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# Endothelial progenitor cells as an angiogenic biomarker for the diagnosis and prognosis of lung cancer

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# Endothelial progenitor cells as an angiogenic biomarker for the diagnosis and prognosis of lung cancer

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## ABSTRACT

**Background**: Angiogenesis is mediated by endothelial progenitor cells (EPCs) derived from bone-marrow. In this prospective study, we tried to investigate the clinical utility of circulating EPCs in lung cancer (LC) patients.

**Materials and methods**: Flow cytometry technique was used to assess circulating EPCs according to the immuno-phenotype $CD45^{-}$ $CD34^{+}$ $CD133^{+}$ $CD146^{+}$ mononuclear cells.

**Results**: Sixty patients and 30 controls were included in this prospective study. The mean of baseline EPC numbers was significantly higher in LC patients than in controls (p =0.003). Pretreatment EPC values were significantly correlated with primary tumor size (p = 0.05) and

tumor response (p = 0.04). Receiver operating characteristics (ROC) curves were plotted to discriminate EPC numbers between patients and controls. Using ROC analysis, the optimal cutoff value was 125 cells/mL with a sensitivity and a specificity for baseline EPCs of 76.7% and 63.3%, respectively. According to this cutoff value, basal EPC values were significantly correlated with primary tumor size (p = 0.047) and response to chemotherapy (p = 0.034). High EPC levels were significantly associated with longer progression-free survival (PFS) and overall survival (OS) duration (p = 0.0043 and p = 0.02, respectively).

**Conclusion:** Increased baseline EPC values seem to be a useful biomarker for the prediction of prognosis and tumor response in LC patients. Furthermore, high EPC levels at diagnosis might be an indicator of tumor growth and longer survival in LC patients.

**Key words:** endothelial progenitor cells; cancer biomarker; lung cancer; prognosis

## Introduction

Lung cancer (LC) accounts for 12% of all cancers diagnosed worldwide, and Non-small cell lung cancer (NSCLC) represents about 85% of LC cases in the United States [1]. The majority of LC patients have an advanced disease at diagnosis. Despite surgical resection and development of new chemotherapy regimens, most LC patients relapse and the overall prognosis remains poor [2]. Slight prolongation in survival rate has been obtained through better tumor response to platinum-based chemotherapy in NSCLC [3]. However, five-year survival rate was related to disease stage, being 2% when it presents as disseminated disease [4]. Actually, an effective marker that may predict prognosis and disease outcome has become necessary in LC. However, several clinicopathological parameters have been previously proposed as prognostic factors in LC [5].

Better understanding of the pathogenesis tumor is closely related to molecular biological alterations involved in LC. Moreover, vascularization and lymphangiogenesis of tumor arise exclusively from endothelial sprouting. In fact, the key therapeutic strategy is the inhibition of specific cytokines, which are essential for tumor vascularization in order to develop new therapeutic agents used in molecular-driven targeted therapy. It has been also shown that tumor vasculature might arise through vasculogenesis. It is widely accepted that both angiogenesis and vasculogenesis are involved in endothelium-dependent vascularization of tumor microcirculation. Nevertheless, circulating biomarkers capable of predicting clinical response to antiangiogenic drugs are still scanty in malignancies, including LC [6].

Chemotherapy is aimed at shrinking primary tumors, slowing tumor growth, and eradicating cancer cells that may have metastasized to other parts of the body [7]. The landscape of immunotherapy in lung cancer is rapidly evolving. Currently, the use of immune checkpoint inhibitors represent the most encouraging clinical form of NSCLC immunotherapy [8]. It has become the standard of care management for patients with resectable locally advanced and metastatic NSCLC leading to notable enhancement in the reported clinical outcomes.

Bone marrow derived endothelial precursor cells are recruited in vasculogenesis. In addition, it has been found that circulating bone marrow (BM)-derived CD34+ endothelial progenitor cells (EPCs) alone can repopulate bone marrow in vivo to home to sites of neovascularization and differentiate into ECs [9, 10]. However, it has been shown that the presence of mesenchymal stem cells induces EPCs to differentiate into endothelial cells, promoting angiogenesis even without the addition of exogenous growth factors [11]. In fact, bone marrow-derived endothelial progenitors constitute a small heterogenous subpopulation of stem cells. An EPC is best defined as an immature precursor cell that individually displays postnatal vasculogenic activity with the capability of forming new blood vessels in vivo [12]. Circulating EPCs could be divided into two subpopulations: early and late. However, there is a lack of hematopoietic stem cell antigens (CD14 and CD45) on the surface of both subpopulations. The expression of CD133 might be useful for the differentiation between platelet and endothelial microparticles derived from EPCs, while CD31 is a typical endothelial antigen [10]. In fact, CD133 is expressed only on the surface of immature EPCs and is lost in mature endothelial cells (ECs). The number of $CD133^+$ cells represents (28.4 ± 2.4)% of the total $CD34^+$ cells in healthy population. Nevertheless, the quantification of circulating EPCs is quite complicated due to the low count of cells in the peripheral blood, methodological divergences and the lack of agreement on phenotypic identification. However, circulating EPCs cannot be effectively determined by a single surface antigen [13].

In response to vascular injury, EPCs acquire the ability to circulate in the peripheral blood, proliferate and differentiate into mature endothelial cells. Thus, elevated levels of circulating EPCs were observed in myocardial infarction, limb ischemia, wound healing and tumor growth [13, 14]. Consequently, it has been found that EPCs could serve as an indicator of neoangiogenesis in breast cancer for predicting recurrence and progression disease [15]. The extent to which EPCs is incorporated into tumor vasculature has been controversial with high variation of EPCs incorporation [16, 17]. The angiogenic cytokine release of EPCs may support this mechanism to improve neovascularization [18, 19]. However, it has been established that circulating EPCs count might either elevate or even drop after surgery [20].

These discordant results might be explained by the different types of surgical procedures which have been used. Nevertheless, the enumeration of EPCs in blood samples was a promising biomarker for the evaluation of anti-angiogenic therapy in cancer patients. Increased EPC numbers have been found in several malignancies, suggesting that EPC levels might be a useful biomarker to select high-risk factors and to predict tumor progression in cancer patients [21].

