T2DM	614 (302/312)	125/129/48	379/225	122/116/74	360/264	No
Mankoc Ramus [20]	2021	Slovenia	T2DM	797 (217/580)	70/96/51	236/198	180/305/95	665/495	Yes
Sesti (1)	Present study	Brazil	T2DM	731 (422/309)	183/194/45	560/284	132/147/30	411/207	Yes
Sesti (2)	Present study	Brazil	T2DM	302 (128/174)	47/65/16	159/97	76/74/24	226/122	Yes
rs507392				5023 (2281/2742)	TT/TC/CC	T/C	TT/TC/CC	T/C	
Abhary (1) [11]	2010	Australia	T2DM	332 (170/162)	65/78/27	208/132	63/88/11	214/110	No
Abhary (2) [11]	2010	Australia	T1DM	167 (102/65)	40/44/18	124/80	24/30/11	78/52	Yes
Yang [14]	2014	China	T2DM	496 (216/280)	141/65/10	347/85	181/81/18	443/117	No
Song [16]	2015	China	T2DM	782 (444/338)	281/149/14	711/177	217/97/24	531/145	No
Fan [17]	2016	China	T2DM	1193 (397/796)	202/161/34	565/229	463/305/28	1231/361	No
Montesanto [18]	2018	Italy	T2DM	420 (104/316)	48/43/13	139/69	130/146/40	406/226	Yes
Kaur [19]	2021	India	T2DM	614 (302/312)	138/124/40	400/204	132/106/74	370/254	No
Sesti (1)	Present study	Brazil	T2DM	722 (418/304)	181/192/45	554/282	126/148/30	400/208	Yes
Sesti (2)	Present study	Brazil	T2DM	297 (128/169)	47/65/16	159/97	72/72/25	216/122	Yes

SNP: single nucleotide polymorphism, T2DM: type 2 diabetes mellitus, T1DM: type 1 diabetes mellitus, HWE: Hardy–Weinberg equilibrium

First author and reference	Year	Country	Type of diabetes	*n* (cases/controls)	Cases		Controls		HWE
					Genotypes	Alleles	Genotypes	Alleles	
rs551238				5031 (2279/2752)	AA/AC/CC	A/C	AA/AC/CC	A/C	
Abhary (1) [11]	2010	Australia	T2DM	333 (170/163)	65/78/27	208/132	64/88/11	216/110	No
Abhary (2) [11]	2010	Australia	T1DM	167 (102/65)	40/44/18	124/80	24/30/11	78/52	Yes
Yang [14]	2014	China	T2DM	494 (216/278)	141/65/10	347/85	182/79/17	443/113	No
Song [16]	2015	China	T2DM	774 (439/335)	286/140/13	712/166	219/92/24	530/140	No
Fan [17]	2016	China	T2DM	1193 (397/796)	203/156/38	562/232	452/299/45	1203/389	Yes
Montesanto [18]	2018	Italy	T2DM	428 (106/322)	51/42/13	144/68	138/143/41	419/225	Yes
Kaur [19]	2021	India	T2DM	614 (302/312)	130/125/47	385/219	123/117/72	363/261	No
Sesti (1)	Present study	Brazil	T2DM	731 (419/312)	181/188/50	550/288	129/150/33	408/216	Yes
Sesti (2)	Present study	Brazil	T2DM	297 (128/169)	46/66/16	158/98	71/73/25	215/123	Yes

SNP: single nucleotide polymorphism, T2DM: type 2 diabetes mellitus, T1DM: type 1 diabetes mellitus, HWE: Hardy–Weinberg equilibrium

Studies included in our meta-analysis were case-control or cross-sectional studies, and most of them enrolled T2DM patients. Most independent sets of cases and controls were composed predominantly of white subjects of European ancestry [10, 11, 18, 20, present study], while five studies were carried out on Indian [12, 19] and Chinese [14, 16, 17] populations (Table 2). According to NOS, all the previous studies included in the meta-analysis were of good quality, with the total scores ranging from 7 to 9 (Supplementary Table 5).

Quantitative pooled analyses of rs1617640 polymorphism. Pooled estimates for the overall association between the rs1617640 polymorphism and DR revealed a moderate to high between-study heterogeneity (I^2 = 57–76%) in almost all genetic models including all the 14 independent subject sets (Supplementary Table 6). We then sought to identify its source by excluding set #2 of Tong et al. [10], which had the highest weight in the analyses (18–27%), and the three sets with controls deviating from HWE [set #1 of 11, 17, 19]. Exclusion of these studies from the meta-analysis markedly reduced the heterogeneity (I^2 = 12–39%), while most of the pooled estimates were similar to those estimated without removing any study. Although the funnel plots were asymmetric with set #2 by Tong et al. [10] and some others lying outside the boundary line, this asymmetry was not confirmed by statistical analysis (Supplementary Table 6). The forest and funnel plots are provided in Supplementary Figs. 1–48.

