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Normalized Serum EGF Levels as a Potential Biomarker in Non-Small Cell Lung Cancer: The Role of Platelets

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Abstract

Background: Literature reports contradictory findings regarding the capacity of serum EGF concentrations ([EGF]) to differentiate non-small cell lung cancer (NSCLC) patients from healthy individuals. Therefore, the diagnostic capacity of [EGF], suggestive of dependency on this growth factor in NSCLC patients (tumors) and hence indicative of possible response to therapies directed to EGF/EGFR, is still an open question. Inconsistencies likely derive from the lack of harmonization and standardization in methodologies for blood and sera processing.

Methods: A cohort of NSCLC patients was evaluated at diagnosis (25) and after first-line-therapy (18/25). Sera were collected 1 h and 4 h after phlebotomy, controlling the variables influencing [EGF]. EGF was quantified by ELISA. Platelets count was also estimated. The values obtained for several combined and/or normalized by platelets count, variables, were compared to those in selected cohorts of healthy controls.

Results: We found differences between healthy individuals and NSCLC patients in the accessibility of EGF to circulation, but not in the total available EGF stock. Indeed, we observed a higher fraction of free EGF in the circulation of patients and consequently a lower amount of EGF stored in platelets. Interestingly, an aberrant relationship between EGF and platelets counts was also observed, especially in patients with thrombocytosis. Moreover, several EGF-related variables, with enough accuracy for discrimination, were identified. Particularly, those variables normalized by platelets count made more evident the differences between patients and healthy controls. Therefore, they might be potential biomarkers in NSCLC.

Conclusion: Our results suggest the increase in free/accessible EGF in blood circulation as relevant to the biology of NSCLC, most likely because it reflects a higher accessibility of this growth factor for the tumor. They also suggest some of the study variables to be further evaluated on its predictive value, to select good responders to CIMAvax-EGF® or other therapies targeting the EGF/EGFR system.

Keywords: Non-small cell lung cancer; Epidermal growth factor; Epidermal growth factor receptor; Platelets; Stratification; Diagnostic biomarker; Predictive biomarker

Abbreviations: NSCLC: Non-Small Cell Lung Cancer; EGF: Epidermal Growth Factor; EGFR: Epidermal Growth Factor Receptor; SNP: Single Nucleotide Polymorphism

Introduction

Lung cancer (LC) is the leading cause of cancer deaths worldwide [1]. Unlike most cancers, which have witnessed steady increases in survival rates, advances have been slow in LC, for which the 5-year survival rate is about 18% [2]. In Cuba, malignant tumors of trachea, bronchia and lung are a health concern as well, with about 4000 newly cases every year and 5720 deaths in 2017 [3]. However, for non-small cell lung cancer (NSCLC), the most frequent LC type, new immunotherapies have shown a great potential for patients in advanced stages [4]. Moreover, personalized medicine is providing hope by treating patients with drugs that are effective based on specific characteristics of their tumors [5]. The Epidermal Growth Factor (EGF), known to stimulate the growth of several types of epithelial tissue, possesses strong mitogenic activity on tumor cells that converts this ligand in an attractive target for designing antitumor strategies [6].

CIMAvax-EGF® [7] is a proven effective Cuban therapeutic vaccine for advanced NSCLC, which induces anti-EGF antibodies that recognize the EGF in circulation, preventing its binding to EGFR, and then disrupting the associated signal transduction cascade in cancer patients and ultimately cell proliferation. Studies of serum EGF concentrations ([EGF]) in Cuban patients treated with this vaccine revealed that high [EGF] are a factor of bad prognosis for NSCLC and at the same time a predictive biomarker of CIMAvax-EGF® efficacy [8-10]. However, the capacity of those concentrations to discriminate between NSCLC patients and healthy individuals (its diagnostic value), suggestive of dependency on EGF in NSCLC patients (tumors) and hence indicative of possible response to therapies directed to this growth factor or its receptor, is still an open question. There are only a few reports available on this topic, some of which have published discrepant findings [11,12]. This inconsistency of results has been presumably caused by the lack of harmonization and sometimes of

standardization in the methodologies used for blood processing and sera collection. Essentially, the majority of reports do not consider the known dependency between [EGF] and the time of sera separation [13,14]. Neither the type of tubes employed for blood collection, which affects clotting times and thus the release of EGF by platelets [15-17], not even the temperature at which blood coagulates, which also impacts this process. Differences in the selection of controls and patients (the lack of control of confounding factors) have also contributed to discrepant results [12].