Little is known about the potential value of EPCs count in LC patients. Therefore, we assessed the hypothesis that EPCs correlate with clinicopathological factors, and EPCs might predict response to chemotherapy and prognosis in LC patients. The present study aims to document the potential role of pretreatment EPC values as a possible biomarker for predicting outcome in LC patients after double agent platinum-based chemotherapy.

## Materials and methods

This prospective study was conducted at Albairouni University Hospital from February 2017 to August 2020 after the ethical approval of ethics committee of Albairouni University Hospital for clinical research (Ethical Committee approval number: E47-20170123). Written informed consent was obtained from all the individuals after a detailed explanation of the study. All procedures were performed in accordance with the ethical standards prescribed by the Helsinky Declaration of the World Medical Association.

### Patients

We restricted our prospective analyses to newly diagnosed patients with histologically confirmed LC. All the included patients were more than 18 years-old and Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤ 2. All the individuals (patients and controls) were free of inflammatory or ischemic disease, pulmonary fibrosis, wounds or ulcers that might influence the number of EPCs [22]. The included patients were classified as patients either at initial diagnosis (ID) or at recurrence (R). Clinical stage was done according to computed tomography (CT) and bone-scan findings based on the American Joint Committee on Cancer/Union Internationale Contra Cancrum pathological tumor-node-metastases (pTNM) classification [23].

Peripheral blood samples were collected in vacutainer tubes containing potassium ethlyne-diaminetetraacetic acid (EDTA) from patients and healthy individuals. To avoid the effects of chemotherapy or surgical wound on EPCs count, samples were collected 21 days after the last cytotoxic infusion and 3 months after thoracotomy.

**Exclusion criteria**

All individuals (patients and healthy individuals) with diabetes mellitus, recent bleeding, thrombotic events, other malignancy, recent surgery or trauma did not qualify for the study. Patients and controls who received medication, known to influence the mobilization of EPCs from the bone marrow, at the time of blood sampling were also excluded from the study.

**Data analysis**

Detailed clinicopathological variables were prospectively collected for analysis including age, gender, smoking status, weight loss, clinical presentation, histology, tumor-node-metastases (TNM) staging. Patients' clinicopathological variables were correlated with baseline EPC values.

Our population was divided according to smoking history into two groups (current or former smokers and never smokers).

Weight loss was considered for patients who had lost more than 5% of their body weight or less than 2% for those who had a body mass index (BMI) < 20 during the last three months. Otherwise, patients were considered as having no recent weight loss.

According to tumor histology, pretreatment EPCs count was with histological subtypes (squamous cell type versus non-squamous cell types including adenocarcinoma, large cell, small cell carcinoma and neuroendocrine tumors). Furthermore, the included patients were classified according to histological tumor differentiation (well-moderately differentiated versus poorly differentiated grade).

The patients were staged either at early stages (IA to IIIA) or at locally advanced and metastatic stages (IIIB, IV), and then correlated with EPC numbers.

Primary tumor size was determined with CT images by measuring the major axis (a) and the minor axis (b) of the primary tumor using the following formula:

$$ETV = \frac{4}{3} \times \pi \left[ \left( \frac{a}{2} \times \frac{b}{2} \right) \times \left( \frac{a}{2} + \frac{b}{2} \right) \right] \div 2$$

According to the longest diameter of the primary lung mass on CT, our population was divided into patients with long axis more than 40 mm and those with long axis ≤40 mm.

Follow-up data were collected until February 2021. The efficacy of treatment was evaluated according to response evaluation criteria for solid tumors (RECIST) [24]. An objective response (OR) was considered for patients who achieved partial response (PR) or stable disease (SD) after treatment. Progression disease (PD) was defined when the patient

had an increase of more than 20% in the longest diameter of the primary tumor or the appearance of new metastases on CT after chemotherapy. Follow-up duration was calculated as the time from initial inclusion of the patient in the study until the earliest event of interest (disease progression, death or the last date of contact with the patient). Progression-free survival (PFS) duration was determined as the time from the first day of chemotherapy to the date on which the patient progressed or died from any cause. Overall survival (OS) duration was defined as the time from the enrolment of patient until the death or the last visit, in which the patients were referred to palliative therapy due to the degradation in their performance status.

**Enumeration of EPCs by fluorescent-activated cell sorting (FACS) analysis**

Whole blood samples from patients were collected prior to any treatment. Pretreatment samples were taken from the peripheral blood by venipuncture into BD vacutainer plus Plastic EDTA tubes to measure the number of circulating EPCs in the peripheral blood. Quantification of circulating EPCs was performed by FACS analysis according to recommendations provided by Mancuso et al. [25] and previous modified protocols [26, 27].

In brief, one ml of whole blood was washed twice with phosphate buffered saline (PBS) containing 0.3% bovine serum albumin and re-suspended in 1 ml of PBS. A panel of mouse anti-human monoclonal antibodies (mAbs) was used: fluorescein isothiocyanate-labeled APC-anti-CD45 (BD Biosciences, San Jose, CA), PE-anti-CD133 (Miltenyl Biotec GmbH, Bergisch Gladbach, Germany), PerCP-anti-CD34 (BD Biosciences) and R-phycoerythrin-labeled FITC-anti-CD146 (BD Biosciences). According to white blood cells count**,** 100 or 200 µl of washed mixed whole blood (in duplicates) were stained and incubated with mAbs for 30 minutes at 4°C. Stained blood samples were subjected to red blood cells lysis with 2 ml of lysing solution (BD Biosource, Europe SA, Belgium) for 10 minutes in the dark. The cells were washed with PBS and re-suspended in 950 µl of PBS. Then, 50 µl of counting beads (Thermo fisher Invitrogen Count Bright™ absolute counting beads; Ref. No. C36950) were added. Blood samples were carried out on the FACS Claibur flow cytometer (BD Biosciences, USA) and data were analyzed using Cell quest pro software (BD Biosciences, USA). A minimum of 100,000 events of mononuclear cells gate were collected for each sample. Circulating EPCs were identified as $CD45^{-}$, $CD34^{+}$, $CD133^{+}$ and $CD146^{+}$. Auto fluorescence, viability and isotype controls were also processed and analyzed for each specimen. After appropriate gating of EPCs population, the absolute numbers of EPCs in one microliter ( $\mu l$ ) were determined using the following formula:

$$cells/\mu l = \frac{no.\,of\ events \in cell \subset region}{no.\,of\ events \in beads\ region} \times \frac{no.\,of\ beads\ per\ test}{volume\ of\ sample\,(\mu l)} \times dilution\ factor$$

The number of circulating EPCs in the whole blood was expressed as counts per ml of blood. We determined the number of EPCs as $CD45^{-}CD34^{+}CD133^{+}CD146^{+}$ cells in the whole blood by flow cytometry. Figure 1 shows a representative FACS analysis of baseline EPCs from a patient with LC at diagnosis as well as from a healthy individual.

