INTRODUCTION
Breast Cancer (BC) is the highest recorded cancer amongst women, affecting over 2 million women each year. Globally, it is the second most common type of cancer that comprises 30% of all new cancer cases among women in the US [1].

Hanahan D and Weinberg RA, postulated the six initial ‘Hallmarks of Cancer’ that establish the molecular and cellular basis of cancer [2]. Induction of angiogenesis is one of these crucial hallmarks for tumour development. In-depth studies on angiogenesis are required to progressively interpret the current knowledge underlying cancer growth, detection and treatment.

Proper risk stratification of BC patients is an important strategy in monitoring tumour progression and improving tumour treatment outcome and survival. It helps in prioritising stratification strategies based on awareness of staging markers that promotes a proper choice of an adequate treatment protocol. Treatment of choice for BC that is positive for progesterone and oestrogen receptors (PR+ and ER+) is hormonal therapy. However, de novo failure to respond to this treatment strategy or acquired resistance following primary response to treatment remains a clinical challenge. Failure of treatment may be the consequence of an improper risk stratification of cancer patients or lack of understanding of the cross-talk between key players of tumour pathogenesis including sex hormones and growth factors [3]. Hence, elucidation of these factors can ensure improvement of treatment strategies.

Currently, the key prognostic biomarkers in early-stage BC are tumour size, grade, Lymph Node (LN) status, ER, PR and Human Epidermal Growth Factor Receptor-1 (HER2) status [4-6]. These markers are used mainly to identify patients who are eligible for hormonal therapy or/and adjuvant chemotherapy. Nevertheless, further studies are required to identify diagnostic, prognostic or predictive biomarkers to improve the therapeutic outcome.

Scientific evidence reports an angiogenic-dependent nature of BC, where angiogenesis plays an important role in cancer growth and metastasis [7,8]. Expression of angiogenic factors by BC cells has been vastly studied at the molecular (mRNA) or functional (protein) level in both node-negative and positive breast cancers [9-11]. These findings rationalise the need for advanced studies on angiogenic growth factors to identify possible targets for antiangiogenic therapy, for eliminating resistant tumour cells that cannot be defeated by anti-hormone treatment [12,13].

In recent times the key regulators of angiogenesis are VEGF and basic Fibroblast growth factor (bFGF), which have received much attention [14-16]. VEGF and its isoforms are potent regulators of the angiogenesis signalling pathway that provide nutrients and oxygen required for tumour growth [12,17,18]. Clinical studies have shown that BC patients expressing increased VEGF levels failed to respond to hormone therapy or suffered early recurrence, indicating that VEGF may be responsible for anti-hormone resistance [19,20].

Majority of the published research work reports the tissue expression of VEGF and its isoforms in BC cells. However, the cellular origin and biochemical nature of VEGF remains poorly understood [21]. In this context, the current work aimed to evaluate the role of VEGF as a prognostic factor in BC patients.

ABSTRACT
Introduction: Basal Vascular Endothelial Growth Factor (VEGF) levels are elevated in the serum of Breast Cancer (BC) patients. Positive correlation has been reported between VEGF expression and microvessel density in primary BC sections. The relapse-free survival rate of patients with VEGF-poor tumours was significantly higher than that of VEGF-rich tumours, suggesting that expression of VEGF may be associated with poor BC prognosis.

Aim: To evaluate the role of VEGF as a prognostic factor in breast cancer patients.

Materials and Methods: This case-control study was conducted on BC patients admitted to the Oncology Unit or the Outpatient Clinics, Faculty of Medicine, Alexandria, Egypt. Clinically diagnosed 75 BC patients were divided into Group IA: 45 patients diagnosed with early BC Stage I and II; and Group IB: 30 patients diagnosed with advanced BC Stage III and IV. Apparently, healthy 60 females (relatives/companions) were recruited as the control group (Group II). Serum VEGF levels were measured by Enzyme-linked immunosorbent assay (ELISA). Serum VEGF data were expressed as median (min-max). Qualitative data were analysed using Chi-square test and Fisher’s-Exact test or Monte Carlo correction. Quantitative data were analysed using Mann Whitney (Z) test and Kruskal Wallis (H) tests. Spearman’s coefficient (r) was performed to determine correlation of serum VEGF with age, tumour size and grade. Binary logistic regression was done to predict the probability of several clinical parameters with respect to the VEGF measurement. Significance level was set at 5%.

