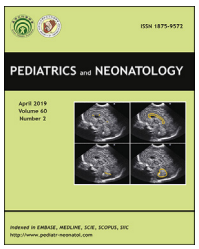
Pediatrics and Neonatology (2019) 60, 129—134

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.pediatr-neonatol.com



Review Article

Association of VEGFA polymorphisms with necrotizing enterocolitis in Chinese Han population

Xiaoyan Gao [a,1], Fei Ma [b,1], Hu Hao [b], Yiheng Dai [a], Weidong Liu [a], Xin Xiao [b,***,2], Pingmin Gao [a,c,**,2], Sitao Li [b,*,2]

[a] *Department of Neonatology, Southern Medical University Affiliated Maternal and Child Health Hospital of Foshan, Foshan, China*
[b] *Department of Neonatology, The Sixth Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China*
[c] *Foshan Institute of Fetal Medicine, Southern Medical University Affiliated Maternal and Child Health Hospital of Foshan, Foshan, China*

Received Dec 27, 2017; received in revised form May 16, 2018; accepted Jul 5, 2018
Available online 19 July 2018

Key Words
necrotizing enterocolitis;
preterm infants;
single-nucleotide polymorphism;
VEGFA

Background: To examine whether polymorphisms in the *VEGFA* gene lead to low VEGFA production in peripheral blood and increased risk of NEC in the Chinese Han population.
Material and methods: Thirty NEC patients and 80 control subjects were enrolled. Six *VEGFA* single-nucleotide polymorphisms (SNPs) were performed using the SEQUENOM MassARRAY platform assay. The concentration of VEGFA in the plasma was measured using an enzyme-linked immunosorbent assay.
Results: The rs699947 and rs833061 VEGF-A SNPs were found to be associated with low plasma levels and high risk of NEC.
Conclusion: Our results suggested that, if validated in larger studies, screening for *VEGFA* SNPs and plasma levels might be useful as a risk factor for NEC in the future.

Abbreviations: HWE, Hardy—Weinberg Equilibrium; LBW, Low birth weight; LD, Linkage disequilibrium; MAF, Minor allele frequencies; NEC, Necrotizing enterocolitis; NICU, neonatal intensive care unit; OR, odds ratio; SNPs, Single-nucleotide polymorphisms; VEGFA, Vascular endothelial growth factor A.

* Corresponding author. Department of Neonatology, The Sixth Affiliated Hospital of Sun Yat-sen University, No. 26 Yuancun Erheng Road, Tianhe District, Guangzhou, 510655, China.
** Corresponding author. Foshan Institute of Fetal Medicine, Southern Medical University, Affiliated Maternal and Child Health Hospital of Foshan, No. 11 Renmin, West Road, Chancheng District, Foshan, 528000, China
*** Corresponding author. Department of Neonatology, The Sixth Affiliated Hospital of Sun Yat-sen University, No. 26 Yuancun Erheng Road, Tianhe District, Guangzhou, 510655, China, Fax: +86 0 20 3877 7850.
E-mail addresses: fsgaopm666@126.com (P. Gao), txiaoxin1049@163.com, sitaoli918@126.com (S. Li).
[1] These authors contributed equally to this work.
[2] These authors contributed equally to this work as senior authors.

https://doi.org/10.1016/j.pedneo.2018.07.002

1. Background

Necrotizing enterocolitis (NEC) is a catastrophic gastrointestinal disease that is a major mortality in premature neonates who survive the first few days after birth.[1] Although the underlying pathogenesis of the disease remains undefined, several contributing factors have been identified such as microbial dysbiosis, formula feeding, and excessive inflammation.[2] Therefore, a unifying concept is proposed that NEC occurs when damaged immature intestinal mucosal barrier permits luminal bacteria to translocate across the epithelial barrier into the lamina propria, triggering a cascade of inflammatory events and progressive tissue damage.[3] It is clear that the intestinal microcirculation plays a role in the events above.[4,5]

Vascular endothelial growth factor (VEGF) is an evolutionarily highly conserved glycoprotein that not only controls microvascular development through powerful vasculogenesis and angiogenesis but also plays an important role in cell proliferation, migration, survival and permeability.[6] The loss of one *VEGF* single allele in mice significantly impairs vessel development, leading to severe growth delay and mortality at midgestation.[7,8] In contrast, VEGF expression is elevated in term animals compared to fetal stages in sheep jejunum, indicating a larger role during postnatal development.[9] In fact, VEGF was also found in biologically high concentrations in human and mouse milk,[10–12] which is known to protect against NEC. In mouse intestine, diminished VEGF expression was linked with heightened risk of NEC.[13]