**Statistical analysis**

The study was analyzed using the statistical package for the social sciences (SPSS Inc., Chicago, IL, USA) version 18.0. Data were reported as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine the differences in EPC levels between sample types. Continuous variables were analyzed by independent *t*-test, while Pearson's chi-square test was used to analyze categorical variables. The Mann-Whitney test was used to assess the association between EPC value and clinicopathologial variables. The cutoff point for EPC levels to distinguish between LC patients and healthy individuals was determined using the receiver operating characteristic (ROC) curve by considering the sensitivity and specificity value. The area under curve (AUC) was calculated in order to estimate the diagnostic accuracy with 95% confidence interval (CI) for the survival rates. Kaplan–Meier method with log-rank test was used to determine the difference in survival curves according to baseline EPC levels and tumor response after chemotherapy. Multivariate analysis of prognostic variables was performed using Cox's regression model with 95% confidence interval (CI). A probability of ($p$-value ≤ 0.05) was considered statistically significant.

**Results**

***Demographic and clinical patients' characteristics***

Sixty patients (51 male, 9 female) with a mean age of 58 years (range, 43–74 years), and 30 healthy volunteers (25 male, 5 female) with a mean age of 53 years (range, 39–62 years) were prospectively included in this study. Four patients were excluded from the study due to the absence of EPCs quantification at diagnosis. Moreover, patients who did not meet the inclusion criteria of the study were also excluded (n = 2). Histopathological findings showed that there were 24 patients with squamous cell carcinoma (SCC), 25 with adenocarcinoma, 5

with large cell carcinoma (LC) and 6 with small cell lung carcinoma (SCLC). Seven patients underwent surgery. Forty-seven patients with inoperable NSCLC received platinum-based chemotherapy, of whom nine patients with locally advanced disease received radiotherapy in combination with conventional chemotherapy. The included patients were at ID (n = 32) or at R (n = 28). No significant difference was observed in pretreatment EPC numbers between patients at ID and those at R (mean ± SEM: 236 ± 34 *vs.* 308 ± 58, p = 0.28). However, the mean of baseline EPC numbers in patients either at early or advanced stages was significantly higher than that in controls (mean ± SEM: 231 ± 60 and 321 ± 56 vs. 121 ± 16; p = 0.006 and 0.0157, respectively) (Fig. 2). Additionally, the mean of basal EPC numbers was significantly higher in SCLC patients than that in controls (mean ± SEM: 280 ± 22 vs. 121 ± 16, p = 0.0002). Overall, baseline EPC values were significantly higher in all LC patients than in controls (p = 0.002). However, the mean of basal EPC values was not significantly correlated neither with tumor histology nor with Pathological Tumor-Node-Metastasis (pTNM) stage (p = 0.35 and p = 0.84, respectively). Moreover, pretreatment EPC values were not significantly correlated with the other patients' characteristics including age (p = 0.11), gender (p = 0.167), smoking history (p = 0.2), recent weight loss (p = 0.99), and tumor differentiation (p = 0.86).

By contrast, baseline EPC numbers were significantly correlated with estimated tumor volume (p = 0.05). In addition, basal EPC values were significantly correlated with tumor diameter ($r^2$ = 0.104, p = 0.005) (Fig. 3). Regarding tumor response, follow up data was available for 43 patients. Data analysis showed that pretreatment EPC values were significantly higher in patients with OR (n = 26) than in those with PD (n = 17) after treatment (mean ± SEM: 366 ± 61 vs. 189 ± 50, $p$ = 0.04). Table 1 summarizes the main clinical characteristics of the participants and their relationship with baseline EPC numbers.

ROC curves for survival rates were designated and the area under the curves (AUC) was calculated (threshold with sensitivity and specificity). Based on ROC curves, a cutoff point was provided to discriminate patients from healthy subjects. For baseline EPCs count, the value was 125 cells/mL with 76.7% sensitivity and 66.3% specificity, and the AUC was 0.731 (p = 0.0001) (Fig. 4). According to this optimal value, there were 46 patients with high EPC levels and 14 patients with low EPC levels. As illustrated in Table 2, our results show that pretreatment EPC values were significantly associated with primary tumor size (p = 0.047) and treatment response (p = 0.028). However, no significant association was found between baseline EPC levels and the other prognostic parameters including gender (p = 0.349) smoking status (0.06) estimated weight loss (p = 0.577), clinical presentation (p = 0.395),

tumor differentiation (p = 0.494) and pTNM staging (p = 0.52). Multivariate analysis (including standard prognostic variables such as smoking status, histological types or tumor stage) also indicated that baseline EPC numbers predicted outcome independent of other variables (p = 0.023, Tab. 3). Data analysis showed 30 and 13 patients with high basal and low basal EPC values, respectively. Kaplan-Meier curves with log rank test revealed that patients with high baseline EPC numbers exhibit significantly longer PFS duration than those with low basal EPC levels (197 vs. 83 days, log rank test, p = 0.0043). Similarly, patients with high pretreatment EPC levels had better OS than those with low EPC levels at diagnosis (315 vs. 149 days, log-rank test, p = 0.021).

