Abstract. Background: Vascular endothelial growth factor A (VEGFA) is essential in tumour angiogenesis, and polymorphisms in the VEGFA gene have been associated with breast cancer prognosis. The human epidermal growth factor receptor 2 (HER2) is overexpressed in breast tumours and is also associated with angiogenesis. We investigated the possible prognostic impact of VEGFA single nucleotide polymorphisms (SNPs) in patients with HER2-positive primary breast cancer. Patients and Methods: DNA was isolated from venous blood samples from 116 HER2-positive patients and genotyped for VEGFA -2578C>A, -1498T>C, -1154G>A, -634G>C, -7C>T and +936C>T SNPs using the TaqMan® SNP Genotyping Assay. Results: The -2578C>A and -634G>C genotypes were associated with tumour size, p≤0.014. In univariate analysis -2578CC, -634CC and -7CC genotypes were associated with inferior recurrence-free survival (p≤0.028) but in cox multivariate analysis, only the -634CC genotype remained an independent prognostic factor (p=0.008). Conclusion: The VEGFA -634CC genotype was found to be associated with an inferior prognosis for patients with HER2-positive breast cancer.

Vascular endothelial growth factor A (VEGFA) is a very potent pro-angiogenic growth factor, and is essential for tumour growth and metastasis (1). VEGFA expression is induced by hypoxia (2) and the protein interacts with its membrane-bound tyrosine kinase receptors, VEGFR1 and VEGFR2. Ligand binding mediates intracellular tyrosine kinase phosphorylation and activates intracellular downstream pathways (3), which induces vascular permeability, and endothelial cell proliferation, migration and survival (4, 5). VEGFA and its receptors are expressed in a wide range of tissues and are highly expressed in malignant breast tumours and other solid tumour types (6). Several studies have reported an association of poor prognosis with VEGFA overexpression (7), but the clinical relevance is still debatable (8).

This might, in part, be explained by the challenge of reliable VEGFA measurements. Immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) are based on measurement of proteins, which are unstable and prone to degradation in cases of inappropriate conservation. Several IHC and ELISA methods have been applied to tumour tissue (9), serum and plasma samples (6), but no validated standardized methods have been established to quantify VEGFA. In contrast, DNA is rather resistant to degradation and assays of DNA produce robust measurements. Germline polymorphisms can be measured in DNA-containing blood samples. Hence, no tumour tissue needs to be available and the minimal invasive procedure makes SNP analyses very attractive.

Single nucleotide polymorphisms (SNPs) are the inherited variation of a specific nucleotide in the germline DNA sequence appearing with a frequency above 1% in a population. The VEGFA gene is located on the short arm of chromosome 6 and several SNPs have been identified in the promoter region, in the 5’ untranslated region (UTR) and in the 3’UTR. These gene regions are sensitive to hypoxia and regulate VEGFA gene expression (10, 11), while polymorphisms of this gene have been shown to alter VEGFA protein expression (12). VEGFA SNPs have also been associated with breast cancer risk (13-15), tumour characteristics (16, 17), prognosis (16, 18-21), and plasma and serum levels of VEGFA (22, 23).

The epidermal growth factor receptor (EGFR) family consists of four structurally related tyrosine kinase receptors. One of these receptors, human epidermal growth factor
receptor 2 (HER2), is overexpressed in 15-30% of breast tumours and is associated with tumour progression and adverse breast cancer prognosis (24). VEGFA and HER2 share some of the same intracellular tyrosine kinase pathways and several studies have demonstrated the significance of EGFR in tumour angiogenesis (25-27). For example, Laughner et al. (28) showed that HER2 signalling increased VEGFA mRNA expression, and Blackwell et al. showed that HER2 gene amplification was correlated with increased angiogenesis, as measured by microvessel density (MVD) (29). Furthermore, Konecny et al. (30) showed that a larger proportion of HER2/neu overexpressing tumours expressed high levels of VEGFA compared to tumours not overexpressing HER2/neu and that high VEGFA expression in combination with high HER2/neu expression was associated with an unfavourable prognosis.