The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PlGF).[14] VEGFA is mainly involved in angiogenesis, and its gene is located on chromosome 6p21.3, and its coding region spans over 14 kb.[15] An association between *VEGFA* variants and perinatal complications, in particular NEC, was described.[16] However, the study was limited by its inability to account for multiple testing in analysis.[16] In the present study, we investigated the association of *VEGFA* SNPs (rs699947, rs833061, rs2010963, rs3025020, rs3025039, and rs6900017) with the risk of NEC and VEGFA plasma levels in the Chinese Han population.

2. Materials and methods

2.1. Study subjects

This preliminary study included 30 preterm neonates with NEC (Bell stage II, n = 25; Bell stage III, n = 5) according to the modified Bell's criteria,[17] born in the Sixth Affiliated Hospital, Sun Yat-sen University and the Foshan Women and Children's Hospital between August 2014 and December 2016. During this period, another 80 gestational age (GA)-, birth weight (BW)- and gender-matched preterm neonates admitted to the neonatal intensive care unit (NICU) without NEC were recruited as control subjects (Supplementary Table S1). This study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Sixth Affiliated Hospital, Sun Yat-sen University and the Foshan Women and Children's Hospital. Written informed consent was obtained from the parents of each participant. When the patients were treated for NEC through bowel rest, decompression, and antibiotic therapy or direct surgical intervention, 0.5-ml blood samples were taken and stored in EDTA for total genomic DNA extraction and plasma preparation. In the control subjects, blood samples were taken after written informed consent was obtained.

2.2. Plasma VEGFA measurement

Blood plasma was prepared by centrifugation of anticoagulant blood tubes at 1000 × *g* for 10 min at 4 °C, and it was stored in 200-μl aliquots at −80 °C. Samples were tested for VEGFA using the human VEGFA sandwich enzyme-linked immunosorbent assay (ELISA; CUSABIO, Wuhan, China). The assay sensitivity was 25.30 pg/mL, and inter-assay and intra-assay precision (CV %) were all less than 8%.

2.3. *VEGFA* genotyping

We selected polymorphisms in the *VEGFA* gene with a minor allele frequency (MAF) of >5% in the Chinese Han population. DNA was extracted from whole blood using the FlexiGene DNA Kit (Qiagen, Hilden, Germany). The selected SNPs included the following: rs699947, rs833061, rs2010963, rs3025020, rs3025039, and rs6900017, which were genotyped using the SEQUENOM MassARRAY platform (CapitalBio, Beijing, China). The PCR primers for the six selected SNPs are listed in Supplementary Table S2. The genotyping call rate was 100% for the six SNPs.

2.4. Statistical analysis

Statistical analyses were performed using the SHEsis software platform[18] and SPSS v. 20.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were summarized as the mean ± standard deviation (SD) or as the median (interquartile range), and the statistical analysis was performed by unpaired student's t-test, Mann–Whitney U-test, or one-way analysis of variance (ANOVA). Categorical variables, expressed as percentages or ratio, were compared using Pearson's χ^2 test. Allele frequencies, genotype frequencies, and departure from Hardy–Weinberg Equilibrium (HWE) for each SNP between the NEC and control groups were compared by χ^2 test. The Haploview software

package (v. 4.2) and SHEsis software analysis were used to evaluate linkage disequilibrium (LD) and haplotype construction.[18,19] Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by logistic regression analysis. All statistical tests were two-tailed, and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. *VEGFA* allele and genotype analysis

Basic information of the six tested SNPs is shown in Table 1. In both NEC patients and control group, all tested SNPs conformed to Hardy–Weinberg equilibrium ($P > 0.05$). Among the six analyzed SNPs, there existed two loci exhibited an association with NEC. The minor allele frequencies (MAF) of rs699947 and rs833061 were significantly higher in patients than in control group ($P = 0.021$ and 0.037, respectively).

Significant differences in the distribution of rs699947 and rs833061 genotypes were seen between NEC patients and control group (Table 2). We observed the alleles 'A' of rs699947 and 'C' of rs833061 increased the NEC risk. In the codominant model, the genotypes 'C/A' of rs699947 and 'T/C' of rs833061 increased NEC risk by 3.21- and 3.04-fold, respectively; in the dominant model, the genotypes 'C/A–A/A' of rs699947 and 'T/C–C/C' of rs833061 increased the same NEC risk by 3.29-fold; in the additive model, the alleles 'A' of rs699947 and 'C' of rs833061 increased NEC risk by 2.09- and 2.20-fold, respectively.