In this work, [EGF] and platelets counts were quantified in 25 NSCLC patients, using previously standardized methodologies for blood processing and sera separation [14]. Platelet´s contribution to [EGF] was studied in patients, showing a clear difference to what is reported for healthy individuals. Several combined and platelets-normalized EGF-related variables were proposed and evaluated on its capacity to discriminate between patients and healthy individuals. We identified variables with a clear diagnostic value and a simple biological interpretation. Moreover, we used them in an attempt to infer the EGF-dependency in tumors of individual patients. We suggest to further evaluate some of these variables, on its predictive value to select good responders to treatment with CIMAvax-EGF® vaccine or other therapies targeting the EGF/EGFR system.

Materials and Methods

Study design

The [EGF] were assessed in NSCLC patients at the time of diagnosis (To) and 4-6 weeks after the first-line-therapy (T1).

Phlebotomy and sera separation

These procedures were performed according to González-Pérez et al. [14]. Each phlebotomy provided two sera, separated at 1 h and 4 h after venipuncture, and therefore two [EGF]: $[EGF]_{1h}$ and $[EGF]_{4h}$, respectively.

Patient and control cohorts: Ethical aspects

Naive patients, with a cytohistological confirmation of NSCLC, a performance status ECOG<3, and a measurable disease according to RECIST version 1.1 [18], were recruited from October 2014-May 2016, at Hermanos Ameijeiras Hospital (HAH) in Havana. Those with brain metastasis and chronic diseases other than hypertension and heart disease were not enrolled in the study. Staging was determined according to the 7th edition of the TNM system [19]. A group of 25 patients was evaluated at diagnosis (NSCLC1) and a subgroup of 18 was additionally evaluated after treatment (NSCLC2) (Table 1 and Supplementary Table S1). Therapy consisted on 3-6 cycles of Cisplatin or Carboplatin, administered with Etoposide, Vinblastine or Paclitaxel, every 21 days, in combination or not with radiotherapy (23-60 Gy in 20 fractions over 4 weeks, Table 1). Two healthy control cohorts, balanced by age and gender to match the NSCLC groups (HC1 and HC2 in Supplementary Table S1 and S2), were randomly picked out from a panel of healthy Cuban donors [14]. The members of the third control group HC3 were selected from the same panel, based on the availability of their platelets count.

The study was approved by the Ethical Committee of HAH. The sera and patient´s data were collected in compliance with Helsinki Declaration.

Primary variables

Platelets count and serum EGF concentrations were considered primary variables. Based on a previous study [14], the EGF measured at the first hour of coagulation $[EGF]_{1h}$, is known to be the EGF more accessible to circulation. Therefore, in this study the $[EGF]_{1h}$ is interpreted as a good estimate of the actual concentration of free EGF in blood circulation (Supplementary Figure S1). Conversely, the EGF measured at 4 h $[EGF]_{4h}$ is very close to the concentration plateau achieved by the progressive EGF release by platelets during aggregation (Supplementary Figure S1). Consequently, the $[EGF]_{4h}$ is interpreted as the average total stock of EGF in the blood sample of an individual.

Measurement of primary variables

[EGF]: The [EGF] were assessed with the UMELISA EGF® kit [20], whose standard is calibrated against the WHO IS EGF 91/530 (NIBSC). Additionally, in a previous study we verified a very good correlation between the UMELISA kit and the widely used Human EGF Immunoassay Quantikine® ELISA (R&D Systems, Minneapolis, MN, USA) (Supplementary Figure S2).

