Association of angiogenesis and inflammation-related gene functional polymorphisms with early-stage breast cancer prognosis

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Abstract. Genetic variations in inflammation- and angiogenesis-related genes may alter the coded protein level and impact the pathogenesis of breast cancer (BC). The present study investigated the association of functional single nucleotide polymorphisms (SNPs) in the VEGFA, IL-1β, IL-1α and IL-6 genes with the early-stage BC phenotype and survival. Genomic DNA and clinical data were collected for 202 adult Eastern European (Lithuanian) women with primary I-II stage BC. Genotyping of the SNPs was performed using TaqMan SNP genotyping assays. Nine VEGFA, IL-1β, IL-1α and IL-6 polymorphisms were analysed. The VEGFA and IL-6 haplotypes were inferred using Phase software. Patients were prospectively followed-up for recurrence, occurrence of metastasis and mortality until April 30, 2019. All studied genotypes were in Hardy-Weinberg equilibrium and had the same distribution as the 1,000 Genomes project Phase 3 dataset for European population. Significant associations of the studied SNPs with clinicopathologic variables were observed between IL-1α rs1800587 C allele and larger primary tumour size; IL-6 rs1800797 A allele, rs1800797 GA genotype, rs1800795 C allele, IL-6 (rs1800797-re1800795) AC diplotype and hormonal receptor-positive disease; IL-6 rs1800797 A allele and HER2 negative status. In univariate Cox survival analysis, IL-1α rs1800587 CC and IL-6 rs1800797 GG genotype carriers exhibited worse disease-free survival (DFS), metastasis-free survival (MFS) and overall survival (OS). The IL-6 rs1800795 GG genotype was associated with worse OS. IL-6 (rs1800797, rs1800795) GG/GG diplotype carriers had shorter MFS and OS. Multivariate Cox survival analysis revealed that the IL-1α rs1800587 CC genotype was an independent negative prognostic factor for DFS, MFS and OS, and the IL6 GG/GG diplotype was an independent negative prognostic factor for MFS and OS. According to the present study, functional SNPs in the IL-1α and IL-6 genes may contribute to the identification of patients at higher risk of BC recurrence, development of metastases and worse OS among early-stage patients with BC.

Introduction

Breast cancer (BC) is one of the most commonly diagnosed cancers and the leading cause of cancer death among women worldwide (1). Improved diagnostic capabilities has led to an increased rate of BC identified at an early stage (2). However, despite early diagnosis and treatment, the rate of recurrence and metastasis following radical treatment remains disappointingly high, and survival varies considerably between patients with closely matching tumour characteristics.

Inflammation and angiogenesis are the main drivers of cancer. These processes are tightly interconnected in the sense that many pro-inflammatory proteins possess proangiogenic properties and vice versa. The formation of new blood vessels in malignant tumours ensures the supply of nutrients and oxygen, hence promoting the spread of tumour cells (3). Microvessel density is a pivotal risk factor for metastasis and a predictor of poor BC prognosis (4).

The vascular endothelial growth factor A (VEGFA, or VEGF) is the best-known proangiogenic molecule. VEGFA
mediates the growth of new blood vessels by binding to the endothelial cell surface receptors. It promotes endothelial cell proliferation, migration and the formation of tubular structures (5). Another mechanism of tumour neovascularization is the so-called inflammatory angiogenesis. Such pro-inflammatory cytokines as interleukin-1β (IL-1β), interleukin-1α (IL-1α) and interleukin-6 (IL-6) through a variety of signalling pathways promote endothelial cell migration and proliferation, contributing to tumour angiogenesis that facilitates the survival of cancer cells (6,7). Apart from stimulating angiogenesis, VEGFA, IL-1β, IL-1α and IL-6 are also involved in inflammatory processes. These proteins may prevent apoptosis and promote cancer cell proliferation, differentiation, migration and metastasis.

The association between the above-mentioned proteins and BC prognosis was demonstrated by several authors. The elevated serum level of VEGFA in metastatic BC patients is linked to worse progression-free survival (PFS) and overall survival (OS) (8). Another cytokine, IL-6, induces epithelial-mesenchymal phenotype and therapeutic resistance in BC cells (9). Higher circulating levels of IL6 were observed in more advanced stages of the disease (10). It was also found that high tumour co-expression of the VEGF and IL-6 family cytokines significantly lowers the human epidermal growth factor receptor 2 (HER2) negative BC survival (11). Moreover, higher expression of pro-inflammatory IL-1β cytokine is correlated with higher BC stage and significantly worse survival (12). In addition, IL-1α acts as a pro-inflammatory molecule itself and also promotes the activity of IL-1β, resulting in an increased growth of BC cells and tumour progression (13).

Common polymorphisms in proinflammatory and proangiogenic cytokine genes may influence their coded protein production and play a role in the course of BC. The polymorphisms with proved functional activity are VEGFA (rs6999477, rs833061, rs25648, rs1005230), IL-1β (rs1143634, rs16944), IL-1α (rs1800587) and IL-6 (rs1800795, rs1800797) (14-21). Candidate gene studies as well as moderate-sized genome-wide association studies (GWAS) highlighted the important role of these polymorphisms in BC risk and aggressiveness (22-32), although substantial heterogeneity across studies exists. The currently available results are inconsistent in terms of different ethnicity and cancer stage; therefore, it is necessary to further investigate the role of VEGFA, IL-1β, IL-1α and IL-6 gene polymorphisms in breast carcinogenesis and cancer progression.

This paper describes a cohort study that aimed to examine the contribution of VEGFA, IL-1β, IL-1α and IL-6 gene polymorphisms to the clinicopathologic features and survival in a homogeneous group of Eastern European (specifically, Lithuanian) early-stage BC patients.