3.2. LD and haplotype analyses

Next, we analyzed the LD and frequency of each haplotype. Limited LD was found between the six *VEGFA* variants tested, and one block was identified; the block (1.1 kb) contained rs699947 and rs833061 ($D' = 0.896$, $r^2 = 0.786$) (Fig. 1). Among the four possible haplotypes, two were identified with frequencies of ≥ 0.05 in both patients and control subjects and the haplotype 'A-C' was found to increase risk of NEC by 1.93-fold (Table 3).

3.3. VEGFA plasma levels

Since the concentrations of plasma VEGFA were reduced in NEC patients compared to that in control group

Table 1 Basic information of candidate *VEGFA* SNPs in NEC patients and control subjects.

SNP	Allele		Control		NEC		*P*	OR (95% CI)
	1[a]	2	MAF	HWE *P*	MAF	HWE *P*		
rs699947	A	C	0.300	0.670	0.467	0.261	0.021	2.04 (1.11–3.76)
rs833061	C	T	0.300	0.670	0.483	0.461	0.011	2.18 (1.19–4.01)
rs2010963	C	G	0.319	0.947	0.300	0.794	0.617	0.92 (0.48–1.75)
rs3025020	T	C	0.350	0.376	0.300	0.019	0.485	1.26 (0.66–2.38)
rs3025039	T	C	0.350	0.058	0.300	0.334	0.659	1.20 (0.53–2.72)
rs6900017	T	C	0.150	0.468	0.100	0.543	0.547	0.73 (0.26–2.04)

NEC, necrotizing enterocolitis; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.
[a] Allele 1 is a minor allele.

Table 2 Associations between the SNP genotypes of *VEGFA* and the risk of NEC.

SNP	Model	Genotype	Control (n, %)	Total NEC (n, %)	OR (95% CI)	*P*
rs699947	Codominant	C/C	40 (50%)	7 (23.3%)	1	
		C/A	32 (40%)	18 (60%)	3.21 (1.20–8.64)	0.018*
		A/A	8 (10%)	5 (16.7%)	3.57 (0.90–14.14)	0.14
	Dominant	C/C	40 (50%)	7 (23.3%)	1	
		C/A–A/A	40 (50%)	23 (76.7%)	3.29 (1.27–8.52)	0.0099*
	Recessive	C/C–C/A	72 (90%)	25 (90%)	1	
		A/A	8 (10%)	5 (10%)	1.80 (0.54–6.01)	0.35
	Log-additive	–	–	–	2.09 (1.10–3.95)	0.021*
rs833061	Codominant	T/T	40 (50%)	7 (23.3%)	1	
		T/C	32 (40%)	17 (56.7%)	3.04 (1.12–8.21)	0.025*
		C/C	8 (10%)	6 (20%)	4.29 (1.14–16.18)	0.061
	Dominant	T/T	40 (50%)	7 (23.3%)	1	
		T/C–C/C	40 (50%)	23 (76.7%)	3.29 (1.27–8.52)	0.0099*
	Recessive	T/T–T/C	72 (90%)	24 (80%)	1	0.18
		C/C	8 (10%)	6 (20%)	2.25 (0.71–7.14)	
	Log-additive	–	–	–	2.20 (1.17–4.13)	0.012*

*$P < 0.05$.