This interaction between VEGFA and EGFR seems to be mediated through an indirect effect of other pro-angiogenic growth factors, matrix metalloproteinases (MMP), facilitating endothelial cell migration and invasion (25) and regulation of the hypoxia-responsive element in the VEGFA gene promoter region (31).

We hypothesized that VEGFA gene variation may have different influences on biological functions in patients with HER2-positive tumours. Therefore, the aim of the present study was to investigate the possible prognostic impact of VEGFA SNPs in patients with HER2-positive primary breast cancer.

Patients and Methods

Patients. This study included 116 patients with breast cancer who were identified as having a positive HerceptTest and/or HER2 (FISH) amplification of more than 2.0 in the time period between 2004 and 2009 at the Department of Pathology, Vejle Hospital. All patients were enrolled in a biomarker protocol either at the time of operation, or during the follow-up at the Department of Surgery or Department of Oncology, Vejle Hospital. All patients signed a written consent form. The study was approved by the Ethical Committee of Southern Denmark (project identification number VF-20040101 and VF-20040017) and complies with the Danish “Act on Processing of Personal Data” (Act no. 429 of 31st May 2000).

According to the national guidelines of the Danish Breast Cancer Group (DBCGR), adjuvant chemotherapy was administered to all pre-menopausal women, post-menopausal women below 60 years of age and patients with oestrogen receptor (ER)-negative tumours. Standard adjuvant chemotherapy was cyclophosphamide, epirubicin and fluorouracil before January 2007, and epirubicin combined with cyclophosphamide followed by docetaxel after January 2007. Patients were offered adjuvant targeted treatment with trastuzumab as standard treatment or according to experimental protocols.

Patients underwent radiotherapy at a dose of 48 Gy/24 fractions (before January 2009), or 50 Gy/25 fractions (following January 2009) in cases of breast-conservation surgery and/or axillary lymph node macrometastases. Patients aged 50 years or younger underwent additional boost dose radiotherapy of 10 Gy/5 fractions to the tumour bed.

Computed tomography of the chest and abdomen, chest X-ray, or bone scintigraphy was performed to exclude distant metastases. Patients were followed-up with routine check-ups every six months. Mammograms and ultrasound were offered every year in cases of breast-conservation surgery and every second year to patients treated with mastectomy. Clinicopathological data were obtained from the pathology reports at the Department of Clinical Pathology, Vejle Hospital, Denmark and clinical data were obtained from the patient files.

Histopathology and IHC. Diagnostic tumour tissue was routinely formalin-fixed (24 to 72 h), paraffin-embedded, and immunostained with Autostainer Link 48 (Dako), then evaluated according to the WHO classification of breast tumours (32). Tumour grade was scored according to modified Bloom Richardson criteria (33) and axillary lymph node metastasis was evaluated by the revised American Joint Committee staging system (34). Axillary dissection was performed if single-cell infiltration, micro- or macrometastases were present in sentinel node biopsies, but only micro- and macrometastases were regarded as node positivity. IHC staining of ER was performed routinely and tumours were classified as ER-positive when at least 10% of the cell nuclei stained positively. IHC staining of HER2 was performed with HerceptTest™ (Dako, Copenhagen, Denmark) and scored as recommended by the American Society of Clinical Oncology (35). A score of 0 and 1+ was regarded as HER2-negative, and 3+ regarded as HER2-positive. A score of 2+ was supplemented with fluorescent in situ hybridization (HER2 FISH pharmDX™ kit; Dako) and amplification of greater than 2.0 was regarded as HER2-positive.

Selection of VEGFA polymorphisms. VEGFA candidate polymorphisms were selected using the public National Center for Biotechnology Information (NCBI) SNP database and available literature reporting associations with breast cancer risk, VEGF expression, tumour aggressiveness, breast cancer prognosis and treatment response (13-20, 22, 36, 37). SNP -2578C>A (rs699947), -1498T>C (rs833061), -1154G>A (rs1570360) and -634G>C (rs2010963) were chosen for genotyping in the promoter region of the VEGFA gene. SNP -7C>T (rs25648) was selected in the 5’UTR and +936C>T (rs3025039) in the 3’UTR.