**Materials and methods**

**Patients.** The study consisted of 202 adult Lithuanian women with primary I-II stage BC. All patients were treated in the Hospital of Lithuanian University of Health Sciences Kaunas Clinics. The exclusion criteria were other malignancies, significant comorbidities and/or incomplete medical documentation. Surgery and adjuvant therapy were chosen by the clinicians, based on pathomorphologic characteristics and validated prognostic factors. The patients were followed until 30 April, 2019 (censoring date).

**Candidate polymorphisms.** The genes and polymorphisms known to modulate inflammation and angiogenesis were selected. The selection criteria included: i) functional single nucleotide polymorphisms (SNPs) in the VEGFA, IL-1β, IL-1α and IL-6 genes predicting alterations in the protein level; ii) SNP relevant to outcomes in other settings; and iii) SNP with a minor allele frequency greater than 15% in the study population. We selected nine SNPs: The VEGFA gene rs6999477, rs833061, rs25648, and rs1005230; the IL-1β gene rs1143634 and rs16944; the IL-1α gene rs1800587; and the IL-6 gene rs1800795 and rs1800797.

**Assay methods.** Peripheral blood samples from the study population were collected in 2009-2017. For genomic DNA extraction from peripheral blood leukocytes, a commercially available DNA extraction kit (i.e., Thermo Fisher Scientific, Lithuania) was used. The DNA was stored at -20°C prior to usage.

Genotyping of the selected polymorphisms was performed at the Dr. K. Janusauskas Laboratory of Genetics of the Institute of Biology Systems and Genetic Research of Lithuanian University of Health Sciences Kaunas Clinics. The SNPs of the target genes were estimated by using TaqMan SNP Genotyping Assays (C_8311602_10, C_1647381_10, C_791476_10, C_8311612_10, C_1839697_20, C_1839695_20, C_9546517_10, C_1839943_10, C_1839943_10; Applied Biosystems; Thermo Fisher Scientific, Inc.). The polymerase chain reaction was performed in a reaction volume of 25 µl containing template DNA (2 ng), 2X TaqMan Universal Master Mix II, no UNG (Applied Biosystems; Thermo Fisher Scientific, Inc.)-12,5 µl, 20X TaqMan SNP Genotyping Assay methods.

**Study design.** A prospective cohort study was conducted at the Hospital of Lithuanian University of Health Sciences. For the case selection, information on primarily BC patients was retrieved from the hospital’s Pathology Department. The patients who fulfilled the inclusion criteria and signed the informed consent document (approved by the Kaunas Regional Ethics Committee for Biomedical research; Protocol number BE-2-10) were enrolled in the study and their peripheral blood samples were obtained. The characteristics of clinical and pathological features and the course of the disease were
obtained for all study subjects. The date of histological BC verification was time zero in the survival analysis. The endpoints of interest were disease-free survival (DFS), metastasis-free survival (MFS) and OS. We checked for associations of SNPs with the known BC prognostic factors and survival endpoints. The guidelines for the reporting of tumour marker prognostic studies were applied while conducting the study (33,34).

Statistical analysis. The allele frequency distributions of the investigated SNPs were compared with the European population data from the 1000 Genomes project phase 3 database (35). For each SNP a Hardy-Weinberg equilibrium was assessed by using Pearson's chi square and Fisher's exact tests. The Haploview v4.1 software was used to check for the linkage disequilibrium between SNPs (36). The VEGFA and IL-6 haplotypes were inferred from the tested SNPs by Bayesian methods as implemented in the Phase software (v2.1; Department of Statistics, University of Washington, Seattle, WA, USA) (37,38). The SNPs were analysed under genotype, allelic and haplotype (for IL-6 and VEGFA SNPs) models. The associations of polymorphisms with clinicopathologic variables were evaluated by Pearson's Chi-square or Fisher's exact test. The Bonferroni-corrected \( \alpha \) level was used in the association analysis for multiple comparisons. Moreover, the Cox proportional hazards model was used to estimate the prognostic factors for DFS, MFS and OS. In addition, multivariate analysis was used to determine the interdependency of genotypes and other known prognostic factors, such as age, tumour differentiation grade, tumour size, lymph node status, oestrogen receptor status, progesterone receptor status and human epidermal growth factor receptor 2 (HER2) status. Hazard ratios (HR) and their 95% confidence intervals (CI) were recorded for each tested marker. Finally, the Kaplan-Meier analysis with the log-rank test was applied to compare the survival of the patients with different genotypes. Statistical significance was set at 5% (\( P<0.05 \)). Statistical analysis was performed using SPSS for Windows v20.0 (Released 2011; IBM Corp).

Results

Sample characteristics. A total of 202 Lithuanian women with early-stage BC were included in the current analysis. The frequency distributions of clinical and tumour biological factors are shown in Table I. For all study participants, primary treatments included surgery (100%), chemotherapy (77%), hormone therapy (71%), trastuzumab (19%) and radiation therapy (97%).

All the patients were genotyped for a panel of nine SNPs: The VEGFA gene rs699947, rs833061, rs25648 and rs1005230; the IL-1\( \alpha \) gene rs1143634 and rs16944; the IL-1\( \beta \) gene rs1800587; the IL-6 gene rs1800795 and rs1800797. The genotypes were found to be in Hardy-Weinberg equilibrium in all the nine SNPs. A strong linkage disequilibrium between four VEGFA and two IL-6 polymorphisms was confirmed (Fig. 1). Our cohort had similar allele distribution to that of the 1000 Genomes project phase 3 for European population. The genotype and allele frequency data is presented in Table II.