## Discussion

Angiogenesis is the formation of new blood vessels originating from an existing microvasculature [28, 29]. This process occurs during pathological conditions such as cancer. There is evidence that vascular endothelial growth factor (VEGF) and other angiogenic factors stimulate the release of EPCs from the bone marrow. Circulating EPCs perform an important role in tumor angiogenesis and thus represent a new biomarker of neoangiogenesis. In fact, their numbers were elevated in the peripheral blood of patients with breast cancer and other tumor affections [16]. These results support the idea that tumor angiogenesis is accelerated, and greater numbers of EPCs are mobilized from bone marrow (and, thus, are found in the peripheral blood) in NSCLC patients compared with those in healthy controls [30–32]. Our results were consistent with these findings in LC patients at early and advanced stages.

The relationship between clinicopathological variables and prognosis has not been well established in LC. However, clinicopathological variables were correlated with prognosis in NSCLC patients [33, 34]. In particular, gender and clinical stages might be risk factors in NSCLC patients [30]. In addition, it has been found that there was a significant association between the common prognostic factors (age, male gender, smoking, growth factors) and reduced EPC levels in the blood circulation [35]. Dome et al. found that there was no significant association between pretreatment EPC levels and various prognostic factors in NSCLC patients [36]. Similarly, serum levels of VEGF were associated with adverse clinicopathological variables including advanced disease stage, positive nodal status and poor performance status [34, 37]. Similar to our findings, they found that pretreatment EPC numbers predicted outcome independent of other prognostic variables. These findings support

the idea that pretreatment EPC numbers may predict clinical behavior in LC patients. Nevertheless, accurate prognosis and relevant therapy decisions rely on establishing the accurate staging of NSCLC. However, evidence indicates that increased EPC numbers were correlated with tumor stage in NSCLC, in which patients with IIIB–IV stage and SCLC had significantly higher EPC numbers than those with NSCLC stage I–IIIA [30]. However, mononuclear cells were isolated by ficoll density gradient centrifugation in this study**.** Then, EPCs were characterized by triple staining using antibodies against $CD133^+$, $CD34^+$ and VEGFR2. It seems that the rate of incorporated EPCs in NSCLC tissue is inevitably underestimated. This is due to the fact that CD34 and VEGFR2 are expressed on EPCs and on mature endothelial cells lining the tumor vasculature, in conjunction with the fact that CD133 expression continuously decreases on the cell surface of circulating EPCs and is lost once EPCs differentiate into more mature endothelial cells in the endothelial tube [37]. However, it is difficult to conclude that vascularization in human NSCLC is exclusively the result of EPC incorporation into the preexisting endothelial tube. Hilbe et al. reported that $CD133^+$ EPCs, which are involved in vasculogenesis, can regulate tumor growth in humans [38]. They also found that the number of $CD133^+$ EPC cells seems to contribute to the formation of capillaries in solid tumors including NSCLC. Unfortunately, the available data are sparse and controversial, which is probably due to the wide variety in the detection methods for circulating EPCs, non-standardized isolation techniques, and the various immunophenotypes of circulating EPCs. Therefore, using a different method of EPC detection could provide discordant results in terms of EPC count. Although circulating EPCs represent a new biomarker of neoangiogenesis, their number was reduced in cancer patients at stage IV compared to those at either stage I, II, or III [39]. This result may be due to an angiogenesis occurring during a distant metastasis, not EPCs in angiogenesis [40]. However, the approach of the current study was to use four concurrent markers ($CD45^-$, $CD34^+$, $CD133^+$, $CD146^+$) to increase the accuracy of detecting circulating EPCs. In fact, the role of EPCs was found to vary following the release into the blood and differentiation into ECs at different clinical stages. Baseline EPC numbers were less in the peripheral blood of patients with gastric carcinoma at stage IV compared with those at other stages [41]. Thus, it was speculated that EPCs are more likely to gather around the tumor tissues to differentiate into ECs. It should be noted that high pretreatment EPC values lost their significance when adjusted for cancer stage [42]. In fact, the complex pathway of angiogenesis may not be accurately reflected by a single endogenous factor. Moreover, multiple factors associated with tumor status in late-stage cancer patients might affect circulating EPC numbers. Hence, EPC numbers may be

influenced by these factors rather than tumor stage. These findings may explain why baseline EPC values were not correlated with tumor stage in our study.

Tumor angiogenesis occurs when there is a local imbalance between proangiogenic and antiangiogenic factors [43]. Formation of tumor blood vessels is regulated by angiogenesis-promoting factors and angiogenesis inhibitory factors. However, increased VEGF expression has been observed in a heterogeneous group of NSCLC and SCLC patients [32], suggesting that VEGF partly reflects the extent of tumor-related angiogenesis. It was found that circulating EPCs were able to migrate and home to the local endothelium, contributing to neovasculature [10, 44]. Circulating EPCs were involved in tumor angiogenesis and mobilized from bone marrow into sites of tumor neovascularization [45]. The contribution of circulating EPCs in tumor angiogenesis and disease progression may be due to their ability to form new vessels and by secretion of porcine agents that direct tumor cells to distant sites [13]. They can also participate in tumor angiogenesis and tumor tissue vasculature [44]. It should be noted that increased post-treatment EPC numbers suggests unfavorable response to chemotherapy, and it may promote tumor development in cancer patients [14]. Therefore, EPCs could potentially serve as a surrogate marker of tumor angiogenesis status [43]. These findings might explain why high baseline EPC levels in LC patients are associated with increased primary tumor size and diameter in our study.

It has been found that post-treatment EPC levels were lower in responders than in non-responders after treatment. It is possible that high $CD133^{+}VEGF^{+}$ EPC levels reflect less normalized tumor vessels and a chemotherapy response that is worse than in patients with low EPC levels [46], but the included NSCLC patients in this study were treated with chemotherapy combined with bevacizumab. Consequently, circulating EPCs might be an indicator of tumor dissemination and regression in cancer patients [47]. It was hypothesized that EPCs are mobilized by multiple factors and the level of EPCs is reflected by the tumor angiogenesis status in cancer patients [46]. However, Rhone et al. (2019) revealed a significantly higher incidence of disease relapse in breast cancer patients with low pretreatment EPC values compared to those with high baseline levels [48]. In addition, no significant relationship was found between CD133 expression and tumor malignancy levels, which may be due to the continuously decreasing expression of this marker on the cell surface of circulating EPCs. These findings might partially explain these discordant results regarding the potential clinical value of baseline EPC levels to predict tumor response in cancer patients. Further clinical studies are required to validate this explanation.