Table 3 shows the overall and subgroup analyses after removing the three subject sets in which the genotype frequencies were not in agreement with HWE. The G allele was associated with a reduced risk of DR under the dominant, heterozygous additive, and overdominant genetic models. Following the standard recommendations for meta-analysis of genetic association studies, we also stratified the analyses by the degree of DR, type of diabetes, and ethnicity including only the studies whose genotype frequencies met the HWE in the control group. Again, the G allele was associated with both PDR and NPDR, under the overdominant and recessive models, respectively, and with DR among patients with T1DM under all the genetic models. Regarding the subjects of non-Asian ancestry, the G allele was associated with the reduced risk of DR under the dominant, heterozygous additive, and overdominant models. On the other hand, no association between the rs1617640 polymorphism and DR was detected in patients with T2DM or Asian ancestry (Table 3). The forest plots are shown in Supplementary Figs. 49–84.

Table 3
Pooled estimates for the association between the *EPO* rs1617640 polymorphism and DR

Subgroup and genetic model	*n* (cases/controls)	Heterogeneity		Pooled OR (95% CI)	
		I^2 (%)	P*	Fixed Model	Random Model
Overall (*n* = 11)					
Dominant	6977 (3593/3384)	62	0.003	0.78 (0.70–0.87)	0.82 (0.68–0.98)
Recessive	6977 (3593/3384)	55	0.013	0.86 (0.75–0.99)	0.88 (0.71–1.10)
Homozygous additive	3845 (2025/1820)	65	0.002	0.72 (0.61–0.84)	0.78 (0.58–1.04)
Heterozygous additive	5855 (3053/2802)	50	0.030	0.80 (0.71–0.89)	0.82 (0.69–0.97)
Overdominant	6977 (3593/3384)	7	0.375	0.88 (0.79–0.97)	0.88 (0.79–0.97)
Allele contrast	13954 (7186/6768)	67	< 0.001	0.85 (0.79–0.92)	0.88 (0.77–1.01)
PDR (*n* = 8)					
Dominant	4843 (2130/2713)	74	< 0.001	0.74 (0.65–0.84)	0.79 (0.60–1.04)
Recessive	4843 (2130/2713)	65	0.006	0.91 (0.78–1.06)	0.90 (0.68–1.21)
Homozygous additive	2592 (1186/1406)	75	< 0.001	0.73 (0.60–0.88)	0.76 (0.50–1.15)
Heterozygous additive	3929 (1730/2199)	66	0.004	0.74 (0.64–0.85)	0.78 (0.61–1.01)
Overdominant	4843 (2130/2713)	38	0.127	0.82 (0.73–0.93)	0.84 (0.72–0.98)
Allele contrast	9686 (4260/5426)	77	< 0.001	0.85 (0.78–0.93)	0.88 (0.73–1.06)
NPDR (*n* = 4)					
Dominant	2438 (1043/1395)	72	0.014	0.84 (0.71–0.99)	0.90 (0.65–1.26)
Recessive	2438 (1043/1395)	25	0.260	0.63 (0.49–0.82)	0.68 (0.48–0.95)
Homozygous additive	1329 (560/769)	60	0.055	0.57 (0.43–0.76)	0.69 (0.41–1.15)
Heterozygous additive	2152 (945/1207)	56	0.076	0.89 (0.75–1.07)	0.94 (0.71–1.25)
Overdominant	2438 (1043/1395)	0	0.633	1.02 (0.87–1.20)	1.02 (0.87–1.20)
Allele contrast	4876 (2086/2790)	73	0.010	0.82 (0.73–0.93)	0.90 (0.69–1.16)
T2DM (*n* = 8)					
Dominant	4851 (2247/2604)	35	0.152	0.90 (0.79–1.02)	0.90 (0.76–1.06)
Recessive	4851 (2247/2604)	31	0.180	1.00 (0.85–1.18)	0.99 (0.79–1.22)
Homozygous additive	2777 (1322/1455)	35	0.151	0.91 (0.74–1.12)	0.90 (0.69–1.17)
Heterozygous additive	4076 (1896/2180)	32	0.174	0.89 (0.78–1.02)	0.89 (0.75–1.06)
Overdominant	4851 (2247/2604)	23	0.243	0.91 (0.81–1.03)	0.91 (0.79–1.05)
Allele contrast	9702 (4494/5208)	38	0.124	0.95 (0.87–1.04)	0.95 (0.84–1.07)
T1DM (*n* = 3)					
Dominant	2126 (1346/780)	4	0.353	0.58 (0.48–0.70)	0.58 (0.47–0.71)
Recessive	2126 (1346/780)	24	0.269	0.63 (0.50–0.80)	0.66 (0.49–0.90)
Homozygous additive	1068 (703/365)	41	0.183	0.47 (0.36–0.62)	0.52 (0.34–0.78)
Heterozygous additive	1779 (1157/622)	0	0.569	0.62 (0.50–0.76)	0.62 (0.50–0.76)