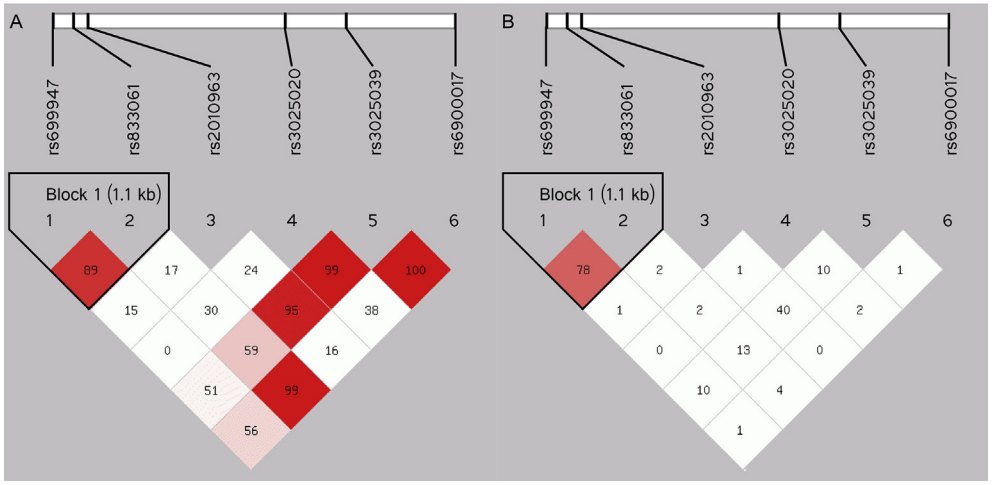


Figure 1 Linkage disequilibrium (LD) plot generated by SHEsis analysis. LD is displayed as (A) pairwise D′ values and (B) r^2 values. Different colors represent different ranges of D′ and r^2 values.

Table 3 Haplotype association analysis of rs699947 and rs833061 in *VEGFA* gene.

Haplotype	Frequency			$\chi 2$	*P*-value	OR (95% CI)
	All	Case	Control			
C-T	0.641	0.477	0.700	4.156	0.042	1.93
A-C	0.327	0.394	0.300			(1.02—3.65)

(Supplementary Table S1), and the genotypes 'C/A' and 'C/A—A/A' of rs699947 and the 'T/C' and 'T/C—C/C' of rs833061 increased NEC risk (Table 2), we hypothesized that the above-mentioned genotypes may contribute to decreased plasma VEGFA levels in patients. In line with our hypothesis, the plasma VEGFA levels were significantly lower in both patients and control group with the above-mentioned genotypes than the 'C/C' of rs699947 and 'T/T' of rs833061; significantly, these were lower in patients than in control group (Fig. 2).

4. Discussion

While the exact pathophysiology of NEC remains elusive, evidence shows that alternated intestinal microcirculation may contribute to the pathogenesis of NEC.[20,21] VEGFA, as a key regulator of vascular vasculogenesis and angiogenesis, plays an important role in the development and maintenance of microvascular networks.[6] Although a preliminary study demonstrated that *VEGFA* variant was associated with NEC,[16] the peripheral blood levels of VEGFA in NEC patients have not previously been examined. Our findings revealed that NEC patients had significantly reduced levels of peripheral blood VEGFA; this effect is intensified by a linkage variants of *VEGFA* (rs699947 and rs833061), showing that

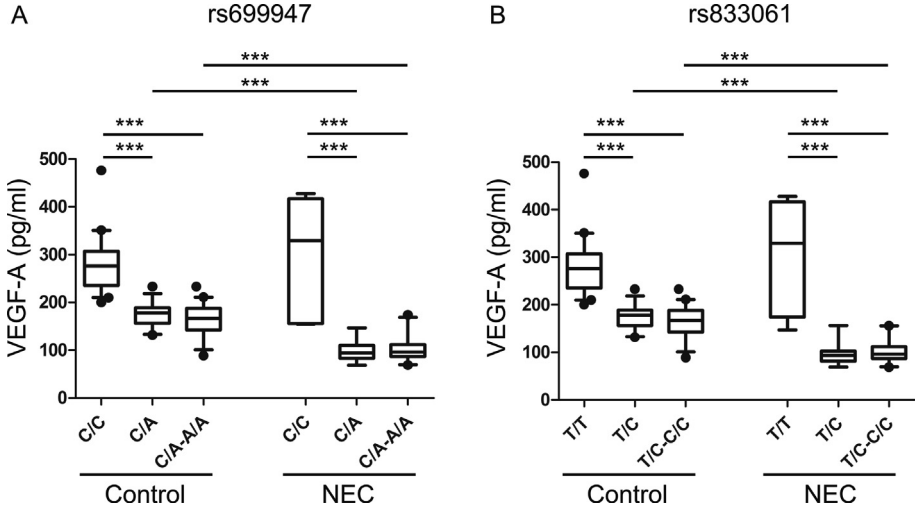


Figure 2 Association between *VEGFA* genotypes and the levels of plasma VEGFA[a]. NEC, necrotizing enterocolitis; VEGFA, vascular endothelial growth factor A. [a]Plasma VEGFA determined by ELISA; results expressed as pg/ml. ***$P < 0.001$. The box shows the medians (solid bar), interquartile ranges (IQRs) (box), and 95th and 5th percentiles (whiskers).