Inferential analysis. The data on associations between the analysed polymorphisms and clinicopathologic tumour features is shown in Tables SI-SVI. In the single-locus analysis, the genotype model revealed a significant link between the IL-6 rs1800797 genotypes and the oestrogen receptor (\( P=0.005 \)) and the progesterone receptor (\( P=0.007 \)) status (Bonferroni adjusted; significant \( p \) value <0.008). The allelic model showed that the A allele of this SNP is associated with positive oestrogen receptors (\( OR=2.23 ; 95\% \ CI: 1.19-4.08; \ P=0.014 \)). Patients carrying the IL-6 rs1800797 A allele were also predisposed to higher rates of HER2 negative BC (\( OR=2.21 ; 95\% \ CI: 1.07-4.57; \ P=0.042 \)). Additionally, another IL-6 polymorphism, rs1800795, in both genotype and allelic models was linked to oestrogen receptor positive BC. Specifically, 72% of patients carrying the IL-6 rs1800795 C allele had oestrogen receptor positive disease, compared to 58% of non-carriers (\( OR=1.92 ; 95\% \ CI: 1.04-3.57; \ P=0.04 \)).

Linkage disequilibrium analysis showed a high degree of disequilibrium between the two IL-6 SNPs (\( r^2=0.89 \)), meaning that the associations were not independent. As such, haplotype analysis was performed to further explore the relationship between IL-6 variations and other prognostic factors. Phasing revealed three possible IL-6 (rs1800797, rs1800795) haplotypes: GG (51.7%), AC (45.5%) and GC (2.8%). It was further demonstrated that the AC haplotype was positively associated with oestrogen receptor positive BC (HR 2.13; 95% CI 1.14-3.98; \( P=0.018 \); Table SII).

| Factor                              | Patients, % |
|-------------------------------------|-------------|
| Age at diagnosis, years             |             |
| <50 years                           | 65          |
| ≥50 years                           | 35          |
| Tumour size, cm                     |             |
| <2                                  | 64          |
| 2-5                                 | 36          |
| Lymph node status                   |             |
| Positive                            | 55          |
| Negative                            | 45          |
| Grade                               |             |
| G1 and G2                           | 78          |
| G3                                  | 22          |
| ER status                           |             |
| ER positive                         | 68          |
| ER negative                         | 32          |
| PR status                           |             |
| PR positive                         | 60          |
| PR negative                         | 40          |
| HER2 status                         |             |
| HER2 positive                       | 19          |
| HER2 negative                       | 81          |

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.
By analysing the associations between the \textit{IL-1\textalpha} SNP and clinicopathologic factors (Table SIII), we found a link between the \textit{IL-1\textalpha} rs1800587 SNP C allele and larger primary tumour size (comparing tumours sized <2 vs. 2-5 cm) (OR 4.91; 95% CI: 1.09-22.01; P=0.022).

Other analysed polymorphisms of \textit{IL-1\beta} and \textit{VEGFA} revealed no associations with the evaluated prognostic factors in neither genotype nor allelic models (Tables SIV-SVI). Due to the strong linkage disequilibrium, four \textit{VEGFA} polymorphisms were also analysed in the haplotype model. Three main haplotypes were identified: CCTC (45.5%), ATCC (33.4%) and ATCT (17.6%), as well as several rare variants. None of the \textit{VEGFA} haplotypes was associated with clinicopathologic prognostic variables.

\textbf{Survival analysis.} In the mean follow-up time of 67 months (range 28-202), progression of the disease was observed for 33 patients. Of those who progressed, 28 had distant metastases. Twenty-two patients with progressive disease died, all due to cancer. The Kaplan-Meier survival analysis showed that the \textit{IL-1\alpha} rs1800587 SNP is associated with early-stage breast cancer DFS, MFS and OS. In particular, patients homozygous for the C allele (CC) had worse survival rates than patients homozygous and heterozygous for the A allele (CT and TT) (Fig. 2A-C).

The univariate Cox regression analysis (presented in Tables III-V) revealed that \textit{L-1\alpha} rs1800587 CC genotype carriers had 2.48 times higher risk of disease recurrence (95% CI: 1.09-22.01; P=0.022).

Other analysed polymorphisms of \textit{IL-1\beta} and \textit{VEGFA} revealed no associations with the evaluated prognostic factors in neither genotype nor allelic models (Tables SIV-SVI). Due to the strong linkage disequilibrium, four \textit{VEGFA} polymorphisms were also analysed in the haplotype model. Three main haplotypes were identified: CCTC (45.5%), ATCC (33.4%) and ATCT (17.6%), as well as several rare variants. None of the \textit{VEGFA} haplotypes was associated with clinicopathologic prognostic variables.

\begin{table}
\centering
\begin{tabular}{lcccc}
\textbf{Gene} & \textbf{Polymorphism} & \textbf{Study allele and genotype frequencies} \\
 & & (1000 Genomes Project Phase 3 database allele frequencies) & \\
\hline
\textit{VEGFA} & rs699947 & A, 0.53 (0.50) & C, 0.47 (0.50) & AA, 0.28 \\
 & rs833061 & T, 0.47 (0.50) & C, 0.53 (0.50) & TT, 0.21 \\
 & rs25648 & C, 0.81 (0.83) & T, 0.19 (0.17) & TT, 0.03 \\
 & rs1005230 & T, 0.52 (0.50) & C, 0.48 (0.50) & TT, 0.28 \\
\textit{IL-6} & rs1800795 & C, 0.47 (0.42) & G, 0.53 (0.58) & CC, 0.24 \\
 & rs1800797 & A, 0.46 (0.41) & G, 0.54 (0.59) & AA, 0.23 \\
\textit{IL-1\beta} & rs1143634 & G, 0.73 (0.75) & A, 0.27 (0.25) & GG, 0.52 \\
 & rs16944 & G, 0.66 (0.65) & A, 0.34 (0.35) & GG, 0.43 \\
\textit{IL-1\alpha} & rs1800587 & C, 0.69 (0.71) & T, 0.31 (0.29) & CC, 0.48 \\
\hline
\end{tabular}
\caption{Allele and genotype frequencies of analysed single nucleotide polymorphisms in the study population and in European population data from the 1000 Genomes Project Phase 3 database.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Linkage disequilibrium data for \textit{VEGFA, IL-1\beta} and \textit{IL-6} single nucleotide polymorphisms. Numerical values are given of D' values, whereas the colors are given to encode r2 (dark grey encodes high r2). VEGFA, vascular endothelial growth factor A; IL, interleukin.}
\end{figure}
Table III. Cox’s univariate model for IL-1β and IL-1α SNPs.