Results: The median VEGF serum levels showed significant difference between the three studied groups (p<0.001). Serum VEGF was significantly increased in both Groups IA/IB patients compared to healthy controls (p<0.001); and were significantly higher in advanced than early BC patients (p<0.001). Serum VEGF was significantly associated with tumour size, tumour grade, Oestrogen Receptor (ER), Progesterone Receptor (PR), luminal tumour, and triple negative BC. BC cases who developed metastasis expressed significantly higher serum VEGF (Group IA: p=0.001; Group IB: p=0.024).

Conclusion: Serum VEGF may serve as an independent prognostic biomarker in BC patients that merits prospective validation.

Keywords: Biological markers, Cancer work-up, Enzyme-linked immunosorbent assay, Tumour staging.
of VEGF by Immunohistochemistry (IHC) [9,21]. However, the assessing of circulating soluble serum biomarker as VEGF would be of considerable benefits. Clinical implementation of serum VEGF biomarker analysis in BC work-up will be cost effective, rapid and reliable test over more subjective approaches as IHC that entails arduous procedures.

Therefore, the present study was designed to evaluate the serum VEGF levels and its’ role at time of initial diagnosis of BC and after 6 months following treatment as a potential prognostic factor in different BC intrinsic subtypes and stages. The inclusions of serum VEGF measurement in the initial tumour work-up will help to risk stratify BC patients and identify patients at high risk of developing metastasis or relapse.

MATERIALS AND METHODS
The present case-control study was conducted from January 2016 to January 2017. Ethics Committee approval was obtained from the Faculty of Medicine Alexandria University (approval no: 0103645). All the procedures followed were in accordance with Helsinki Declaration, 1964 and its later amendments. BC patients admitted to the Oncology Unit or visiting the Outpatient Clinics, Main Alexandria University hospitals, Faculty of Medicine, Alexandria, Egypt were recruited. Purpose and procedure of the study was explained to all the participants, and informed consent was obtained before commencement of the study.

During this one year study, a total of 1500 specimens were received at the Histopathology Department for examination. Amongst them, 75 (95% CI, 80% statistical power) cases positive for BC by histopathological analysis were selected for the study. Based on the diagnosis, the samples were divided into; Group IA: 45 patients diagnosed with early BC stage I and II, mean age 44.67±11.09 years; Group IB: 30 patients diagnosed with advanced BC stage III and IV mean age 55.27±11.67 years. Apparently, healthy 60 females (relatives/companions) were recruited as the control group (Group II), mean age 40.67±11.9 years.

Inclusion Criteria
BC patients have histologically proven positive, adequate venous blood samples available for evaluation, normal cardiac imaging and no other diagnosed malignancies were included.

Exclusion Criteria
Patients diagnosed with liver, renal (laboratory) or heart failure (cardiac imaging) or patients with chronic inflammatory diseases including autoimmune diseases (patient’s history) or viral infection (laboratory); HBV, HCV, and HIV were excluded. Patients suffered from uncontrolled DM (HbA1C) or uncontrolled hypertension (laboratory); HBV, HCV, and HIV were excluded. Patients diagnosed with liver, renal (laboratory) or heart failure (cardiac imaging) or patients with chronic inflammatory diseases including autoimmune diseases (patient’s history) or viral infection (laboratory); HBV, HCV, and HIV were excluded. Patients suffering from uncontrolled DM (HbA1C) or uncontrolled hypertension (medical history and follow-up) were also excluded.

For all the BC patients, a thorough family and clinical history were taken. Clinical examination for tumour size, LN examination, and routine laboratory investigations were carried out. Radiological examination for BC diagnosis included Ultrasonography (USG) and mammography for both breasts. For detection of metastasis, chest X-ray, abdominal and pelvis USG, CT for chest, abdomen, pelvis, brain (when indicated), and bone scanning were done.

Histopathological Examination
The excised tumour tissues and LN (s) were examined histopathologically, where the tumour tissue sections were diagnosed and clinicopathological parameters such as age, histological type, size, tumour grade, lymphatic or vascular invasion, LN metastasis, ER, PR, and HER2 status were recorded for each case [Table/Fig-1].