n = number of independent sets of cases and controls. *Computed by Q-test. Statistically significant association estimates are shown in bold, considering the most appropriate model for each analysis (fixed- or random-effects). OR: odds ratio, 95% CI: 95% confidence interval, PDR: proliferative DR, NPDR: non-proliferative DR

Subgroup and genetic model	*n* (cases/controls)	Heterogeneity		Pooled OR (95% CI)	
		I^2 (%)	P*	Fixed Model	Random Model
Overdominant	2126 (1346/780)	0	0.841	0.80 (0.67–0.96)	0.80 (0.67–0.96)
Allele contrast	4252 (2692/1560)	30	0.241	0.69 (0.61–0.78)	0.71 (0.59–0.84)
Non-Asian (*n* = 8)					
Dominant	5002 (2594/2408)	68	0.003	0.74 (0.65–0.83)	0.78 (0.62–0.99)
Recessive	5002 (2594/2408)	67	0.004	0.84 (0.71–0.98)	0.90 (0.67–1.21)
Homozygous additive	2577 (1382/1195)	75	< 0.001	0.70 (0.58–0.84)	0.77 (0.53–1.13)
Heterozygous additive	4241 (2227/2014)	53	0.037	0.75 (0.65–0.85)	0.77 (0.63–0.95)
Overdominant	5002 (2594/2408)	4	0.399	0.83 (0.74–0.94)	0.83 (0.74–0.94)
Allele contrast	10004 (5188/4816)	74	< 0.001	0.82 (0.76–0.90)	0.87 (0.73–1.04)
Asian (*n* = 3)					
Dominant	1975 (999/976)	0	0.681	0.94 (0.76–1.17)	0.94 (0.76–1.17)
Recessive	1975 (999/976)	0	0.571	0.92 (0.71–1.20)	0.92 (0.71–1.20)
Homozygous additive	1268 (643/625)	0	0.785	0.82 (0.55–1.21)	0.82 (0.55–1.21)
Heterozygous additive	1614 (826/788)	0	0.602	0.97 (0.78–1.22)	0.97 (0.78–1.22)
Overdominant	1975 (999/976)	0	0.599	1.00 (0.82–1.20)	1.00 (0.82–1.20)
Allele contrast	3950 (1998/1952)	0	0.743	0.95 (0.81–1.10)	0.95 (0.81–1.10)

n = number of independent sets of cases and controls. *Computed by Q-test. Statistically significant association estimates are shown in bold, considering the most appropriate model for each analysis (fixed- or random-effects). OR: odds ratio, 95% CI: 95% confidence interval, PDR: proliferative DR, NPDR: non-proliferative DR

Quantitative pooled analyses of rs507392 and rs551238 polymorphisms. In relation to the rs507392 polymorphism, five of the nine subject sets initially eligible for the meta-analysis did not meet the HWE [set #1 of 11, 14, 16, 17, 19]. Removing these studies from the meta-analysis eliminated the between-study heterogeneity, while the association estimates remained statistically non-significant. With fewer data available, subgroup analyses including only the studies that met the HWE were restricted to T2DM patients (who were all of non-Asian ancestry), and no association was observed in this group (Table 4 and Supplementary Figs. 85–102).

Table 4
Pooled estimates for the association between the *EPO* rs507392 polymorphism and DR