Platelets count

Blood collected into EDTA-containing test-tubes vacutainer (Becton Dickinson, UK) was assayed in hematological analyzer Mindray BC-3200 (impedance method). We used the reference interval $150\text{-}400 \times 10^9$ platelets/L, employed at HAH and commonly accepted in clinical laboratories [21], with thrombocytosis defined as a counting above 400×10^9 platelets/L. Note that, although data reported seem to indicate an associated to aging decrease in platelets count and also statistically significant differences between counting in women and men [22-24], these differences have no practical importance [25] and do not account for separate norms for women and men [26]. Moreover, these differences usually oscillate in the range of variability of platelet's count estimation ($\leq 6\%$ for Mindray BC-3200, according to Peng et al. [27]).

Combined variables

The calculated ratio $r=[EGF]_{1h}/[EGF]_{4h}$ is interpreted as the EGF fraction from the total stock which is available in circulation. Then, the difference $d=[EGF]_{4h}-[EGF]_{1h}$ is interpreted as a measure proportional to the EGF stored in platelets (not available to circulation).

Normalized variables

We defined several EGF-related variables normalized by platelets count, expressed in grams of EGF per platelet. The $[EGF]_{1h}$/platelets/L is interpreted as the average EGF contributed to circulation per platelet. The $[EGF]_{4h}$/platelets/L is interpreted as the average total stock of EGF per platelet. Finally, d/platelets/L is interpreted as the average EGF stored per platelet (not in circulation).

Discriminatory capacity of different variables

The capacity of variables to discriminate between patients (NSCLC1, n=25) and healthy controls (HC3, n=15) was assessed by the nonparametric method for univariate analysis ROC (Receiver Operating Characteristic) [28,29]. The discrimination accuracy achieved by each studied variable was evaluated by the area under curve (AUC) and the associated p value. For variables with higher/lower values in patients than in controls, the AUC represents the

probability that a randomly evaluated patient will have a lower/higher value than a randomly evaluated control. The p value tests the null hypothesis that the AUC is truly equal to 0.50 (indicating the test is not better at diagnosing than chance). AUC values >0.70 with p<0.05 identify the variables with significant discriminatory capacity. A significant p-value in pairwise comparisons of AUCs means the variable with the highest AUC diagnoses significantly better than the others.

Inference of EGF-dependency in NSCLC

The EGF-dependency of the tumors in different patients was inferred from their stratification by those variables that were found discriminatory in ROC analysis. An optimal cut-off value $C^{(>/<)}$ (decision threshold) was determined for each variable, by maximizing simultaneously sensitivity- Se (%) and specificity-Sp (%) [30]. It was assumed that patients correctly identified by the ROC test (above $C^>$/ below $C^<$) are those with values more anomalous for the evaluated variable, being therefore correctly discriminated with respect to healthy individuals. Consequently, it was inferred that the variables able to better achieve the discrimination might be those chosen to evaluate the stratification of patients, regarding their potential response to treatment with therapies targeting the EGF/EGFR system.

Statistics

Unpaired Student's t-test was used to compare means ($\alpha=0.05$). Confounding factors were controlled by design (matching) and by analysis (stratification). Statistical correlations and some associations were assessed with the Pearson's correlation coefficient-R and by ANOVA or contingency analysis. The statistical packages Statgraphics Centurion XVI, version 16.1.11 and GraphPad Prism 5 were used.

Results

Serum EGF levels and patient´s clinicopathological characteristics

Table 1 shows the clinicopathological characteristics of the 25 NSCLC patients included in our study, the majority of which classified into metastatic state (60% stage IV) or stage III (36%). These high percentages were expected, because NSCLC is mostly diagnosed at advanced stages. The majority of patients (2/3) was 60 years or older (mean age 62.52 years), which was expected because LC mainly occurs in older people. The predominant skin color was white (76%), in correspondence with its higher presentation inside our population (>64% according to the latest Cuban census about population and housing [31]). The proportion of white skin with other (3:1), the ratio men:women, and the predominant ECOG, were very similar to those found in a previous Cuban study [10].