| Reference SNP ID | Model       | Genotype/allele/ haplotype | Patients n | PFS Univariate hazard ratio (95% CI) | P-value | MFS Univariate hazard ratio (CI) | P-value | OS Multivariate hazard ratio (95% CI) | P-value |
|------------------|-------------|-----------------------------|------------|--------------------------------------|---------|----------------------------------|---------|--------------------------------------|---------|
| **IL-1β rs1143634** | Genotype   | AA                          | 12         | 1                                    | 0.043   | 1                                | 0.062   | 1                                    | 0.271   |
|                  |            | GA                          | 85         | 0.443 (0.094-2.100)                  | 0.305   | 0.945 (0.116-7.688)              | 0.360   | 0.773 (0.093-6.421)                  | 0.811   |
|                  |            | GG                          | 105        | 1.243 (0.292-5.291)                  | 0.768   | 2.555 (0.343-19.048)             | 0.958   | 1.667 (0.220-12.630)                 | 0.621   |
|                  | Allelic    | A allele carriers           | 97         |                                      |         | 2.490 (0.944-5.246)             | 0.056   | 2.686 (0.932-6.102)                  | 0.058   |
|                  |            | A allele non carriers       | 105        |                                      |         | 1                                |         |                                      |         |
|                  |            | G allele carriers           | 190        |                                      |         | 1                                |         |                                      |         |
|                  |            | G allele non carriers       | 12         |                                      |         | 1.171 (0.279-4.913)             | 0.829   | 0.563 (0.076-4.148)                  | 0.573   |
| **IL-1β rs16944** | Genotype   | AA                          | 20         |                                      |         | 1                                |         |                                      |         |
|                  |            | GA                          | 95         | 1.657 (0.387-7.096)                  | 0.496   | 1.386 (0.320-6.005)              | 0.663   | 1.183 (0.264-5.287)                  | 0.826   |
|                  |            | GG                          | 87         | 0.974 (0.216-4.396)                  | 0.973   | 0.763 (0.165-3.533)              | 0.729   | 0.814 (0.173-3.834)                  | 0.794   |
|                  | Allelic    | A allele carriers           | 115        |                                      |         | 1.623 (0.302-1.294)             | 0.201   | 0.573 (0.259-1.267)                  | 0.169   |
|                  |            | A allele non carriers       | 87         |                                      |         | 0.623 (0.302-1.294)             | 0.201   | 0.573 (0.259-1.267)                  | 0.169   |
|                  |            | G allele carriers           | 182        |                                      |         | 1                                |         |                                      |         |
|                  |            | G allele non carriers       | 20         |                                      |         | 0.754 (0.180-3.155)             | 0.70    | 0.926 (0.220-3.905)                  | 0.917   |
| **IL-1α rs1800587** | Genotype | TT                          | 18         |                                      |         | 1                                |         |                                      |         |
|                  |            | CT                          | 87         | 0.805 (0.174-3.729)                  | 0.782   | 1.292 (0.159-10.506)            | 0.811   | 1.015 (0.122-8.428)                  | 0.989   |
|                  |            | CC                          | 97         | 2.067 (0.486-8.794)                  | 0.326   | 3.891 (0.521-29.034)            | 0.185   | 2.664 (0.352-20.187)                 | 0.343   |
|                  | Allelic    | T allele carriers           | 105        |                                      |         | 1                                |         |                                      |         |
|                  |            | T allele non carriers       | 97         | 2.480 (1.199-5.114)                  | 0.014a  | 3.122 (1.372-7.104)             | 0.007a  | 2.632 (1.072-6.461)                  | 0.035a  |
|                  |            | C allele carriers           | 184        |                                      |         | 1                                |         |                                      |         |
|                  |            | C allele non carriers       | 18         | 0.703 (0.168-2.940)                  | 0.630   | 0.392 (0.053-2.888)             | 0.358   | 0.551 (0.074-4.096)                  | 0.560   |

*Significant. Unadjusted hazard ratios for PFS, MFS and OS with each of the SNPs in genotype and allelic model. IL, interleukin; PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival; SNP, single nucleotide polymorphism.
Table IV. Cox’s univariate model for *IL-6* SNPs.