Subgroup and genetic model	*n* (cases/controls)	Heterogeneity		Pooled OR (95% CI)	
		I^2 (%)	P*	Fixed Model	Random Model
Overall (*n* = 9)					
Dominant	5023 (2281/2742)	2	0.414	1.04 (0.93–1.17)	1.04 (0.92–1.17)
Recessive	5023 (2281/2742)	79	< 0.001	0.95 (0.79–1.16)	0.99 (0.63–1.54)
Homozygous additive	3029 (1360/1669)	76	< 0.001	0.97 (0.79–1.19)	1.00 (0.64–1.55)
Heterozygous additive	4545 (2064/2481)	0	0.622	1.06 (0.94–1.20)	1.06 (0.94–1.20)
Overdominant	5023 (2281/2742)	26	0.216	1.06 (0.94–1.19)	1.05 (0.92–1.21)
Allele contrast	10046 (4562/5484)	60	0.010	1.01 (0.93–1.11)	1.00 (0.86–1.16)
Only in HWE (*n* = 4)					
Dominant	1606 (752/854)	0	0.568	0.96 (0.78–1.18)	0.96 (0.78–1.18)
Recessive	1606 (752/854)	0	0.921	1.00 (0.73–1.37)	1.00 (0.73–1.37)
Homozygous additive	866 (408/458)	0	0.986	0.98 (0.70–1.37)	0.98 (0.70–1.38)
Heterozygous additive	1408 (660/748)	0	0.414	0.95 (0.77–1.19)	0.95 (0.77–1.19)
Overdominant	1606 (752/854)	7	0.360	0.96 (0.78–1.18)	0.96 (0.77–1.19)
Allele contrast	3212 (1504/1708)	0	0.888	0.98 (0.84–1.14)	0.98 (0.84–1.14)
T2DM (*n* = 3)					
Dominant	1439 (650/789)	0	0.369	0.96 (0.77–1.20)	0.96 (0.77–1.20)
Recessive	1439 (650/789)	0	0.789	0.99 (0.71–1.39)	0.99 (0.71–1.40)
Homozygous additive	773 (350/423)	0	0.929	0.98 (0.69–1.41)	0.98 (0.69–1.41)
Heterozygous additive	1270 (576/694)	29	0.247	0.96 (0.76–1.21)	0.97 (0.73–1.29)
Overdominant	1439 (650/789)	36	0.207	0.97 (0.78–1.20)	0.98 (0.74–1.31)
Allele contrast	2878 (1300/1578)	0	0.729	0.98 (0.83–1.15)	0.98 (0.83–1.15)

n = number of independent sets of cases and controls. *Computed by Q-test. OR: odds ratio, 95% CI: 95% confidence interval, HWE: Hardy–Weinberg equilibrium

Regarding the rs551238 polymorphism, the genotype frequencies in controls were not in agreement with HWE in four of the nine subject sets [set #1 of 11, 14, 16, 19]. The overall pooled analyses including or excluding these studies revealed no association between the rs551238 and DR in any genetic model, even among T2DM patients or non-Asians (Table 5 and Supplementary Figs. 103–126).

Table 5
Pooled estimates for the association between the *EPO* rs551238 polymorphism and DR

Subgroup and genetic model	*n* (cases/controls)	Heterogeneity		Pooled OR (95% CI)	
		I^2 (%)	P*	Fixed Model	Random Model
Overall (*n* = 9)					
Dominant	5031 (2279/2752)	0	0.595	1.02 (0.91–1.15)	1.02 (0.91–1.15)
Recessive	5031 (2279/2752)	69	0.001	0.98 (0.81–1.18)	0.98 (0.68–1.40)
Homozygous additive	3056 (1375/1681)	66	0.003	0.98 (0.80–1.20)	0.98 (0.68–1.40)
Heterozygous additive	4520 (2047/2473)	0	0.698	1.04 (0.92–1.18)	1.04 (0.92–1.18)
Overdominant	5031 (2279/2752)	9	0.362	1.04 (0.92–1.16)	1.03 (0.91–1.17)
Allele contrast	10062 (4558/5504)	43	0.084	1.01 (0.92–1.10)	1.00 (0.89–1.13)
Only in HWE (*n* = 5)					
Dominant	2816 (1152/1664)	20	0.288	1.07 (0.92–1.25)	1.06 (0.88–1.27)
Recessive	2816 (1152/1664)	13	0.329	1.20 (0.93–1.55)	1.20 (0.91–1.58)
Homozygous additive	1625 (656/969)	22	0.277	1.22 (0.94–1.60)	1.20 (0.88–1.64)
Heterozygous additive	2526 (1017/1509)	10	0.348	1.03 (0.88–1.22)	1.03 (0.86–1.23)
Overdominant	2816 (1152/1664)	0	0.405	1.00 (0.85–1.17)	1.00 (0.85–1.17)
Allele contrast	5632 (2304/3328)	24	0.260	1.08 (0.96–1.22)	1.07 (0.93–1.23)
T2DM (*n* = 4)					
Dominant	2649 (1050/1599)	36	0.194	1.08 (0.92–1.27)	1.07 (0.86–1.32)
Recessive	2649 (1050/1599)	33	0.214	1.22 (0.94–1.59)	1.20 (0.86–1.67)
Homozygous additive	1532 (598/934)	38	0.184	1.25 (0.94–1.65)	1.22 (0.84–1.75)
Heterozygous additive	2388 (933/1455)	29	0.238	1.04 (0.88–1.24)	1.04 (0.84–1.28)
Overdominant	2649 (1050/1599)	22	0.278	1.01 (0.86–1.19)	1.01 (0.83–1.22)
Allele contrast	5298 (2100/3198)	40	0.171	1.09 (0.97–1.24)	1.07 (0.91–1.27)
Non-Asian (*n* = 4)					
Dominant	1623 (755/868)	0	0.537	0.96 (0.78–1.18)	0.96 (0.78–1.18)
Recessive	1623 (755/868)	0	0.881	1.02 (0.75–1.38)	1.02 (0.75–1.38)
Homozygous additive	887 (415/472)	0	0.964	0.99 (0.72–1.38)	1.00 (0.72–1.38)
Heterozygous additive	1416 (658/758)	1	0.386	0.95 (0.76–1.18)	0.95 (0.76–1.18)
Overdominant	1623 (755/868)	13	0.329	0.95 (0.78–1.17)	0.96 (0.76–1.20)
Allele contrast	3246 (1510/1736)	0	0.857	0.98 (0.84–1.14)	0.98 (0.84–1.14)