Reports about variation of [EGF] in different subsets of NSCLC patients are scarce and controversial. Therefore, despite our small sample size, we report in Table 1 the average [EGF] measured at 1 h and 4 h, in naive patients with different clinicopathological characteristics. Note that, as it is explained in section Materials and Methods, $[EGF]_{1h}$ most likely estimates the concentration of free EGF in circulation, and $[EGF]_{4h}$ corresponds to the total stock of EGF. Despite the overall small sample size, with the ratios of sampling obtained and $\alpha=0.05$, statistical powers > 0.99 were reached in the

significant differences found between [EGF] in the groups compared. Interestingly, there were no differences between most patient´s strata regarding [EGF]. As Lemos-González et al. [11], we did not find association between [EGF] and the stages III and IV of the disease, patient´s gender or age, although a tendency to decrease was appreciated for $[EGF]_{4h}$ in older patients (61-78 years), as it was previously described for healthy individuals [14,32,33]. However, significant differences were found between T3 and T4 status analyzing $[EGF]_{4h}$ (p=0.0103), but not when $[EGF]_{1h}$ were compared (p=0.2740). The evaluation of nodal involvement revealed as well, a tendency of [EGF] to decrease at N3 level, as compared to N0 and N2, but without statistical significance.

Serum EGF levels before/after first-line therapy

Figure 1A illustrates that in NSCLC patients [EGF] at 1 h and 4 h were highly variable, at diagnosis (To), as well as after first-line therapy (T1). EGF concentrations at 4h were higher than those at 1h, on average (Figure 1A) and also in the majority of patients, with a few exceptions that showed $[EGF]_{4h}<[EGF]_{1h}$ (Figures 1B and 1C). Overall, in qualitative terms, EGF concentrations in NSCLC patients were highly variable, just as reported earlier in healthy individuals [14,34,35]. Moreover, they usually increased with the time of sera separation, just as described for normal subjects [13,14].

Therapy reduced the average [EGF] in patients, but significantly only circulating levels ($[EGF]_{1h}$, Figure 1A). That decrease was clearly patient-dependent and not described by a simple relation of proportionality. Therefore, while we found a significant, although non-strong, correlation between [EGF] at 4 h and 1 h at diagnosis To (NSCLC1, n=24: R=0.5120, p=0.0105), in treated patients T1 this correlation was clearly lost (NSCLC2, n=18: R=0.2506, p=0.3159).

Serum EGF levels in NSCLC patients and controls

Figure 2 shows the direct comparison of [EGF] between NSCLC patients and groups of healthy individuals well-balanced by age and gender (HC1 and HC2). There were no differences between these cohorts (Figure 2A) regarding the average total stock of EGF ($[EGF]_{4h}$), neither before (To) nor after chemoradiotherapy (T1). However, the average concentration of free EGF in serum ($[EGF]_{1h}$) was significantly higher in naive- but not in treated-patients (Figure 2B). To further compare healthy controls and NSCLC patients, we used the combined variables $r=[EGF]_{1h}/[EGF]_{4h}$ and $d=[EGF]_{4h}-[EGF]_{1h}$, which offer different, but complementary information (see Materials and Methods). We found very significant differences between controls and patients at diagnosis by the variable r (Figure 2C), which were stronger than those found by comparing $[EGF]_{1h}$ (Figure 2B). Additionally, r also caught some difference after treatment, although it was not statistically significant. The variable d however, captured the difference at To similarly to $[EGF]_{1h}$ and also at T1 (Figure 2D).

Summarizing, our results show differences between healthy individuals and NSCLC patients regarding the accessibility of EGF to circulation, but not regarding the total stock of EGF. Certainly, we observed a higher fraction of free EGF in the circulation of patients (r) and consequently a lower amount of EGF stored in platelets (d). These results suggest that the increase in free/accessible EGF in blood circulation is relevant to the biology of NSCLC, most likely because it reflects a higher accessibility to this tumoral growth factor.