| Reference SNP ID | Genotype/allele/haplotype | Patients, n | Genotype | PFS Univariate hazard ratio (95% CI) P-value | MFS Univariate hazard ratio (95% CI) P-value | OS Univariate hazard ratio (95% CI) P-value |
|------------------|---------------------------|-------------|----------|--------------------------------------------|---------------------------------------------|----------------------------------------|
| *IL-6* rs1800795 | Genotype CC               | 48          | Genotype | 1                                           | 0.239                                       | 1                                       |
|                  | CC                         |             |          | 0.239                                       | 1                                           | 0.160                                   |
|                  | GC                         | 95          |          | 0.484                                       | 0.983 (0.336-2.881) 0.972                   | 1.216 (0.321-4.560) 0.779               |
|                  | GG                         | 49          |          | 0.119                                       | 2.053 (0.736-5.761) 0.174                   | 2.842 (0.798-10.193) 0.109              |
| Allelic C allele carriers | 143          | 1          |          | 1                                           | 1                                           | 1                                       |
|                  | C allele non carriers      | 49          |          | 0.114                                       | 2.074 (0.983-4.375) 0.055                   | 2.484 (1.075-5.737) 0.033*              |
|                  | G allele carriers          | 144         |          | 1                                           | 1                                           | 1                                       |
|                  | G allele non carriers      | 48          |          | 0.245                                       | 0.716 (0.272-1.888) 0.499                   | 0.552 (0.163-1.866) 0.339               |
| Allelic A allele carriers | 141          | 1          |          | 1                                           | 1                                           | 1                                       |
|                  | A allele non carriers      | 61          |          | 0.245                                       | 0.716 (0.272-1.888) 0.499                   | 0.552 (0.163-1.866) 0.339               |
|                  | G allele non carriers      | 46          |          | 0.339                                       | 0.610 (0.232-1.606) 0.317                   | 0.458 (0.135-1.548) 0.209               |
| *IL-6* rs1800797 | Genotype AA               | 46          | Genotype | 1                                           | 0.170                                       | 1                                       |
|                  | AA                         |             |          | 1                                           | 1                                           | 1                                       |
|                  | GA                         | 95          |          | 0.296                                       | 1.173 (0.406-3.441) 0.771                   | 1.536 (0.418-5.783) 0.528               |
|                  | GG                         | 61          |          | 0.067                                       | 2.361 (0.841-6.627) 0.104                   | 3.174 (0.858-11.355) 0.077              |
| Allelic A allele carriers | 141          | 1          |          | 1                                           | 1                                           | 1                                       |
|                  | A allele non carriers      | 61          |          | 0.245                                       | 0.716 (0.272-1.888) 0.499                   | 0.552 (0.163-1.866) 0.339               |
|                  | G allele carriers          | 156         |          | 1                                           | 1                                           | 1                                       |
|                  | G allele non carriers      | 46          |          | 0.245                                       | 0.716 (0.272-1.888) 0.499                   | 0.552 (0.163-1.866) 0.339               |
| *IL-6* ts1800797- rs1800795 | Diplole AC/AC            | 48          |          | 0.339                                       | 0.610 (0.232-1.606) 0.317                   | 0.458 (0.135-1.548) 0.209               |
|                  | AC/AC                      |             |          | 1                                           | 0.292                                       | 1                                       |
|                  | GG/AC                      | 80          |          | 0.328                                       | 1.125 (0.383-3.305) 0.830                   | 1.141 (0.382-5.434) 0.589               |
|                  | GG/GG                      | 63          |          | 0.143                                       | 1.953 (0.695-5.485) 0.204                   | 2.555 (0.711-9.161) 0.150               |
| Haplotype AC haplotype carriers | 136          | 1          |          | 1                                           | 1                                           | 1                                       |
|                  | AC haplotype non carriers  | 60          |          | 0.094                                       | 2.142 (1.021-4.514) 0.045*                  | 2.455 (1.062-5.661) 0.035*              |
|                  | GG haplotype carriers      | 149         |          | 1                                           | 1                                           | 1                                       |
|                  | GG haplotype non carriers  | 48          |          | 0.219                                       | 1.438 (0.546-3.791) 0.462                   | 1.894 (0.560-6.401) 0.304               |

*Significant. Unadjusted hazard ratios for PFS, MFS and OS with each of the SNPs in genotype, allelic and haplotype models. IL-6, interleukin-6; PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival; SNP, single nucleotide polymorphism.
risk of disease recurrence, metastasis and death (Table IV; Fig. 3A and B).

In a multivariate Cox regression model including age at diagnosis, tumour size, lymph node status, tumour differentiation grade, oestrogen receptor, progesterone receptor and HER2 status, the IL-1α rs1800587 CC genotype remained a significant predictor of poor DFS, MFS and OS. Furthermore, we confirmed a negative prognostic value of the IL-6 rs1800797-GG/CC diplotype on DFS, MFS and OS (Table VI). Finally, other polymorphisms and VEGFA haplotypes were not associated with any of the survival endpoints.

Discussion

In the present study, we investigated the associations between nine functional SNPs in four cytokine genes (i.e., VEGFA, IL-1α, IL-1β and IL-6) and the clinicopathologic profiles and survival rates in a group of Lithuanian women with early-stage BC. We found an association between the IL-6 SNPs and the oestrogen receptor positive, progesterone receptor positive and HER2 negative status, and a link between IL-1α SNP and larger primary tumour size. Furthermore, we confirmed a negative prognostic value of the IL-6 rs1800797-GG/CC diplotype on DFS, MFS and OS and of the IL-1α rs1800587 CC genotype on DFS, MFS and OS in a highly homogeneous group of patients.

Several authors have demonstrated that carrying the IL-6 rs1800795 G or IL-6 rs1800795 C alleles is associated with higher IL-6 protein production (19‑21). Specifically, IL-6 is a cytokine which is considered a prognostic marker as well as a potential therapeutic target for BC patients. This cytokine acts through several pathways, regulating the proliferation, apoptosis and metabolism of BC cells. However, the most important role of IL-6 in breast carcinogenesis is its potential to induce breast metastasis by enhancing angiogenesis and tumour cell migration (39). According to previous studies, IL-6 rs1800797 and rs1800795 polymorphisms appear to be biologically important; however, the data on their clinical importance is still heterogeneous.

The association of IL-6 polymorphisms with BC survival was analysed in several studies (Table VII). Snoussi et al (32) and DeMichele et al (27) analysed patients with non-metastatic stage I‑III BC. In both studies the IL-6 rs1800795 GG genotype was associated with decreased DFS and OS. In DeMichele et al (27) the presence of at least one copy of the haplotype rs1800797G-rs1800796G-373(10A/11T)-rs1800795G was associated with worse DFS. Snoussi et al (32) also found a significant link between IL-6 rs1800797 GG polymorphism and decreased DFS and OS. However, in DeMichele et al (28), where only locally advanced stage III patients with more than 10 positive lymph nodes were studied, the results were not replicated. Specifically, there was no impact of IL-6 polymorphisms on DFS and OS in the study population. In the same study, only an unplanned sub-analysis showed an association of the rs1800795 GG and rs1800797 GG genotypes with lower DFS but not OS in the oestrogen receptor positive patient subgroup. Our study is the first to confirm the prognostic value of IL-6 polymorphisms (namely, the IL-6 GG/GG diplotype) on DFS and OS in the study population.