It has been reported that NSCLC patients with low baseline EPC levels have prolonged PFS duration compared to those with high basal EPC numbers [32]. Using a cutoff value of 375 pg/ml (the mean value for serum VEGF levels), NSCLC patients with high VEGF levels had reduced OS and PFS [33, 35]. In addition, Dome et al. (2006) found that the low circulating EPCs group showed longer PFS time than the high circulating EPCs group [36]. In this study, $CD34^+$ and $VEGFR2^+$ cells were determined as circulating EPCs. However, $CD34^+$ and $VEGFR^+$ are also expressed by mature CECs [46]. However, the standardization of EPCs definition is still critical. On the other hand, it has been found that OS in NSCLC patients with high pretreatment EPC values (above the median value of 1000 cells/ml) was significantly shorter compared to OS in patients with low pretreatment EPC levels [30]. According to the optimal cutoff value for baseline EPCs in our study, LC patients with pretreatment EPC levels (above 125 cells/mL) had longer PFS and OS. These discordant results might be due to the cutoff value considered in each study. In fact, the mean or the median value was considered as cutoff points in some clinical studies [49]. In our study, the optimal cutoff value for baseline EPCs levels was determined according to ROC curves. This analysis appears to be more accurate for the determination of prognostic significance of circulating EPCs in LC patients. Nevertheless, the relevant mechanisms that may explain this discordance are still unclear. The present study has several limitations including the heterogeneity of our population, the lack of some follow-up data and the relatively small sample size. Thus, our results might be biased and validated studies with a lager sample size are needed to confirm our findings.

## Conclusions and perspectives

Our results suggest pretreatment EPCs as a potential diagnostic tool in the clinical setting of LC. Furthermore, baseline EPC levels could be useful for the prediction of prognosis and survival in LC patients. However, it has yet to be determined whether EPCs might be a surrogate marker to monitor the efficacy of chemotherapy regimens in LC. Therefore, a further large-scale clinical study is required to confirm our results and to clarify whether circulating EPC levels could be used as a surrogate marker to evaluate treatment response in LC patients treated with double-agent platinum-based chemotherapy.

## Conflict of interest

Authors declare no conflict of interests.

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None declared.

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**Ethical declaration**

The ethical approval to conduct this study has been granted by the ethical committee of Al-Bairouni University Hospital.

**Authors' contributions**

F.N. performed the analysis. H.S. and H.A. contributed to the study's conception and design. F.N. and G.A. participated in data interpretation. N.A. and I.B., participated in the collection of data. A.J., as technician, analyzed acquisition data, A.I. participated in the collection of literature. F.N. and A.I. revised critically the manuscript. All authors have read and approved the final manuscript.

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**Table 1.** Baseline endothelial progenitor cells (EPC) values and clinical characteristics of lung cancer (LC) patients

| **Clinical characteristics** | **N° (%)** | **EPCs value Mean ± SEM** | **p-value** |
|---|---|---|---|
| **Patients** | | 270 ± 33 | 0.002* |
| Healthy controls | | 121 ± 16 | |
| **Sex** | | | |
| Male | 51 (85) | 283 ± 37 | 0.0167* |
| Female | 9 (15) | 175 ± 47 | |
| **Age** | | | |
| ≤ 60 years | 34 (56.7) | 328 ± 58 | 0.115 |
| > 60 years | 26 (43.3) | 224 ± 36 | |
| **Smoking status** | | | |
| Smokers | 47 (78.3) | 292 ± 39 | 0.2 |
| Non-smokers | 13 (21.7) | 190 ± 54 | |
| **Estimated weight loss** | | | |
| Recent weight loss | 14(42.4) | 275 ± 41 | 0.99 |
| None | 19(57.6) | 275 ± 71 | |
| **Clinical presentation** | | | |
| ID | 32 (53.3) | 236 ± 34 | 0.28 |
| R | 28 (46.7) | 308 ± 58 | |
| **Histology** | | | 0.35 |
| SCC | 17 (31.5) | 322 ± 62 | |
| Non-SCC | 37 (68.5) | 250 ± 45 | |

| | | | |
|---|---|---|---|
| **Tumor differentiation** | | | |
| Moderately/well | 13 (39.4) | 255 ± 71 | 0.86 |
| Poorly | 20 (60.6) | 280 ± 41 | |
| **p-TNM stage** | | | |
| Early | 7 (11.7) | 290 ± 59 | 0.84 |
| Advanced | 53 (88.3) | 269 ± 36 | |
| **Response to chemotherapy** | | | |
| PR/SD | 26 (60.4) | 366 ± 61 | 0.04* |
| PD | 17 (39.6) | 189 ± 50 | |

*p < 0.05; SEM — standard error of the mean; EPCs — endothelial progenitor cells; ID — initial diagnosis; R — relapse; SCC — squamous cell carcinoma; TNM — tumor–node–metastases; PR — partial response; SD — stable disease; PD — progression disease