DNA isolation. Venous blood samples were collected in 3-ml EDTA-containing tubes preoperatively, or during follow-up. Blood samples were stored temporarily at −20°C for 24-72 h, followed by storage at −80°C. Genomic DNA for SNP analysis was isolated from 300 μl whole blood on a Maxwell 16 System using Maxwell® 16 Blood DNA Purification Kit (#AS1010; Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions, with the exception that elution was performed in 350 μl.

Genotyping. SNPs were genotyped using TaqMan® SNP Genotyping Assay (Applied Biosystems, Carlsbad, California, USA) according to the manufacturer’s instructions, with 2 μl purified DNA and genotyping Master Mix in a total volume of 10 μl (-2578C>A, -1498T>C, -1154G>A and +936C>T), or 20 μl (-634G>C and -7C>T). PCR amplification was performed with a 7900 HT Real-time PCR system (Applied Biosystems, Carlsbad, California, USA) at 50°C for 2 min and at 95°C for 10 min, followed by 40-50 cycles at 95°C for 15 s and 60°C for 1 min. Finally, fluorescence endpoint reading was performed and the allelic discrimination plot was provided.
visualized with the SDS software (Applied Biosystems, Carlsbad, California, USA). Each plate contained two non-DNA template controls and a positive control for each genotype. The quality value threshold was >95.0%.

The -1154G>A SNP were not recalled successfully for two patients and consequently sequenced. Forward (TTT TCA GCC TGT GAA CCT TG) and reverse (GAT CCT CCC CGC TAC CAG) primers were designed in-house using Primer3 Input, version 0.4.0 (available at http://primer3.sourceforge.net/) (38). AmpliTaq Gold® (Applied Biosystems, Carlsbad, California, USA) was used for PCR amplification, with 2 μl purified DNA, 0.5 μM forward primer and 0.5 μM reverse primer, 2.0 μl dimethyl sulfoxide (DMSO), 1.5 mM MgCl2, 0.2 mM dNTP, 0.04 U Gold Taq and 2.5 μl buffer at 95°C for 10 min and 37 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s, followed by 72°C for 10 min. Unused primers and nucleotides were removed from the PCR product with ExoSAP-IT (USB; Affymetrix, Santa Clara, California, USA). The PCR sequencing was performed with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, USA) using 1 μl PCR product, 1.5 μl BigDye sequencing buffer, 1 μl Terminator RR mix, 1 μl 1.6 μM forward or reverse primer, and 5.5 μl sterile H2O with cycling conditions as follows: 96°C for 1 min and 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

Ten microliters of PCR product were added to 45 μl of SAM solution (BigDye® XTerminator™ Purification Kit; Applied Biosystems, Carlsbad, California, USA) and to 10 μl BigDyeX Terminator (BigDye® X Terminator™ Purification Kit; Applied Biosystems, Carlsbad, California, USA) and the sequencing product was visualized by a 3130 Genetic Analyser (Applied Biosystems, Carlsbad, California, USA). A positive control for each genotype and a non-DNA template control were processed.

Statistics. Medians were compared using the Mann Whitney U-test and proportions were compared by the chi-square test or Fisher’s exact test, when appropriate. Chi-square test was also used to test for concordance with Hardy Weinberg equilibrium. Recurrence-free survival (RFS) was defined as the time from operation to the earliest radiological or pathological confirmation of local or distant recurrence, or death from any cause according to Hudis et al. (39). Univariate RFS was calculated by the Kaplan Meier method and survival curves were compared with log-rank statistics. Multivariate RFS analysis was performed by Cox proportional hazard regression adjusted for genotype, adjuvant chemotherapy, tumour size (≤>20 mm), axillary lymph node status and dichotomized histopathological tumour grade (grade 1 versus grade 2, 3 and unknown). Age at diagnosis, ER status, adjuvant radiotherapy, adjuvant endocrine therapy and adjuvant targeted treatment were excluded from the model because inclusion did not change the model considerably. Haplotypes were created from SNP with significant prognostic impact in univariate analysis using PHASE, version 2.1 (Matthew Stephens, Seattle, USA; http://www.stat.washington.edu/stephens/) (40, 41). However, haplotype analysis did not contribute any new information or new classification of patients in terms of prognostically favourable or unfavourable groups because of a high degree of linkage disequilibrium. p-Values <0.05 were considered statistically significant and all tests were two-sided. Statistical analyses were performed using the NCSS statistical software, version 07.1.15 (NCSS Statistical Software, Kaysville, Utah, USA), except for testing interaction and the proportional hazard assumption, which were analysed with Stata/IC 10.0 (StataCorp LP, College Station, Texas, USA).