VEGFA gene polymorphisms play an important role in the aetiology of NEC.

Genetic factors modulating liability or severity of NEC have been widely investigated.[22] Similarly to previous studies suggesting the role of genetic polymorphisms in the aetiology of NEC, we demonstrated that the minor 'A' allele of rs699947 and 'C' allele of rs833061 increased NEC risk in the Chinese Han population. In the codominant and dominant genetic models, the genotypes 'C/A' and 'C/A—A/A' of rs699947 and the 'T/C' and 'T/C—C/C' of rs833061 were shown to be more strongly associated with the development of NEC. This was partially in agreement with Banyasz et al.'s study of a Hungarian population that demonstrated a significant association only between rs699947 (−2578C > A) and NEC, but no association between rs833061 (−460T > C) and NEC.[16] The discrepancies in the association of these *VEGFA* variants with NEC is likely to be attributed to ethnic differences, and the sample size of each of these studies, as well as the differences in inclusion and exclusion criteria of the studied population.

Interestingly, further analysis indicated that the plasma VEGFA levels were lower in both patients and control group with the genotypes 'C/A' and 'C/A—A/A' of rs699947 and 'T/C' and 'T/C—C/C' of rs833061 than the 'C/C' of rs699947 and 'T/T' of rs833061; significantly, these were lower in patients than in control group. Consistent with this phenomenon, recent studies have demonstrated that the levels of VEGFA in intestine are reduced in human and experimental NEC, which might contribute to NEC pathogenesis.[13] Similarly, VEGFA overexpression through plasmids administered subcutaneously seems to be a promising approach in the treatment of experimental NEC.[23] Significantly, through LD and haplotype analyses, we found that there existed a high LD between rs699947 and rs833061, and the haplotype 'C/A' increased NEC risk by 1.93-fold. This was confirmed by the plasma levels of VEGFA, which were associated with the genetic variants of rs699947 and rs833061, respectively.

The rs2010963, rs3025020, rs3025039, and rs6900017 SNPs had no association with NEC in the Chinese Han population. In contrast, Banyasz et al.'s study demonstrated association of rs2010963 with low birth weight (LBW) infants in Hungary.[16] The main reason for this discrepancy is the different compared studied population. Our results were similar to the findings by Marwa et al., as they did not find any association between rs2010963, rs13385 and rs2074613 and preeclampsia in Tunisia.[24] Our results were also different from Kreiner-Moller et al. and Manso et al. as they demonstrated that rs6900017 was associated with lung function and stroke, respectively.[25,26] Although NEC, lung function and stroke were all associated with angiogenesis, the discrepancies in the association of rs6900017 with the diseases above is likely to be attributed to the differences of specific diseases.

To the best of our knowledge, this is the first study to investigate the association of SNPs in the *VEGFA* gene and its plasma levels with NEC simultaneously. However, there are some limitations that must be considered. In the NEC patients, we were unable to test the association of the major and minor genotypes of rs699947 and rs833061 with NEC severity because of the relatively small sample size. In addition, the 95% CI ranges for the OR of the association between rs699947, rs833061 and NEC were quite wide; this was, in part, owing to the small sample size of patients. Therefore, further research is needed with increased sample size for verification.

In conclusion, this study investigated the potential association between *VEGFA* gene polymorphisms, VEGFA plasma levels, and the development of NEC in preterm neonates. We found that the linkage variants of *VEGFA* (rs699947 and rs833061) were associated with decreased plasma VEGFA levels and that they represented a risk factor for NEC. These findings suggest that testing for the *VEGFA* rs699947 and rs833061 SNPS and/or VEGFA blood plasma levels would provide valuable information for NEC risk assessment.

Conflicts of interest

The authors declare no financial or commercial interests exist.

Acknowledgements

This work was supported by the Medical Scientific Research Foundation of Guangdong Province, China (A2014704), and the Science and Technology Foundation of Guangzhou, China (201604020154).