n = number of independent sets of cases and controls. *Computed by Q-test. OR: odds ratio, 95% CI: 95% confidence interval, HWE: Hardy–Weinberg equilibrium

Quantitative pooled analyses of EPO haplotypes. The combined analysis, including seven sets of cases and controls, showed that the haplotype carrying the minor alleles (GCC) was not associated with DR in comparison to the haplotype carrying the major alleles (TTA), regardless of whether the genotype frequencies were in HWE in the control groups (Supplementary Figs. 127 and 128).

Discussion

In our case-control study, we did not find evidence of an association between *EPO* gene polymorphisms and DR in patients with T2DM from Southern Brazil. However, the meta-analysis showed that the G allele of the rs1617640 was associated with protection for both PDR and NPDR. In the subgroup analyses by type of diabetes and ancestry, the G allele was also associated with a decreased risk of DR (PDR + NPDR) among patients with T1DM and among those of non-Asian ancestry. No other associations were detected.

Regarding the rs1617640 polymorphism, the findings of our case-control study are in accordance with most of the previous individual studies, which reported no association between this genetic variant and DR in Indian [12], Chinese [14, 16], and Italian T2DM patients [18], as well as in five different cohorts of subjects of white European ancestry with T1DM [11, 13, 15]. The rs1617640 polymorphism was also not associated with time to development of severe DR in a large cohort of T1DM patients followed for over 15 years (from the WESDR + DCCT/EDIC studies) [15]. However, other studies have found opposing results [10, 11, 17, 19, 20]. The T allele was associated with an increased risk of PDR in three European-American cohorts of T2DM and T1DM patients from different geographic areas in the United States [10] and with DR in North Indians with T2DM [19]. On the other hand, the G allele was associated with the increased risk of DR in T2DM patients from Australia [11], China [17], and Slovenia [20]. Thus, differences in ethnicity and type of diabetes do not seem to explain the discrepancies between these studies.

When all the available genotype data were pooled in the meta-analysis, the between-heterogeneity was substantial, and the random-effects model revealed no association between the rs1617640 polymorphism and DR. The asymmetry seen in the funnel plots, and not confirmed by statistical test, can be attributed to neither publication bias nor small study effects [34–37] because most of the studies did not find an association between the rs1617640 variant and DR, and two of the subject sets that lied outside the majority of funnel plots and had shown the strongest association had sample sizes of more than 1100 individuals [set #2 of 10, 17]. Apart from this, genotype frequencies were not in HWE among controls in three studies [set #1 of 11, 17, 19]. Departures from HWE may occur due to several reasons other than genotyping errors, such as population stratification and selection bias in the enrollment of controls [34, 35]. Despite these considerations, HWE has been used as the main parameter of post-genotyping quality control in association studies.

Therefore, following the standard recommendations for meta-analyses of gene-disease associations [34–36], we removed the sets in which genotype frequencies were in Hardy–Weinberg disequilibrium to repeat the overall analysis and perform the subgroup analyses for the rs1617640 polymorphism to identify the possible causes of between-study heterogeneity. Heterogeneity was still moderate to high for most genetic models in the PDR, NPDR, and non-Asian subgroup analyses, while it was low or null among T2DM, T1DM, and Asian patients. The subject set #2 of Tong et al. [10], which contributed 20.6% of the total sample size in the overall meta-analysis, seemed to be the main factor contributing to the heterogeneity across the studies and also contributed to a statistically significant association between the rs1617640 polymorphism and DR in PDR, NPDR, T1DM, and non-Asian subgroups.