Quantification of VEGF at Diagnosis and after Treatment
Serum samples were collected from all the study subjects at the time of diagnosis (before any surgical intervention or starting any treatment protocols) and after 6 months of treatment. Sera were obtained by centrifugation and samples were stored at -80°C until the analysis of VEGF levels. Serum VEGF levels were measured by using the double antibody sandwich ELISA (Human VEGF PicoKine ELISA Kit, USA; Catalog No. EK0539) according to the manufacturers’ instructions. Optical density absorbance was read at 450 nm and human VEGF concentration of the samples was calculated from the standard curve. Serum VEGF reference range was taken to be 31.2 pg/mL-2000 pg/mL.

STATISTICAL ANALYSIS
Data were analysed using IBM’s Statistical Package for the Social Sciences (SPSS) software, Version 20.0. (Armonk, NY: IBM Corp) [22,23]. Parametric quantitative data were expressed using mean, Standard Deviation (SD) and median. Significance level was set at 5%. Qualitative data were analysed using Chi-square test and Fisher’s-Exact test or Monte Carlo correction for Chi-square when more than 20% of the cells have expected count less than 5. F-test (ANOVA) was used to compare normally distributed quantitative variables and Post-Hoc test (LSD) for pairwise comparisons. Mann-Whitney (S) test and Kruskal Wallis (H) test were used for comparing abnormally distributed quantitative variables between the studied groups. Spearman Coefficient (ρ) was used to correlate between two distributed abnormally quantitative variables. Binary logistic regression was done to predict the probability of a certain nominal variable, given the VEGF measurement.

RESULTS
Clinical data for the studied BC cases (Group IA and IB) including tumour size, grade, histopathological examination and BC intrinsic subtypes are presented in [Table/Fig-2].

Analysis of median VEGF serum levels showed significant difference between the three studied groups (p<0.001) [Table/Fig-3]. The median VEGF serum level was significantly increased in Group IB than Group IA (Median=583 (160-940.4) and 290.4 (59.8-650) IU/mL, respectively). In the controls, the median VEGF serum level was 61.7 (35-96.7) IU/mL. Serum VEGF was significantly increased in both Group IA and Group IB patients, compared to healthy controls (p<0.001 for each), and was significantly increased in advanced (Group IB) than early (Group IA) BC patients (p<0.001).

Association between serum VEGF and different clinic-pathological parameters are presented in [Table/Fig-4]. Median serum VEGF levels showed significantly higher values as the tumour size increased (p=0.017) and the tumour grade advanced (p=0.008). Serum VEGF levels in ER or PR negative tumours showed significantly higher values than positive tumours (p=0.013, 0.012, respectively).
Studying the relation of serum VEGF levels with intrinsic subtypes showed that VEGF serum levels were significantly lower in luminal tumour subtypes (p=0.013), while significantly higher in triple negative subtype (p=0.005).

<table>
<thead>
<tr>
<th>Tumour size</th>
<th>Group IA (early)</th>
<th>Group IB (advanced)</th>
<th>Test of significance</th>
<th>p</th>
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<tr>
<td>T1</td>
<td>12 (26.7%)</td>
<td>0 (0.0%)</td>
<td>χ²=3.273 MCp&lt;0.001</td>
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<tr>
<td>T2</td>
<td>33 (73.3%)</td>
<td>20 (66.7%)</td>
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<tr>
<td>T3</td>
<td>0 (0.0%)</td>
<td>8 (26.7%)</td>
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<tr>
<td>T4</td>
<td>0 (0.0%)</td>
<td>2 (6.7%)</td>
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<table>
<thead>
<tr>
<th>Tumour grade</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>Lymph nodes</th>
<th>Lympo-vascular Invasion</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>Luminal tumour</th>
<th>HER2-enriched</th>
<th>Triple negative</th>
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<td></td>
<td>6 (13.3%)</td>
<td>22 (46.7%)</td>
<td>30 (66.7%)</td>
<td>21 (46.7%)</td>
<td>36 (80.0%)</td>
<td>33 (73.3%)</td>
<td>15 (33.3%)</td>
<td>36 (80.0%)</td>
<td>6 (13.3%)</td>
<td>4 (13.3%)</td>
<td>3 (6.7%)</td>
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<td>0 (0.0%)</td>
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<td>20 (66.7%)</td>
<td>20 (66.7%)</td>
<td>8 (26.7%)</td>
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<tr>
<td></td>
<td>χ²=1.692</td>
<td>χ²=0.233</td>
<td>χ²=3.376</td>
<td>χ²=3.376</td>
<td>χ²=0.786</td>
<td>χ²=0.386</td>
<td>χ²=0.076</td>
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<td>FEp=0.279</td>
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</table>