### Results

**Patients’ characteristics.** Patients’ characteristics are shown in Table I. The median patient age was 54.5 years and the vast majority had ductal carcinoma. Twenty-five patients (21.6%) received no adjuvant chemotherapy because of age, performance status, or patient’s wish. Forty patients (34.5%) received no adjuvant-targeted treatment because of age, performance status, or unknown HER2 status at the time of administration of adjuvant treatment. Most patients were offered trastuzumab alone, or in combination with lapatinib, or neratinib. Five patients were offered lapatinib only.

### Table I. Clinicopathological characteristics of study patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (range)</th>
<th>Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>54.5 (26.0-84.6)</td>
<td>17 (14.7)</td>
</tr>
<tr>
<td>&lt;40</td>
<td>56 (48.3)</td>
<td>43 (37.1)</td>
</tr>
<tr>
<td>≥60</td>
<td>108 (93.1)</td>
<td>2 (1.7)</td>
</tr>
<tr>
<td>Tumour type</td>
<td></td>
<td>6 (5.2)</td>
</tr>
<tr>
<td>Ductal</td>
<td></td>
<td>13 (11.2)</td>
</tr>
<tr>
<td>Lobular</td>
<td></td>
<td>55 (47.4)</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>61 (52.6)</td>
</tr>
<tr>
<td>Tumour size, mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20</td>
<td>52 (44.8)</td>
<td>63 (54.3)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>5 (4.3)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>Tumour grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>47 (40.5)</td>
<td>69 (59.5)</td>
</tr>
<tr>
<td>2</td>
<td>25 (21.6)</td>
<td>39 (33.6)</td>
</tr>
<tr>
<td>3</td>
<td>35 (29.6)</td>
<td>45 (38.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (6.0)</td>
<td></td>
</tr>
<tr>
<td>Axillary lymph node metastasis, n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>76 (65.5)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40 (34.5)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>76 (65.5)</td>
<td></td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>56 (48.3)</td>
<td>60 (51.7)</td>
</tr>
<tr>
<td>CEF</td>
<td>19 (16.4)</td>
<td></td>
</tr>
<tr>
<td>EC+Doc</td>
<td>97 (83.6)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6 (5.2)</td>
<td></td>
</tr>
</tbody>
</table>

CEF: Cyclophosphamide, epirubicin and fluorouracil; EC+Doc: epirubicin and cyclophosphamide followed by docetaxel. †Seventy-one patients received trastuzumab alone, or in combination with lapatinib, or neratinib. Five patients were offered lapatinib only.

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5.1 years (range 2.6-18.6 years) for patients still alive at the 1st January of 2012. Thirty-six patients had a local or distant recurrence, 18 patients died from breast cancer and two patients died from other causes. In univariate analysis the -2578CC genotype conferred an inferior prognosis compared to -2578CA and -2578AA combined (p=0.028) (Figure 1A and 1D). The -634CC genotype was a significant predictor of adverse prognosis compared to -634CG and -634GG, both individually (p=0.006) and when combined (p=0.001) (Figure 1B and 1E). The -7CC genotype predicted adverse prognosis compared to -7CT and -7TT combined (p=0.015) (Figure 1C and 1F). Axillary lymph node status was the only clinicopathological characteristic that was revealed to be a prognostic indicator of RFS in univariate analysis (p=0.006). Patients who were offered adjuvant chemotherapy had a better prognosis than patients who did not receive adjuvant chemotherapy (p=0.017), but administration of adjuvant-targeted treatment did not significantly improve RFS (p=0.110), although a trend was seen in the Kaplan Meier analysis.