An issue that may raise some criticism regarding the results is the fact that our case-control study did not detect an association between the rs1617640 polymorphism and DR, whereas our meta-analysis revealed an association of the G allele with a reduced risk of DR. This is not unexpected. First, the meta-analysis involves a larger number of subjects, therefore it is more powerful than a single study to detect an association of low magnitude. Second, the association with either one of the two alleles, or the lack of it, may be population-specific. Third, although the type of diabetes did not seem to explain the discrepant results across the individual studies, the meta-analysis showed that the G allele of the rs1617640 polymorphism was associated with a decreased risk of DR under all the genetic models in T1DM patients, while no association was observed in T2DM patients. Thus, the results of our case-control study are actually in agreement with those obtained in the meta-analysis.

Moreover, the findings of our meta-analysis regarding the rs1617640 polymorphism are in line with those reported in a previous study, in which the TT genotype was associated with an increased risk of DR, as compared to the GG genotype, with a similar magnitude of association [41]. In their analyses, without the cohorts of Tong et al. [10], no association between the rs1617640 polymorphism and DR was observed in any genetic model, as well as in T2DM and Asian populations [41]. In addition to four peer-reviewed studies published between 2008 and 2014, also included in our meta-analysis [10–12, 14], the overall analysis performed by Li et al. [41] considered the data from two theses for master's degrees but did not include studies published from 2015 onwards.

Although both meta-analyses indicate the existence of an association between the rs1617640 polymorphism and DR, the actual biological model that describes such relationship is yet to be defined. The G allele was associated with DR under a recessive model in four studies [11, 17, 19, 20]. However, in our overall meta-analysis, the G allele was associated with a decreased risk of DR under the dominant, heterozygous additive, and overdominant genetic models. This is in line with the original report by Tong et al. [10], which suggested an additive allele–dosage effect for the rs1617640 polymorphism. To the best of our knowledge, those authors were the only ones who performed functional assays and prediction analysis to evaluate the effect of the rs1617640 variant on gene expression. The T allele markedly increased the *EPO* expression in cultured human embryonic kidney (HEK) 293 cells, and the computational analysis suggested that the T allele creates a

transcriptional binding site, which likely accounted for the enhanced expression as compared with the G allele. Moreover, vitreous levels of EPO were much higher in non-diabetic subjects with the TT genotype than in those with the GG genotype [10]. Taken together, these findings suggest that high levels of EPO are associated with DR, especially PDR, and the T allele of the rs1617640 is likely a risk factor for DR as it increases the gene expression. This is consistent with experimental evidence showing that exogenous EPO protects against early DR, but it is detrimental in PDR [4–6].

In relation to the rs507392 and rs551238 polymorphisms, available data are scarcer [10, 11, 14, 16–19]. Not all the studies discussed so far have investigated the association of these two genetic variants with DR and, among those that examined such an association, not all reported the genotype data. The findings of the previous studies are varied, even in populations with the same ethnicity. Under the recessive and homozygous additive models, the C allele of both polymorphisms was strongly associated with an increased risk of DR in Australian [11] and Chinese [17] T2DM patients, whereas it was strongly associated with a decreased risk of DR in another population of Chinese T2DM patients [16]. In addition, the C allele of the rs507392 polymorphism was associated with a decreased risk of DR in North Indian T2DM patients, whereas the C allele of the rs551238 was not associated with this complication [19]. In contrast, the C allele of the rs551238 polymorphism was less frequent in patients with DR than in those without this complication, while the rs507392 polymorphism was not associated with DR in the cohort of Utahns (USA) of European ancestry with T2DM [10]. Similar to our case-control study, the rs507392 and rs551238 polymorphisms were not associated with DR in T1DM patients from Australia [11] and in T2DM patients from China [14] and Italy [18]. Our meta-analysis, including either all the studies or only the subject sets in which the control groups met the HWE, revealed no association of the rs507392 and rs551238 variants with DR. It is worth mentioning that Li et al. [41] did not perform the meta-analysis for the rs507392 polymorphism because they considered only the study by Abhary et al. [11] as having DR as an outcome. As for the rs551238 polymorphism, the pooled analysis of three studies (only one common to ours) showed that the A allele was associated with a reduced risk of DR [41].