**Table/Fig-2**: Clinical data for the studied breast cancer cases (group IA/IB).

**Table/Fig-3**: Comparison between the three studied groups according to serum VEGF [IU/mL]. Horizontal bars representing the comparison between serum VEGF levels in each group presented as Median (min-max) and SD. ***: p<0.001 (Table below shows the figure data in numerical values); H: Kruskal Wallis test Sig. bet. grps: significance between groups; p: value for Mann Whitney test for comparing between group IA and group IB; p: value for Mann Whitney test for comparing between group IA and group II; p: value for Mann Whitney test for comparing between group IB and group II; **: Statistically significant at p<0.001.

**Table/Fig-4**: Association between serum VEGF (IU/mL) with different clinic-pathological parameters. VEGF: vascular endothelial growth factor; ER: Oestrogen receptor; PR: progesterone receptor, HER2: human epidermal growth factor receptor 2; ρ: Spearman correlation coefficient; **: Statistically significant at p<0.001.

The probability of predicting a specified clinical or histopathological factor, given the serum VEGF, was studied using binary logistic regression test [Table/Fig-6]. The tested factors were age, tumour size, tumour grade, luminal tumour (BC subtype) and tumour staging (early/advanced stage). The only independent factor detected to significantly correlate with serum VEGF was tumour staging.
(OR: 2.421; 95% CI: 11.9-331.0; p=0.047). To assess the prognostic potential of measuring serum VEGF biomarker in BC, follow-up of the BC patients (Group IA and Group IB) was done for a period of 6 months [Table/Fig-7]. In both groups, cases who developed metastasis were those patients who expressed significantly higher serum VEGF for [Group IA, p<0.001; for Group IB, p=0.024].

**Variables**

<table>
<thead>
<tr>
<th>Group IA</th>
<th>Group IB</th>
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<tbody>
<tr>
<td>Disease free</td>
<td>Metastasis</td>
</tr>
<tr>
<td>(n=36)</td>
<td>(n=9)</td>
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<tr>
<td>Early</td>
<td>266.20</td>
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<tr>
<td>(n=45)</td>
<td>(58.8-376.4)</td>
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<tr>
<td>Group IB</td>
<td>(n=24)</td>
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<tr>
<td>Advanced</td>
<td>461.60</td>
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<td>(n=30)</td>
<td>(826.7-732.0)</td>
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</table>

**DISCUSSION**

Targeted therapies in treating cancer have gained more attention in an attempt to hinder tumour progression and metastasis. Inhibition of tumour angiogenesis process is a proposed strategy for cancer treatment, as angiogenesis is an important regulator of cancer growth, invasion and metastasis. The assessment of angiogenesis by evaluation of Intratumoral Microvessel Density (IMD) using immunohistochemistry is a commonly used technique in BC [9,21]. Nevertheless, IMD quantification technique is an operator dependent technique making the process variable among laboratories, arduous and requires skills. Hence, identifying serological biomarkers, as a less subjective measurement, that can be used to identify high risk BC patients can improve clinical and therapeutic management.

In the present study, it was observed that VEGF serum levels were significantly elevated in BC patients as compared to healthy controls and the levels were significantly much higher in advanced than early BC patients. Therefore, VEGF can be used to identify a subset of breast cancer at higher risk for development of recurrence and distant metastasis. These findings were in accordance with various studies on BC patients and several other types of cancers where increased VEGF levels were recorded in metastatic than localised tumour stages [9,11,22-25]. Ali EM et al., study also reported significantly increased mean serum VEGF in BC patients preoperatively than postoperatively, when compared to benign breast lesions [21].