The above-mentioned studies by Snoussi et al (32) and DeMichele et al (27,28) did not analyse MFS as a survival endpoint. To the best of our knowledge, the only study that evaluated the association between IL-6 polymorphisms and BC metastasis was a case-control study conducted by Abana et al (26). The authors demonstrated the significant link between the IL-6 rs1800795 GG genotype and the development of BC metastases with an OR of 1.52. However, due to case-control design, we have no data on MFS differences. In our study IL-6 GG/GG predicted MFS and OS, but not DFS.

Taking into account the critical role of IL-6 in the development
Table V. Cox’s univariate model for VEGFA SNPs.

| Reference SNP ID | Model | Genotype/allele/ haplotype | Patients, n | PFS | MFS | OS |
|------------------|-------|---------------------------|-------------|-----|-----|----|
|                  |       |                           |             |     |     |     |
|                  |       | Genotype AA               | 56          | 1   | 1   | 0.373 | 1 | 0.538 | 1 | 0.162 |
|                  |       | CA                        | 101         | 1.483 (0.616-3.565) | 0.387 | 1.104 (0.447-2.754) | 0.317 | 1.141 | (0.383-3.414) | 0.813 |
|                  |       | CC                        | 45          | 2.03 (0.76-5.47) | 0.160 | 1.689 (0.613-4.622) | 0.845 | 2.524 | (0.822-7.723) | 0.105 |
|                  |       |                           |             |     |     |     |
|                  |       | Allelic A allele carriers  | 157         | 1   | 1   | 1   |
|                  |       | A allele non carriers     | 45          | 1.568 (0.727-3.383) | 0.251 | 1.585 (0.697-3.604) | 0.272 | 2.322 | (0.973-5.543) | 0.058 |
|                  |       | C allele carriers         | 146         | 0.613 (0.266-1.415) | 0.252 | 0.791 (0.336-1.864) | 0.592 | 1 | 0.651 |
|                  |       | C allele non carriers     | 56          | 1   | 1   | 1   |
|                  |       |                           |             |     |     |     |
|                  |       | Genotype CC               | 56          | 1   | 1   | 0.295 | 1 | 0.430 | 1 | 0.115 |
|                  |       | TC                        | 103         | 1.445 (0.600-3.499) | 0.410 | 1.081 (0.429-2.703) | 0.875 | 1.124 | (0.377-3.344) | 0.842 |
|                  |       | TT                        | 43          | 2.189 (0.813-5.892) | 0.121 | 1.801 (0.652-4.974) | 0.257 | 1 | 0.694 |
|                  |       |                           |             |     |     |     |
|                  |       | Allelic C allele carriers | 159         | 1   | 1   | 1   |
|                  |       | C allele non carriers     | 43          | 1.709 (0.792-3.687) | 0.172 | 1.718 (0.755-3.905) | 0.197 | 2.511 | (0.952-5.994) | 0.058 |
|                  |       | T allele carriers         | 146         | 1   | 1   | 1   |
|                  |       | T allele non carriers     | 56          | 0.609 (0.264-1.405) | 0.245 | 0.786 (0.334-1.853) | 0.583 | 1 | 0.649 |
|                  |       |                           |             |     |     |     |
|                  |       | Genotype TT               | 57          | 1   | 1   | 0.438 | 1 | 0.657 | 1 | 0.268 |
|                  |       | CT                        | 99          | 1.553 (0.646-3.755) | 0.327 | 1.153 (0.465-2.891) | 0.763 | 1.204 | (0.402-3.593) | 0.740 |
|                  |       | CC                        | 46          | 1.894 (0.702-5.083) | 0.207 | 1.584 (0.571-4.355) | 0.381 | 2.281 | (0.753-6.974) | 0.149 |
|                  |       |                           |             |     |     |     |
|                  |       | Allelic C allele carriers | 57          | 1   | 1   | 1   |
|                  |       | C allele non carriers     | 145         | 0.604 (0.262-1.393) | 0.237 | 0.779 (0.331-1.835) | 0.568 | 1 | 0.647 |
|                  |       | T allele carriers         | 156         | 1   | 1   | 1   |
|                  |       | T allele non carriers     | 46          | 2.034 (0.857-4.844) | 0.110 | 1.440 (0.634-3.269) | 0.384 | 2.031 | (0.851-4.844) | 0.110 |
Table V. Continued.

| Reference SNP ID | Model   | Genotype/allele/haplotype | Patients, n | PFS Univariate hazard ratio (95% CI) | P-value | MFS Univariate hazard ratio (95% CI) | P-value | OS Univariate hazard ratio (95% CI) | P-value |
|------------------|---------|----------------------------|-------------|-------------------------------------|---------|--------------------------------------|---------|-------------------------------------|---------|
|                  |         | VEGF A rs25648 Genotype    |             |                                     |         |                                     |         |                                     |         |
|                  |         | CC                         | 131         |                                     | 0.425   |                                     | 0.727   |                                     | 0.602   |
|                  |         | CT                         | 65          | 0.604 (0.271-1.345)                 | 0.217   | 0.796 (0.348-1.820)                 | 0.589   | 0.706 (0.274-1.819)                 | 0.471   |
|                  |         | TT                         | 6           | 1.373 (0.185-10.181)               | 0.756   | 1.706 (0.228-12.794)               | 0.603   | 1.861 (0.246-14.087)               | 0.548   |
|                  |         | Allelic T allele carriers  | 71          |                                     | 1       |                                     | 1       |                                     | 1       |
|                  |         | T allele non carriers      | 131         | 1.553 (0.721-3.343)               | 0.261   | 1.181 (0.534-2.612)               | 0.681   | 1.291 (0.526-3.168)               | 0.577   |
|                  |         | C allele carriers          | 196         |                                     | 1       |                                     | 1       |                                     | 1       |
|                  |         | C allele non carriers      | 6           | 1.593 (0.217-11.703)             | 0.647   | 1.835 (0.248-13.556)             | 0.552   | 2.083 (0.280-15.494)             | 0.474   |