Although the rs1617640, rs507392, and rs551238 polymorphisms were in high LD with each other in T1DM and T2DM patients [10, 11, 14, 16, 19, present study], findings regarding an additional combined effect of the three *EPO* polymorphisms in the susceptibility of DR are also inconclusive. No evidence of an association between *EPO* haplotypes and DR was found in our case-control study as well as in two Chinese T2DM populations [14, 16]. However, the GCC haplotype was reported to be independently associated with an increased risk of DR under a recessive model in T2DM, but not in T1DM, in a white Australian population [11]. In another study of Chinese T2DM patients, the strongest relationship was observed for the carriership of at least one copy of the minor allele of each polymorphism (GCC) in comparison to the homozygosity for the three major alleles [17]. It is worth noting that risk and protective haplotypes were identified in the cohorts studied by Tong et al. [10], and the main difference between them was the rs1617640 polymorphism. Risk haplotypes carried the T allele, while the G allele was present in the protective haplotypes, irrespective of the alleles at the other two polymorphisms [10]. On the other hand, a recent study on North Indians with T2DM reported that the main source of the association between the TTA haplotype and DR was the T allele of the rs507392 polymorphism [19]. However, our meta-analysis detected no association between the GCC haplotype and DR.

In general, the studies included in our meta-analysis can be considered of good quality as suggested by the scoring scale used for this purpose (NOS). However, specific guidelines for genetic association studies have focused on the HWE test in controls as a means of assessing study quality [34–36] and on the phenotyping, blinding, validity of genotyping method, and population stratification [21, 34]. A critical aspect related to the methodological quality of the previous studies is the lack of blinding and re-genotyping as quality control procedures in half of them [16, 17, 19, 20]. In the other studies, at least one procedure to improve the genotyping accuracy was reported, such as the re-genotyping of part of the samples by sequencing [10], sequencing of some samples for each genotype at each polymorphism [11], and genotype reading by two investigators blinded to the sample phenotypes [12]. Other authors, who genotyped the samples for *EPO* polymorphisms using the Sequenom technology, described a battery of quality control tests [14, 18]. In addition, retinopathy grading was reported to have been performed without prior knowledge of genotypes in one study [10].

Population stratification is unlikely to have been a confounding factor [21, 42] in the previous studies since the authors enrolled subjects from populations with a majority ethnic group (> 90%) [10, 11, 14, 16, 17, 19, 20], used the ancestry from a given region as one of the inclusion criteria [10, 18] or matched the cases and controls by ethnicity [12]. In the study by Abhary et al. [11], there was no difference in the allele frequencies of *EPO* polymorphisms among white subjects of European ancestry and non-white subjects of Asian and Middle Eastern ancestry. In our case-control study, the genotype and allele frequencies were virtually identical in white and non-white subjects.

On the other hand, the unavailability of all genotype data that could be incorporated in the quantitative synthesis is the main limitation of our meta-analysis. Some of the previous original studies were published without reporting the genotype frequencies of the polymorphisms under investigation. In addition, data reported in some papers were unclear, inaccurate, or did not match the sample size described in the text or in the tables. Despite our attempt to obtain all the missing genotype data by e-mail, they were still missing for the rs1617640 [13, 15], rs507392, and rs551238 [10] polymorphisms. Hence, we included most, but not all the previous studies that examined the association of

EPO polymorphisms with DR and met the screening criteria. Although we could perform subgroup analyses by the severity of DR, considering NPDR, and PDR as separate outcomes, it was also limited by the lack of this information in most of the eligible studies. In future studies, authors, as well as the reviewers and editors, should be aware that the complete description of genotype frequencies and outcomes is essential to allow for comparisons across the studies and perform pooled association analyses.

Conclusion

Despite some limitations and the negative findings of our case-control study, suggestive evidence provided by our meta-analysis supports *EPO* as a potential gene involved in the susceptibility of DR, with the rs1617640 polymorphism being the most prominent candidate, thus deserving further investigation.

Abbreviations

DR: diabetic retinopathy; NPDR; non-proliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; EPO: erythropoietin; ESRD: end-stage renal disease; T2DM: type 2 diabetes mellitus; PCR, polymerase chain reaction; HWE: Hardy–Weinberg equilibrium; LD: linkage disequilibrium; OR: odds ratio; 95% CI: 95% confidence interval; NOS: Newcastle–Ottawa Scale; T1DM: type 1 diabetes mellitus; SNP: single nucleotide polymorphism.

Declarations

Ethics approval and consent to participate

The study was performed according to the tenets of the Declaration of Helsinki and written informed consent was obtained from all participants. The study was approved by the Human Research Ethics Committee of Universidade Luterana do Brasil – ULBRA (CAAE number: 55236216.2.0000.5349; consolidated review number: 1.553.469; date of approval: May 20, 2016).

Consent for publication

Not applicable.