**Table 2.** Correlation between clinicopathological variables and baseline endothelial progenitor cells (EPCs) levels in patients with lung cancer (LC)

| Variables | **Nº** | **Low EPC levels N = 14 (%)** | **High EPC levels N = 46 (%)** | **Chi-squared** | **p-value** |
|---|---|---|---|---|---|
| **Gender** | | | | | |
| Male | 51 | 11 (18.3) | 40 (66.7) | 0.592 | 0.349 |
| Female | 9 | 3 (5) | 6 (10) | | |
| **Age [years]** | | | | | |
| ≤ 60 | 34 | 12(20) | 21(35) | 5.031 | 0.023* |
| > 60 | 26 | 3(5) | 24(40) | | |
| **Smoking history** | | | | | |
| Former smokers | 47 | 7 (11.7) | 40 (66.7) | 4.057 | 0.06 |
| Never smokers | 13 | 5 (8.3) | 8 (13.3) | | |
| **Estimated weight loss** | | | | | |
| None | 22 | 7 (38.9) | 15 (16.7) | 0.311 | 0.577 |
| Yes | 29 | 6 (36.1) | 23 (8.3) | | |
| **Clinical presentation** | | | | | |
| ID | 33 | 9 (16.7) | 23 (38.3) | 0.358 | 0.39 |
| R | 27 | 6 (8.3) | 22 (36.7) | | |
| **Tumor histology** | | | | | |
| SCC | 17 | 5 (9.3) | 12 (22.2) | 0.12 | 0.73 |
| Non-SCC | 37 | 9 (16.7) | 28 (51.9) | | |

| | | | | | |
|---|---|---|---|---|---|
| **Tumor differentiation** | | | | | |
| Moderately/well | 13 | 4 (12.1) | 9 (27.3) | 1.964 | 0.16 |
| Poorly | 20 | 3 (9.1) | 17 (51.5) | | |
| **Tumor diameter (major axis) [cm]** | | | | | |
| ≤ 4 | 20 | 8 (17.7) | 23 (51.1) | 4.126 | 0.047* |
| > 4 | 25 | 7 (15.6) | 7 (15.6) | | |
| **Staging** | | | | | |
| I–IIIA | 7 | 1 (1.7) | 6 (10) | 0.298 | 0.52 |
| IIIB, IV | 53 | 14 (23.3) | 39 (65) | | |
| **Treatment response** | | | | | |
| PR/SD | 26 | 4 (9.3) | 22 (51.2) | 4.821 | 0.028* |
| PD | 17 | 7 (16.2) | 10 (23.3) | | |

*p < 0.05; EPCs — endothelial progenitor cells; SCC — squamous cell carcinoma; ID — initial diagnosis; R — relapse; PR — partial response; SD — stable disease; PD — progression disease

**Table 3.** Multivariate analysis of standard prognostic variables in lung cancer (LC) patients

| **Prognostic factor** | **Relative risk (95% confidence interval)** | **value** | |
|---|---|---|---|
| Smoking status (nonsmoker *vs.* smoker) | 0.391 (0.113–1.352) | | |
| Histological type (squamous *vs.* non squamous) | 0.857 (0.233–3.159) | 0.514 | |
| p-TNM stage (I–IIIA *vs.* IIIB–IV) | 0.226 (0.042–1.228) | 0.124 | |
| Baseline EPC numbers (Low *vs.* High)† | 5.833 (1.252–27.17) | 0.027***** | |

*****p ≤ 0.05; †cutoff value between low and high baseline EPC levels was defined as 125 EPCs/mL of peripheral blood

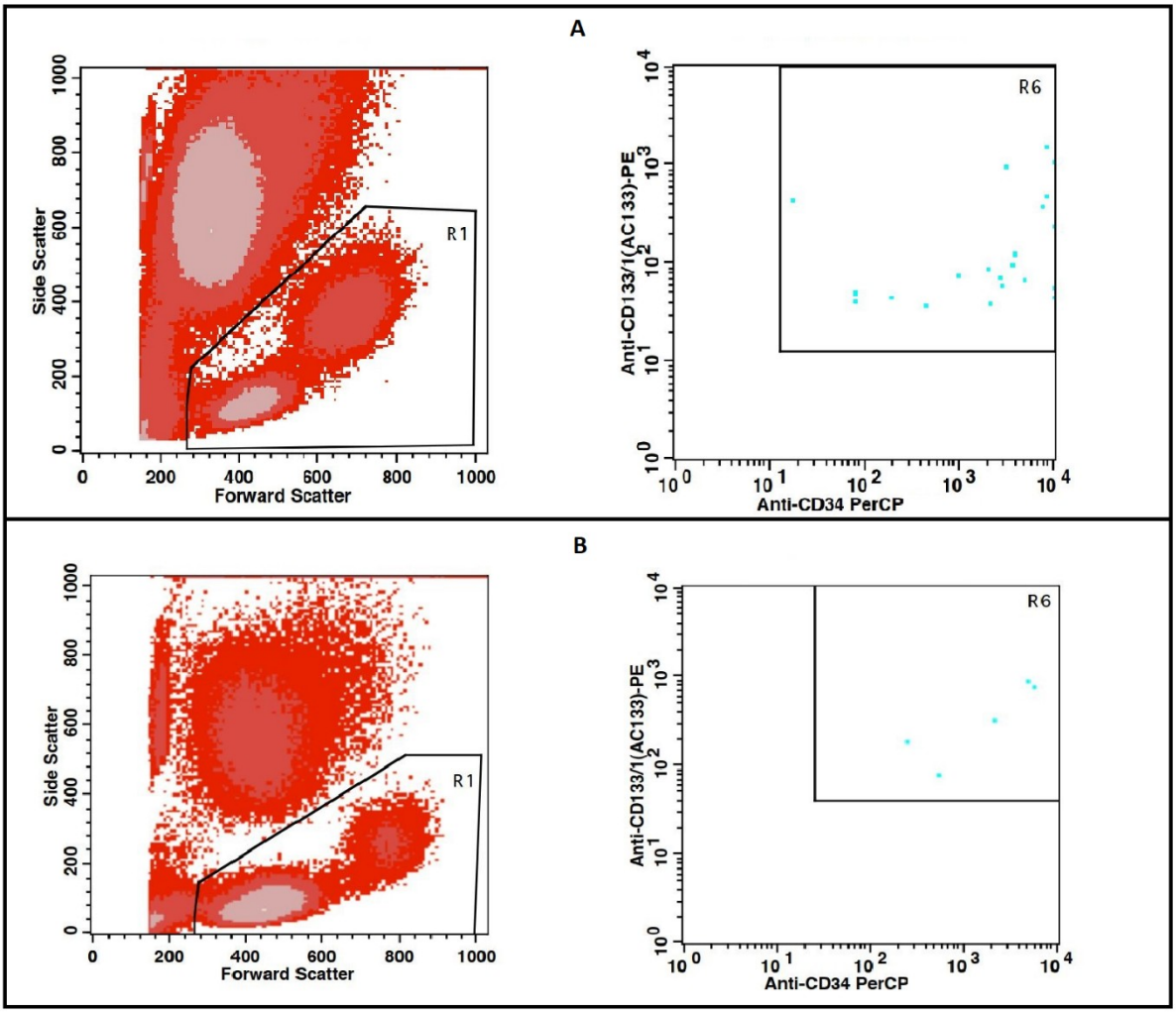


**Figure 1.** Fluorescent-activated cell sorting (FACS) analysis for the quantification of circulating endothelial progenitor cells (EPCs). The analysis gate used to exclude platelets, debris and hematopoetic cells expressing the CD45 antigen versus side scatter (SCC). The gate, restricted to CD45–, CD34+, CD133+, and CD146+ population, was used to enumerate EPCs in the peripheral blood of LC patient (**A**) and healthy individual (**B**)