In multivariate analysis, the -634CC genotype remained an independent prognostic variable not only when -634CG and -634GG were combined in the model (p=0.008), but also when genotypes were entered in the model separately (Hazard ratio (HR)=2.57, 95% confidence interval (CI)=1.05-6.30, p=0.040). SNP -2578C>A and -7C>T failed as independent prognostic variables in multivariate analysis (-2578CC: p=0.056; -7CC: p=0.070). Results from multivariate analyses are shown in Table III. SNPs -1154G>A and +936C>T were not associated with RFS in univariate (-1154GG: p=0.968; +936C>T: p=0.878) nor in multivariate analysis (-1154GG versus GA+AA: HR=1.03, 95% CI=0.53-2.00, p=0.941; +936CC versus CT+TT: HR=0.88, 95% CI=0.38-2.03, p=0.765). No interaction was observed between SNPs, tumour grade, tumour size or axillary lymph node metastasis (p>0.174).

### Discussion

The treatment of breast cancer is based on prognostic and predictive markers. Therefore, reliable markers are needed to improve the selection of patients who are most likely to benefit from treatment, in that modern cancer therapy is expensive and can be associated with severe side-effects. We found that the VEGFA gene polymorphism might have potential as a prognostic marker in HER2-positive breast cancer, and to our knowledge, this is the first study to evaluate VEGFA SNPs in patients with HER2-positive disease.

The VEGFA -2578A allele and the -1498T allele have been associated with increased breast cancer risk (13), whereas the +936T allele has been related to reduced breast cancer risk and low plasma VEGFA levels (14, 15, 22). In an American study, invasive breast cancer, but not in situ cancer, was more frequent for those carrying the -2578C and -1154G alleles (17). Results are, however, inconsistent between studies and between cancer types (10).

We were able to identify five studies dealing with VEGFA SNPs and prognosis in breast cancer (16, 18-21). Kidd et al.

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### Table II. Genotypic and allelic frequencies of vascular endothelial growth factor A (VEGFA) gene.

<table>
<thead>
<tr>
<th>Polymorphism ID</th>
<th>Genotype</th>
<th>Genotypic frequency (No, %)</th>
<th>Allelic frequency (%): Allele A</th>
<th>Allele B</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2578C&gt;A</td>
<td>rs699947</td>
<td>CC 36 (31.0) GA 53 (45.7) AA 27 (23.3)</td>
<td>0.54 0.46</td>
<td></td>
</tr>
<tr>
<td>-1154G&gt;A</td>
<td>rs1570360</td>
<td>GG 62 (53.5) GA 45 (38.8) AA 9 (7.8)</td>
<td>0.73 0.27</td>
<td></td>
</tr>
<tr>
<td>-634G&gt;C</td>
<td>rs2010963</td>
<td>GG 48 (41.4) GC 51 (44.0) CC 17 (14.7)</td>
<td>0.63 0.37</td>
<td></td>
</tr>
<tr>
<td>-7C&gt;T</td>
<td>rs25648</td>
<td>CC 77 (66.4) CT 34 (29.3) TT 5 (4.3)</td>
<td>0.81 0.19</td>
<td></td>
</tr>
<tr>
<td>+936C&gt;T</td>
<td>rs3025039</td>
<td>CC 90 (77.6) CT 25 (21.6) TT 1 (0.9)</td>
<td>0.88 0.12</td>
<td></td>
</tr>
</tbody>
</table>

**Genotypes.** VEGFA SNP -2578C>A and -1498T>C were in 100% linkage disequilibrium. Because -2578C>A was the most cited SNP in the literature, results for VEGFA -2578C>A are shown only. All SNPs were in Hardy Weinberg equilibrium (p>0.500). Genotypic frequencies are shown in Table II.