References

1. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med* 2011;**364**:255—64.
2. Remon JI, Amin SC, Mehendale SR, Rao R, Luciano AA, Garzon SA, et al. Depth of bacterial invasion in resected intestinal tissue predicts mortality in surgical necrotizing enterocolitis. *J Perinatol* 2015;**35**:755—62.
3. Maheshwari A, Kelly DR, Nicola T, Ambalavanan N, Jain SK, Murphy-Ullrich J, et al. TGF-β2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. *Gastroenterology* 2011;**140**:242—53.
4. Crafts TD, Jensen AR, Blocher-Smith EC, Markel TA. Vascular endothelial growth factor: therapeutic possibilities and challenges for the treatment of ischemia. *Cytokine* 2015;**71**:385—93.
5. Watkins DJ, Besner GE. The role of the intestinal microcirculation in necrotizing enterocolitis. *Semin Pediatr Surg* 2013;**22**:83—7.
6. Delcombel R, Janssen L, Vassy R, Gammons M, Haddad O, Richard B, et al. New prospects in the roles of the C-terminal domains of VEGF-A and their cooperation for ligand binding, cellular signaling and vessels formation. *Angiogenesis* 2013;**16**:353—71.
7. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;**380**:439—42.
8. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;**380**:435—9.
9. Holmes K, Charnock Jones SD, Forhead AJ, Giussani DA, Fowden AL, Licence D, et al. Localization and control of expression of VEGF-A and the VEGFR-2 receptor in fetal sheep intestines. *Pediatr Res* 2008;**63**:143—8.
10. Rossiter H, Barresi C, Ghannadan M, Gruber F, Mildner M, Födinger D, et al. Inactivation of VEGF in mammary gland

epithelium severely compromises mammary gland development and function. *FASEB J* 2007;**21**:3994—4004.
11. Vuorela P, Andersson S, Carpén O, Ylikorkala O, Halmesmäki E. Unbound vascular endothelial growth factor and its receptors in breast, human milk, and newborn intestine. *Am J Clin Nutr* 2000;**72**:1196—201.
12. Siafakas CG, Anatolitou F, Fusunyan RD, Walker WA, Sanderson IR. Vascular endothelial growth factor (VEGF) is present in human breast milk and its receptor is present on intestinal epithelial cells. *Pediatr Res* 1999;**45**:652—7.
13. Sabnis A, Carrasco R, Liu SX, Yan XC, Managlia E, Chou PM, et al. Intestinal vascular endothelial growth factor is decreased in necrotizing enterocolitis. *Neonatology* 2015;**107**:191—8.
14. Byrne AM, Bouchier-Hayes DJ, Harmey JH. Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). *J Cell Mol Med* 2005;**9**:777—94.
15. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)* 2005;**109**:227—41.
16. Bányász I, Bokodi G, Vásárhelyi B, Treszl A, Derzbach L, Szabó A, et al. Genetic polymorphisms for vascular endothelial growth factor in perinatal complications. *Eur Cytokine Netw* 2006;**17**:266—70.
17. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. *Pediatr Clin North Am* 1986;**33**:179—201.
18. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005;**15**:97—8.
19. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;**21**:263—5.
20. Downard CD, Grant SN, Matheson PJ, Guillaume AW, Debski R, Fallat ME, et al. Altered intestinal microcirculation is the critical event in the development of necrotizing enterocolitis. *J Pediatr Surg* 2011;**46**:1023—8.
21. Yu X, Radulescu A, Zorko N, Besner GE. Heparin-binding EGF-like growth factor increases intestinal microvascular blood flow in necrotizing enterocolitis. *Gastroenterology* 2009;**137**:221—30.
22. Cuna A, Sampath V. Genetic alterations in necrotizing enterocolitis. *Semin Perinatol* 2017;**41**:61—9.
23. Karatepe HO, Kilincaslan H, Berber M, Ozen A, Saricoban HE, Ustek D, et al. The effect of vascular endothelial growth factor overexpression in experimental necrotizing enterocolitis. *Pediatr Surg Int* 2014;**30**:327—32.
24. Ben Ali Gannoun M, Al-Madhi SA, Zitouni H, Raguema N, Meddeb S, Hachena Ben Ali F, et al. Vascular endothelial growth factor single nucleotide polymorphisms and haplotypes in pre-eclampsia: a case-control study. *Cytokine* 2017;**97**:175—80.
25. Kreiner-Møller E, Chawes BL, Vissing NH, Koppelman GH, Postma DS, Madsen JS, et al. VEGFA variants are associated with pre-school lung function, but not neonatal lung function. *Clin Exp Allergy* 2013;**43**:1236—45.
26. Manso H, Krug T, Sobral J, Albergaria I, Gaspar G, Ferro JM, et al. Evidence for epistatic gene interactions between growth factor genes in stroke outcome. *Eur J Neurol* 2012;**19**:1151—3.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pedneo.2018.07.002.