Availability of data and materials

The genotyping dataset generated during the case-control study are available in the Figshare repository (https://doi.org/10.6084/m9.figshare.16417161). The data used in the meta-analysis are included within the supplementary material (Additional file 4: Fig. S1-S128). Additional data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.F.S. conceived the case-control study and performed the genotyping and statistical analyses. R.C.S. enrolled the patients and performed the DNA extraction and genotyping. E.R.P. enrolled the patients, updated the database, performed the DNA extraction, and supervised the genotyping. D.S. extracted the data from eligible papers and assessed the methodological quality of each study included in the meta-analysis. D.C. enrolled the patients, updated the database, and performed the DNA extraction. L.H.C. contributed to acquire data from patients and supervised the clinical stages of the study. K.G.S. enrolled the patients, updated the database, performed the DNA extraction, supervised the study, contributed funding resources, performed the search literature, study screening, data extraction, methodological quality assessment, and statistical analyses of the case-control study and meta-analysis, interpreted the data, and wrote the paper. All authors reviewed the manuscript for critical comment and approved the submitted version.

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Figures

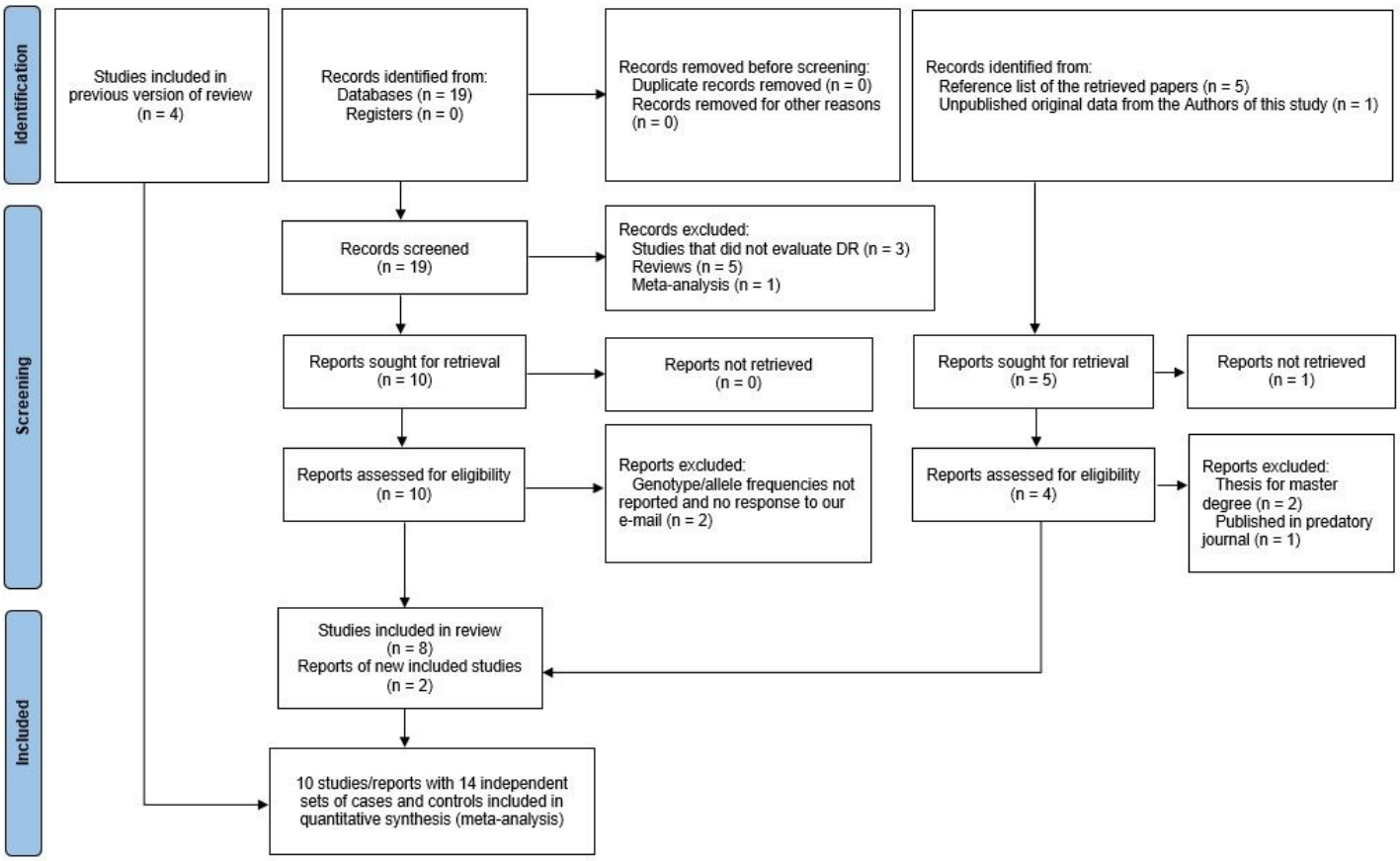

Figure 1

PRISMA flow diagram depicting the results of the search and selection of studies included in the meta-analysis.

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