**Association with clinicopathological characteristics.** The median tumour size was significantly larger for patients with -2578CC genotype (23.5 mm) compared to those with -2578CA and -2578AA combined (20 mm) (p=0.014). The median tumour size for those with -634CC genotype (30 mm) was also significantly larger than those for -634CG and -634GG combined (20 mm) (p=0.001). No difference in tumour size was observed for SNP -1154G>A, -7C>T or +963C>T. No associations were observed between genotypes and age groups (<35, 35-59, ≥60 years), tumour grade or axillary lymph node status (p>0.092). Patients with ER-negative and those with ER-positive tumours were equally distributed between genotypes (p=0.507). Administration of radiotherapy, adjuvant chemotherapy and adjuvant-targeted treatment were also equally distributed between the genotypes (p>0.134).

**Prognostic impact of SNPs.** The median follow-up time was 5.1 years (range 2.6-18.6 years) for patients still alive at the 1st January of 2012. Thirty-six patients had a local or distant recurrence, 18 patients died from breast cancer and two patients died from other causes. In univariate analysis the -2578CC genotype conferred an inferior prognosis compared to -2578CA and -2578AA combined (p=0.028) (Figure 1A and 1D). The -634CC genotype was a significant predictor of adverse prognosis compared to -634CG and -634GG, both individually (p=0.006) and when combined (p=0.001) (Figure 1B and 1E). The -7CC genotype predicted adverse prognosis compared to -7CT and -7TT combined (p=0.015) (Figure 1C and 1F). Axillary lymph node status was the only clinicopathological characteristic that was revealed to be a prognostic indicator of RFS in univariate analysis (p=0.006). Patients who were offered adjuvant chemotherapy had a better prognosis than patients who did not receive adjuvant chemotherapy (p=0.017), but administration of adjuvant-targeted treatment did not significantly improve RFS (p=0.110), although a trend was seen in the Kaplan Meier analysis.

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We were able to identify five studies dealing with VEGFA SNPs and prognosis in breast cancer (16, 18-21). Kidd et al.
evaluated the influence of sequence variants in angiogenic genes and their corresponding receptors [interleukin (IL-10), transforming growth factor β1 (TGF-β1) and VEGF] as predictors of prognosis in 441 node-positive and node-negative breast cancer cases. They found a trend for inferior disease-free survival (DFS) and overall survival (OS) for the \( VEGFA \)-2578CC genotype in both uni- and multivariate analyses, which is in accordance with our results. They also found that the addition of \( VEGFA \)-2578C>A genotype increased the predictive accuracy compared to models with standard clinicopathological diagnostic markers alone. Interestingly, the -634G>C SNP was not associated with either DFS or OS. Furthermore, the -2578A/-1154G/-634G haplotype was associated with a marginally improved prognosis. Surprisingly, the -2578C/-1154G/-634C haplotype was not associated with an increased risk of disease recurrence. Only the -2578C/-1154G/-634G haplotype was significantly related to an adverse prognosis.

In an unselected Swedish population, Jin et al. (16) found that the -634CC genotype was associated with larger tumour size and high tumour grade, whereas low tumour grade was associated with the -2578AA genotype. Furthermore, tumour size and tumour grade increased with increasing copy number of the -2578C/-634C haplotype. No association between genotype and distant metastasis was documented, but only a few recurrences were registered and no survival analysis was performed. We support the findings on the association between -634G>C and tumour size, but we observed no correlation with tumour grade.

In contrast to our findings, Balasubramanian et al. (20) found that the -7CC genotype conferred superior prognosis in univariate analysis compared to -7CT and -7TT in a cohort of 498 Caucasians. A study by Lu et al. (19) is also in conflict with our results. They found that the -634CC genotype was associated with an improved OS compared to -634GG in 1,119 patients in the Shanghai Breast Cancer...
However, consistent with our study, Lu et al. found no association between +936C>T and prognosis, whereas Kripl et al. (18) found that the +936 T allele was protective against recurrence of low-grade tumours only. HER2 positivity is associated with high-grade tumours. Consequently, we observed only very few low-grade tumours and this might explain why VEGFA +936C>T failed as a prognostic marker in our study. In the study by Lu et al., no information on tumour grade was supplied.