Unadjusted hazard ratios for PFS, MFS and OS with each of the SNPs in genotype and allelic model. VEGF A, vascular endothelial growth factor A; SNP, single nucleotide polymorphism; PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival.
Table VI. Cox's multivariate model.

| Variable                        | Patients, n | PFS Multivariate hazard ratio (95% CI) | P-value | MFS Multivariate hazard ratio (95% CI) | P-value | OS Multivariate hazard ratio (95% CI) | P-value |
|---------------------------------|-------------|----------------------------------------|---------|----------------------------------------|---------|---------------------------------------|---------|
| Age at diagnosis, years         |             |                                        |         |                                        |         |                                      |         |
| ≥50                             | 71          | 1                                      |         | 1                                      |         | 1                                    |         |
| <50                             | 131         | 7.523 (0.990-56.690) 0.049<sup>a</sup> |         | 6.081 (0.786-46.619) 0.082              |         | 9.237 (0.983-57.232) 0.091           |         |
| Tumor size, cm                  |             |                                        |         |                                        |         |                                      |         |
| <2                              | 129         | 1                                      |         | 1                                      |         | 1                                    |         |
| 2-5                             | 73          | 1.102 (0.583-2.399) 0.795              |         | 1.018 (0.522-1.824) 0.631              |         | 1.021 (0.529-1.919) 0.669           |         |
| Lymph node status               |             |                                        |         |                                        |         |                                      |         |
| Negative                        | 111         | 2.331 (1.086-4.989) 0.030<sup>a</sup> |         | 2.413 (1.051-5.587) 0.039<sup>a</sup> |         | 2.151 (0.832-5.560) 0.115           |         |
| Positive                        | 91          |                                        |         |                                        |         |                                      |         |
| Grade                           |             |                                        |         |                                        |         |                                      |         |
| G1 and G2                       | 158         | 1                                      |         | 1                                      |         | 1                                    |         |
| G3                              | 44          | 2.281 (0.916-5.719) 0.079              |         | 2.145 (0.815-5.628) 0.123              |         | 1.904 (0.636-5.645) 0.247           |         |
| Estrogen receptor status        |             |                                        |         |                                        |         |                                      |         |
| Positive                        | 137         | 1                                      |         | 1                                      |         | 1                                    |         |
| Negative                        | 65          | 1.182 (0.482-2.939) 0.721              |         | 1.304 (0.496-3.455) 0.594              |         | 1.580 (0.545-4.555) 0.395           |         |
| Progesterone receptor status    |             |                                        |         |                                        |         |                                      |         |
| Positive                        | 121         | 3.088 (1.193-7.993) 0.020<sup>a</sup> |         | 3.284 (1.164-9.228) 0.025<sup>a</sup> |         | 4.670 (1.315-16.435) 0.018<sup>a</sup> |         |
| Negative                        | 82          |                                        |         |                                        |         |                                      |         |
| Human epidermal growth factor   |             |                                        |         |                                        |         |                                      |         |
| receptor 2 status              |             |                                        |         |                                        |         |                                      |         |
| Negative                        | 164         | 1                                      |         | 1                                      |         | 1                                    |         |
| Positive                        | 38          | 2.393 (0.841-6.817) 0.104              |         | 1.904 (0.641-5.621) 0.246              |         | 1.718 (0.518-5.735) 0.376           |         |
| IL-6 haplotype                  |             |                                        |         |                                        |         |                                      |         |
| AC haplotype carriers           | 128         | -                                      |         | 1                                      |         | 1                                    |         |

<sup>a</sup> Denotes statistical significance.
of BC metastasis and our study results, we propose the hypothesis that IL‑6 polymorphisms may predict the development of metastasis rather than loco‑regional relapse.

It is important to mention that, in contrast with the findings of the above‑mentioned studies, Markkula et al (29), who analysed a Swedish cohort of patients with any primary stage non‑metastatic BC, demonstrated that the carriers of the C allele in rs1800795 SNP had a higher risk of early BC events. The authors, however, conducted no DFS, MFS or OS analyses. Furthermore, none of the GWAS studies showed a statistically significant association between IL‑6 polymorphisms and BC survival (23,24). The contrasting findings in IL‑6 polymorphism studies may be due to sampling errors or differences in patient ethnicity. Nevertheless, most of the experimental data and the possible biological pathway support our findings.

IL‑6 also acts as a regulator of oestrogen synthesis and may modulate the tumour cell growth related to the hormone receptor status (40). Hormone‑sensitive cells exhibit a higher response to IL‑6, while ER‑negative cells are suppressed by IL‑6 (41). However, there is no clear mechanism of association between IL‑6 SNPs and the oestrogen receptor positive, progesterone receptor positive and HER2 negative status. Therefore, further studies are needed.

Table VI. Continued.

| Patients, n | Multivariate hazard ratio (95% CI) | Multivariate hazard ratio (95% CI) | Multivariate hazard ratio (95% CI) |
|------------|-----------------------------------|-----------------------------------|-----------------------------------|
| AC haplotype non carriers (GG/GG) | 63 | ‑ ‑ | 2.750 (1.184‑7.690) |
| IL‑1α rs1800587 T allele carriers (CT+TT genotypes) | 105 | 2.704 (1.280‑5.683) | 0.009 |
| T allele non carriers (CC genotype) | 97 | ‑ ‑ | 2.750 (1.184‑7.690) |

Significant ‑, not significant in univariate analysis. Adjusted hazard ratios for PFS, MFS and OS. PFS, progression‑free survival; MFS, metastasis‑free survival; OS, overall survival.