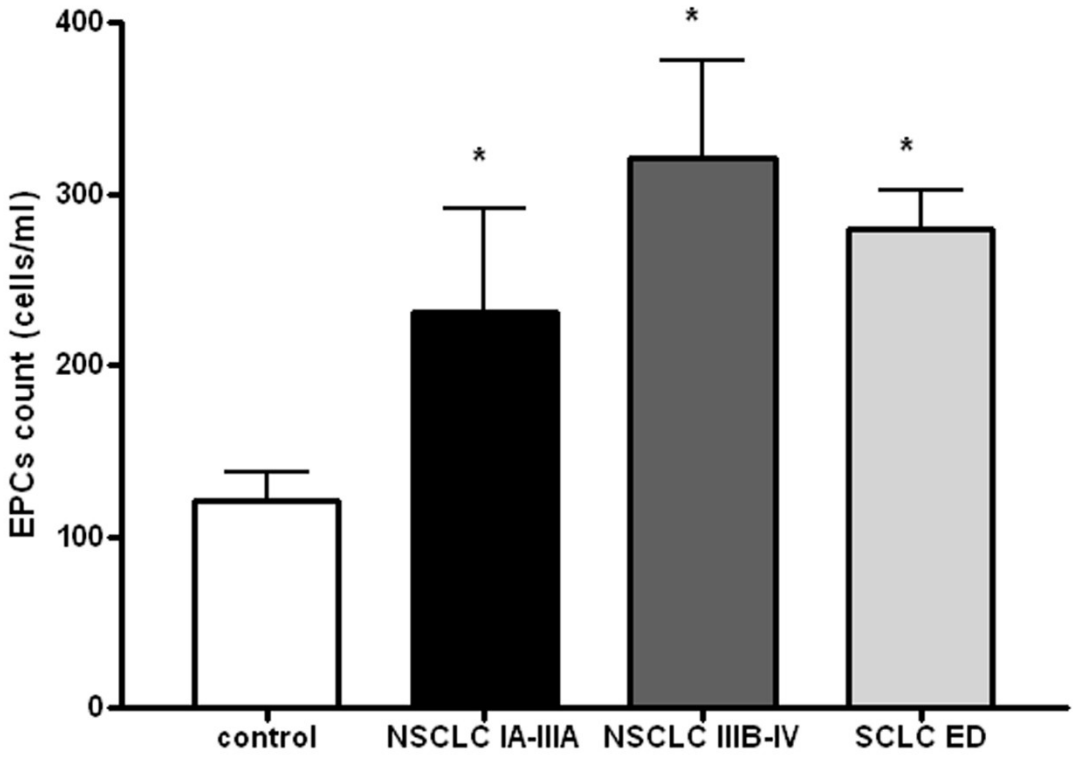


**Figure 2.** Baseline endothelial progenitor cell (EPC) values in lung cancer patients (LC) and in healthy subjects. The number of EPCs was significantly higher in all investigated groups of non-small cell lung cancer (NSCLC) patients with stage of I–IIIA (n = 7), NSCLC with stage of IIIB–IV (n = 49), and extensive disease of SCLC patients (n = 4) compared to healthy individuals as controls (n = 30). The results are expressed as mean ± standard error of the mean (SEM); $p \leq 0.05$ was considered as statistically significant. *$p < 0.001$