Several molecular pathways have been hypothesized to unveil the VEGF’s role in BC tumourigenesis. Linderholm BK et al., showed that the combination of increased VEGF expression levels and mutated p53 has been correlated with poor outcome for BC patients. The study suggested that tumour angiogenesis might be conjoinly regulated by the increased VEGF and the loss of wild type p53 that functions as tumour suppressor gene [26]. However, some studies reported a relation between BRAC-1 mutation and increased VEGF in BC patients. Kawai H et al., study demonstrated that mutated BRCA-1 failed to inhibit VEGF gene transcription activation and protein secretation, mediated through the oestrogen signalling pathway, in a pathogenic mechanism that could promote tumorigenesis and angiogenesis [27]. Hypoxia can induce an increase in the expression of VEGF at molecular (mRNA) and cellular (protein) levels [28]. Bos R et al., investigated the correlation between increased level of the transcriptional factor; Hypoxia inducible factor-1 alpha (HIF-1α), and VEGF in BC patients [29]. They demonstrated that HIF-1α expression increased in poorly differentiated and more aggressive BC lesions compared to well-differentiated lesions, and the increased levels of HIF-1α were significantly associated with increased VEGF [29].

The present study showed positive association between serum VEGF with different clinico-pathological parameters with significantly higher serum VEGF values as the tumour size increased (p=0.017), tumour grade advanced (p=0.008), ER**+** (p=0.013) and PR**+** (p=0.012), while no significant association with HER2 was detected. Additionally, the data showed an increasing trend in serum VEGF with LN involvement or lympho-vascular invasion; however, the differences were not statistically significant. In agreement with the present data, Liu Y et al., reported a positive association of VEGF with tumour size, grade and hormonal receptor in primary invasive BC. However, their study showed positive association with nodal involvement and HER2 expression [22,30]. Goussia A et al., showed positive association of VEGF-A with ER and PR, where high VEGF was detected with negative hormonal receptors. However, their study could not confirm any association with tumour size or LN involvement [31]. In addition, multiple studies could not confirm the association of VEGF with any of the clinico-pathological parameters in invasive BC patients [10,32]. These inconsistent results can be explained in the context of variability of the applied method, as most of the studies were based on IHC staining examination, using different detecting antibodies with different selected cut-offs, in addition to the biological and molecular heterogeneity of studied samples.

Evaluating the relation of serum VEGF levels with BC intrinsic subtypes showed that VEGF serum levels were significantly lower in luminal tumour subtypes (p=0.013), while significantly higher in triple negative subtype (p=0.005). Similar findings were reported by Linderholm BK et al., where VEGF was significantly higher and had shorter survival time in triple negative BC subtype [33]. Liu Y et al., reported significantly lowered VEGF in luminal A subtype, while VEGF was significantly higher in luminal B subtype and HER2- enriched [22]. Goussia A et al., findings are in partial agreement with the present data, with a positive association between low serum VEGF and luminal subtypes, in contrast to high VEGF in HER2-enriched subtypes [31]. Furthermore, the present study was carried out to establish the probability of predicting a specified clinical or histopathological factor with a known serum VEGF. The only independent factor detected to significantly associated with serum VEGF was tumour staging (early/advanced) (p=0.032), where serum VEGF can be used to predict tumour staging. Follow-up of the BC patients over a 6-month period demonstrated that metastasis occurred in those expressing significantly higher pretreatment serum VEGF. These findings are in accordance with Ali EM et al., who reported that higher VEGF was associated with poor survival rates with significantly lower overall or disease free survival in patients having increased pretreatment VEGF serum levels [21].
LIMITATION

Relatively small sample size. Further studies with larger sample size, and extended treatment time frame for VEGF analysis are required. In addition, correlation study with different treatment protocols could provide more informative data. The present authors also recommend the consideration of setting an optimal cut-off point(s) for serum VEGF that could be correlated to different clinical parameters of BC. Furthermore, the study of the regulation of VEGF gene transcription activation and protein secretion will aid in understanding pathogenic mechanisms that can identify possible targets for novel therapy.

CONCLUSION

Breast Cancer (BC) is the main cause of death related to cancer among women all over the world which summons the need for the identification of simple, reliable and cost-effective biomarkers particularly in countries with financial constraints. From the present study, it can be concluded that VEGF could serve as an independent prognostic biomarker in BC patients. BC patients expressing high levels of serum VEGF were associated with advanced tumour grade, presence of distant metastasis and aggressive subtypes. VEGF levels may be used as a predictor of BC staging and can be used to identify BC patients at high risk of developing metastasis or relapse. Adopting serum VEGF in the routine BC work-up would help in proper selection of BC treatment protocol.

REFERENCES