Figure 3. Kaplan‑Meier survival curves for the IL‑6 haplotype (rs1800797, rs1800795). (A) MFS and (B) OS. IL‑6, interleukin‑6; MFS, metastasis‑free survival; OS, overall survival.
Table VII. Studies evaluating the associations of IL-6 rs1800795, IL-6 rs1800797 and IL-1α rs1800587 single nucleotide polymorphisms with breast cancer survival.

| Author, year       | Polymorphism            | Study design | Number (inclusion criteria) | Ethnicity                  | Finding                                                                 | (Refs.) |
|--------------------|-------------------------|--------------|-----------------------------|----------------------------|-------------------------------------------------------------------------|---------|
| Abana et al., 2017 | IL-6 rs1800795          | Case/control| 157/158 (stage IV vs. stage I-III) | Non-Hispanic white       | GG genotype with distant metastasis                                     | (26)    |
| DeMichele et al., 2003 | IL-6 rs1800795          | Cohort       | 80 (stage I-III)            | Various                    | GG genotype with worse DFS and OS                                      | (27)    |
| DeMichele et al., 2009 | IL-6 rs1800795,       | Cohort       | 346 (non-metastatic, ≥10 positive nodes) | Various                    | GG genotypes of both SNPs with worse DFS in estrogen receptor positive subgroup | (28)    |
| Markkula et al., 2014 | IL-6 rs1800795          | Cohort       | 634 (stage I-III)           | No data                    | C allele in estrogen receptor negative cases with early events         | (29)    |
| Snoussi et al., 2005 | IL-6 rs1800795,       | Cohort       | 305 (stage I-III)           | North African              | GG genotypes in both SNPs with worse DFS, but not OS                  | (32)    |
| Grimm et al., 2009  | IL-1α rs1800587         | Cohort       | 262 (stage I-III)           | Caucasian                  | TT genotype with worse DFS and OS                                      | (31)    |
| Escała-García et al., 2019 | GWAS                  | Cohort       | 96.661 (stage I-IV)         | Various                    | C allele with OS in the univariate analysis only                       | (23)    |
| Khan et al., 2017   | GWAS                    | Meta-analysis| 3.136 (stage I-IV estrogen receptor positive) | Various                    | No association of IL-1α and IL-6 SNPs with breast cancer-specific mortality | (24)    |
| Present study      | IL-6 rs1800795,       | Cohort       | 202 (early stage I-II)      | Eastern-European (Lithuanian) | GG/GG diplotype with worse MFS and OS                                 | -       |
|                    | IL-1α rs1800587        |              |                             |                            | CC genotype with worse DFS, MFS and OS                                  |         |

GWAS, genome-wide association studies. IL, interleukin-1.
As far as IL-1α SNP is concerned, Um et al (18) demonstrated that the IL-1α rs1800587 CC genotype is associated with higher transcriptional activity of the IL-1α gene. Overexpression of the IL-1α promotes tumour invasiveness and metastasis by inducing the expression of angiogenic genes and growth factors (12). A large meta-analysis performed by Xia et al (30) showed that the IL-1α rs1800587 C allele and CC genotype are associated with increased cancer risk in general. The studies which investigated the associations between IL-1α rs1800587 polymorphism and BC survival are presented in Table VII. Grimm et al (31) analysed 262 Caucasian BC patients and found that the IL-1α rs1800758 C allele in the univariate model is associated with OS; however, the multivariate model failed to repeat the association. In our study, the multivariate survival analysis confirmed the statistically significant impact of the CC genotype on DFS, MFS and OS. Contrasting data is provided by Snoussi et al (32), who analysed North African BC patients and found that the rs1800587 homozygous TT genotype showed a significant association with reduced DFS and OS rate. However, the allele and genotype frequency of rs1800587 SNP in African population differs substantially from that in the population with European ancestry. This difference may be the cause of the observed inconsistency between these studies. We demonstrated that in an Eastern-European population the IL-1α rs1800758 C allele is associated with more aggressive local disease (i.e., larger tumour size) and for the first time we proposed that the IL-1α rs1800758 CC genotype is an independent negative prognostic biomarker for early-stage BC. As the allele frequencies of the IL-1α rs1800758 SNP in our study correspond to those of the 1000 Genomes project phase 3 database for European population, these findings could potentially be replicated for European population, but larger confirmatory studies are warranted.

Although experimental data suggests that VEGFA and IL-1β play a role in BC, our findings, along with the results from several other studies (22-24), do not suggest that several common polymorphisms in these genes are associated with BC clinical and morphological variables and BC survival rates. Potential limitations of our study include a limited sample size, lack of access to tumour and/or tumour stromal tissue (which would have allowed for the assessment of the presence of SNPs in those tissues), the risk of other confounders, possible gene-gene and gene-environment interactions, and non-random sampling. We also acknowledge that VEGFA, IL-1α, IL-1β and IL-6 measurements were not available in the current study. However, our study supports the relevance of functional IL-6 and IL-1α germline polymorphisms to BC prognosis. Further investigations, preferable on larger cohorts with different ethnic origins, are needed to confirm the results of the current study.

In conclusion, we found an association between the IL-1α rs1800587 C allele and larger primary tumour size. The IL-6 rs1800797 A allele, GA genotype, IL-6 rs1800795 C allele and IL-6 (rs1800797-re1800797) AC diplotype were linked to hormonal receptor positive BC. Additionally, IL-6 rs1800797 A was associated with HER2 negative status. The multivariate IL-1α rs1800587 CC genotype was confirmed as an independent negative prognostic factor for DFS, MFS and OS, and the IL6 GG/GG diplotype for MFS and OS in early-stage BC patients. Our findings confirm the hypothesis that functional SNPs in angiogenesis- and inflammation-associated genes are associated with early-stage BC prognosis in Lithuanian population.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

EK, RU and EJu designed the research study. EK, RU, RI, LP, VR, EJa and EJu performed the research. EK and RU analyzed the data. RU and RI contributed essential reagents or tools. EK wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by Kaunas Regional Ethics Committee for Biomedical Research (protocol no. BE-2-10). Informed consent for participation in the study and use of tissue was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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