Allelic frequencies vary between different ethnic groups, but the genotypic frequencies in the Shanghai Breast Cancer Study (19) were very similar to the genotype distribution in our study. Moreover, the study by Balsubramanian et al. (20) was conducted on Caucasians and, as expected, their genotypic frequencies did not deviate remarkably from ours.

We measured SNPs in DNA from venous blood samples assuming that the germline genotype is identical to the genotype in the tumour, and that inherited variation in the germline is responsible for the biological variations rather than somatic mutation or loss of heterozygosity in the tumour and metastases. This is supported by Schneider et al. (42) who found 100% concordance between VEGFA +936C>T genotype in the tumour, in the metastatic lymph nodes, and in the uninvolved lymph nodes, thus demonstrating that germline SNPs can be obtained from uninvolved axillary lymph nodes, although no comparison to germline DNA obtained from blood samples was made. We measured germline SNPs as did three of the other studies. Only Kidd et al. (21) and Jin et al. (16) determined SNPs in tumour tissue and some of their results were consistent with ours.

Unfortunately, our study was limited to 116 patients, resulting in a low number of patients with the variant allele and the risk that our finding is simply due to chance does exist. We did not correct for multiple testing because of the hypothesis-generating nature of this study.

Some other unresolved issues may contribute to the conflicting results between studies. Substantial differences between adjuvant treatment regimens might also explain the inconsistency between studies because changes in treatment options have improved breast cancer prognosis significantly during the past decades. In the previous studies, all patients were included since before 2001, but only a few of our patients were treated in that time period. In the study by Lu et al. (19), almost every patient was offered adjuvant chemotherapy as opposed to 31% of the patients in the study by Kidd et al. (21) and 78.4% in our study, but no information of the type of chemotherapy was available in either of these studies, nor for the other three studies (16, 18, 20).

The VEGFA signalling pathway is regulated by multiple factors, including the ER (43), and ER-negativity is more
frequent among HER2-positive cases (44). The proportion of ER-negative tumours was considerably higher in our study than in any of the other studies and this may also contribute to the different results between studies. In our study, patients underwent different chemotherapy regimens and different adjuvant treatment modalities, but these were equally distributed between genotypes. Thus, any possible confounding by treatment seems unlike in our study.

Our study was conducted only on patients with HER2-positive cancer; HER2 status was not reported in any of the other studies, presumably because these studies were conducted before HER2 assessment was a routine diagnostic procedure and adjuvant-targeted treatment was introduced. However, according to Kidd et al. (21) HER2 status was available, but the proportion of HER2-positive and -negative cases are not given in the article. Thus, the number of HER2-positive cases and their genotypes are unknown. A completely opposite effect of genotypes in HER2-positive patients alone compared to a cohort with unknown HER2 status seems unlikely, but cannot be excluded. Furthermore, Schneider et al. (36) found that VEGFA SNPs were associated with response and adverse effects to anti-VEGFA treatment introducing SNPs as potential biomarkers for targeted anti-VEGFA treatment (36). In pre-clinical studies, anti-EGFR and anti-HER2 treatment has also been shown to reduce VEGFA expression and reduce angiogenesis (25, 45). The majority of patients in our study were offered adjuvant anti-HER2 treatment, but due to the relatively small sample size and the low variant frequency, subgroup analysis does not seem appropriate. One may speculate how VEGFA gene polymorphisms influence the efficacy of adjuvant anti-HER2 treatment and prognosis.

SNPs are stable parameters in contrast to other tumour markers, which fluctuate during tumour growth and treatment. Furthermore, SNPs can be measured from DNA in blood samples; hence, no tumour tissue needs to be available. The resistance of DNA to degradation and reliable measurements make analysis of SNPs as biomarker candidates very attractive. However, as discussed, results are inconsistent and the role of VEGFA SNPs as biomarkers of clinical relevance is still to be elucidated.

In this hypothesis generating study, the VEGFA -2578CC, -634CC and -7CC genotypes were associated with adverse breast cancer prognosis in patients with primary HER2-positive patients, but only VEGFA -634G>C had any independent prognostic impact in multivariate analysis. The present study encourages further investigation and validation of VEGFA SNPs as prognostic markers in both HER2-positive and HER2-negative breast cancer.

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