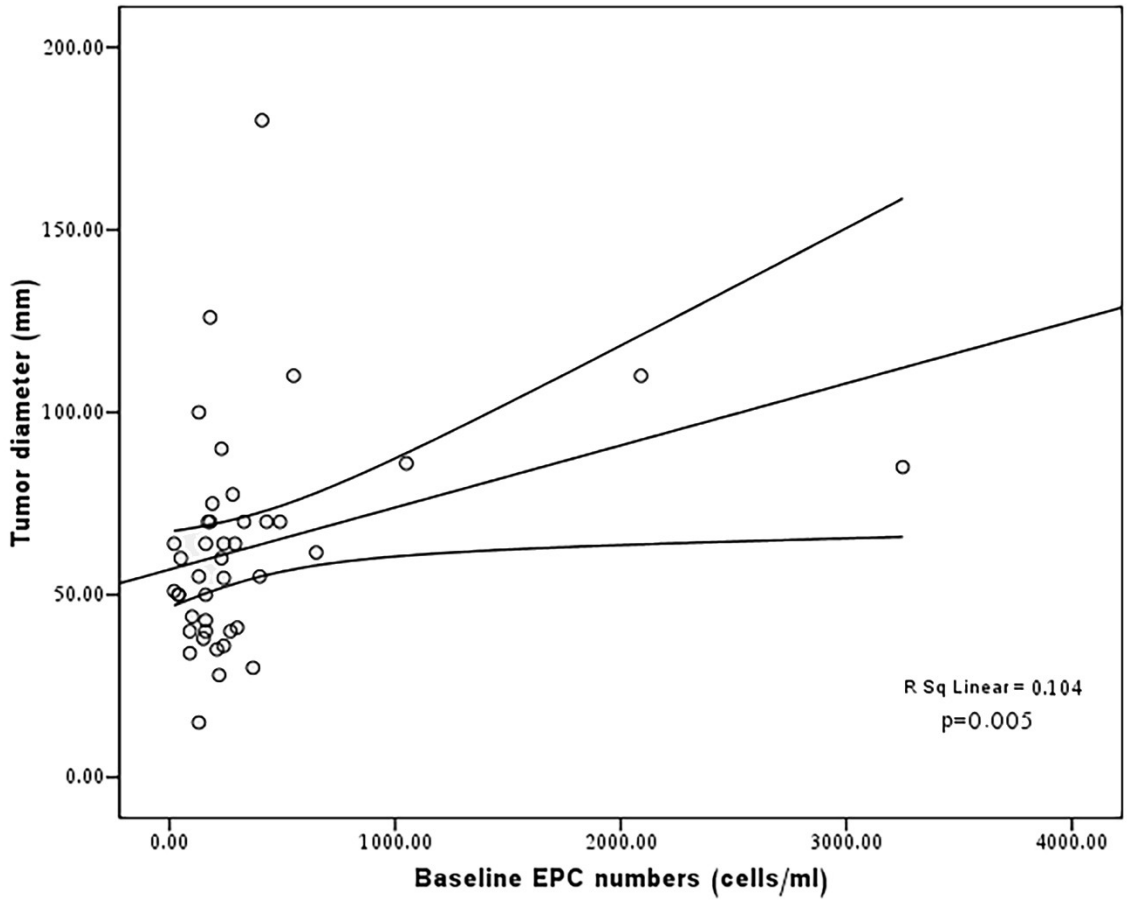


**Figure 3.** Scatter plot shows a significant correlation between baseline endothelial progenitor cells (EPC) values with tumor diameter (Spearman correlation coefficient, r2 = 0.104; p = 0.005)

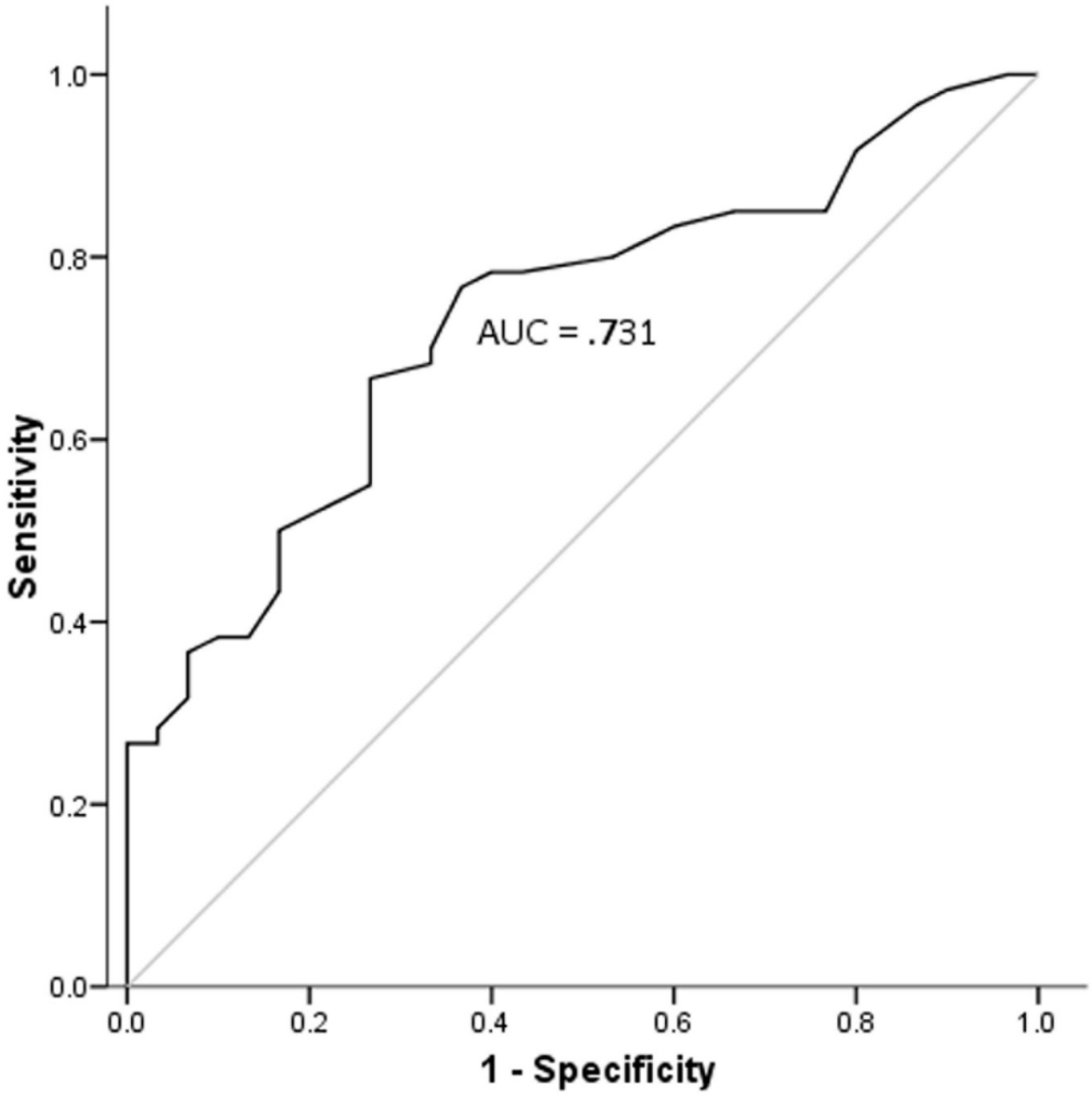


**Figure 4.** A graph showing receiver operating characteristic (ROC) curves for baseline endothelial progenitor cell (EPC) numbers as diagnostic tool in lung cancer patients. The optimal cutoff value between low and high levels was 125 cells/mL of the peripheral blood. According to this value, the sensitivity and specificity for baseline EPC values were 76.7% and 63.3%, respectively. The most accurate indicator for diagnostic accuracy presents an area under the curve (AUC) of 0.731 (p-value = 0.0001). The diagonal reference line acted as an indicator of diagnostic value