| Parameters		NSCLC1, To n (%)[b]	NSCLC2, To n (%)[b]	$[EGF]_{1h}$[a,b] (pg/mL)	$[EGF]_{4h}$[a,b] (pg/mL)
Gender	W	5(20)	3(17)	803 ± 228	1017 ± 172
	M	20(80)	15(83)	627 ± 80	1010 ± 75
Age (years)	46-60	11(44)	9(50)	702 ± 111	1165 ± 69
	61-78	14(56)	9(50)	631 ± 110	902 ± 96
Skin colour	White	19(76)	14(78)	648 ± 98	1029 ± 83
	Other	6(24)	4(22)	706 ± 102	957 ± 109
Primary tumor status	T1	3(12)	3(17)	757 ± 83	987 ± 99
	T2	5(20)	5(28)	555 ± 231	980 ± 200
	T3	4(16)	2(11)	868 ± 285	1364 ± 110
	T4	13(52)	8(44)	618 ± 89	918 ± 77
Nodal status	N0	7(28)	5(28)	853 ± 125	1151 ± 81
	N1	1(4)	0(0)	--	--
	N2	6(24)	6(33)	832 ± 188	1198 ± 130
	N3	11(44)	7(39)	493 ± 114	853 ± 96
Distant metastasis	M0	10(40)	7(39)	660 ± 164	1062 ± 138
	M1	10(40)	7(39)	644 ± 112	936 ± 82
	M1a	3(12)	2(11)	721 ± 86	1132 ± 73
	M1b	2(8)	2(11)	676 ± 27	--
Stage	I	0(0)	0(0)	--	--
	II	1(4)	1(6)	--	--
	III	9(36)	6(33)	589 ± 165	1014 ± 144
	IV	15(60)	11(61)	664 ± 75	974 ± 64
ECOG	1	24(96)	18(100)	--	--
	2	1(4)	0(0)	--	--
Platelets (× 10^9/L)	≤ 400	16(64)	12(67)	709 ± 93	967 ± 70
	>400	9(36)	6(33)	579 ± 140	1100 ± 149
Primary tumor Size (mm)	≤ 35	7/22(32)	6(33)	685 ± 115	1114 ± 57
	>35	15/22(68)	12(67)	653 ± 100	977 ± 87
1st line-therapy	CT	25(100)	10(56)	--	--
	CRT	8(32)	8(44)	--	--
# of cycles	≤ 3	8/24(33)	3(17)	--	--
	4-6	16/24(67)	15(83)	--	--
W: Women; M: Men; CT: Chemotherapy; CRT: Chemoradiotherapy; Other: Negroes and mulattos; SME: Standard Mean Error; [a]Mean [EGF] ± SME; [b]Rounded values					

Table 1: Demographic and clinicopathological characteristics of NSCLC patients at diagnosis: Its relation with serum [EGF].
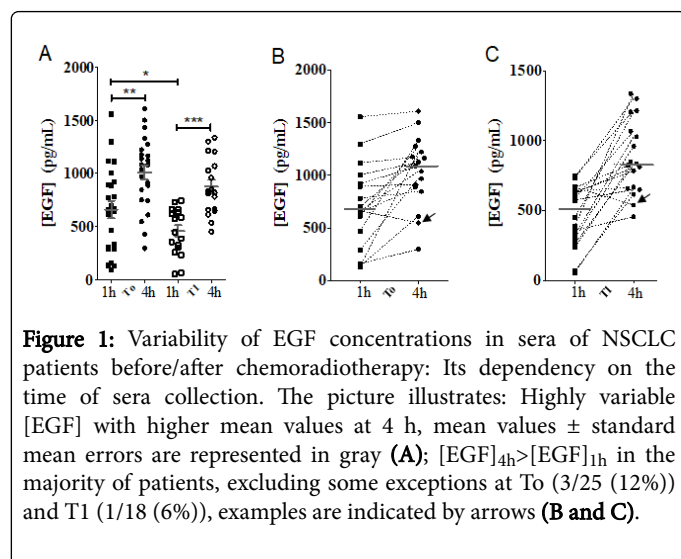
Figure 1: Variability of EGF concentrations in sera of NSCLC patients before/after chemoradiotherapy: Its dependency on the time of sera collection. The picture illustrates: Highly variable [EGF] with higher mean values at 4 h, mean values ± standard mean errors are represented in gray (A); $[EGF]_{4h} > [EGF]_{1h}$ in the majority of patients, excluding some exceptions at To (3/25 (12%)) and T1 (1/18 (6%)), examples are indicated by arrows (B and C).
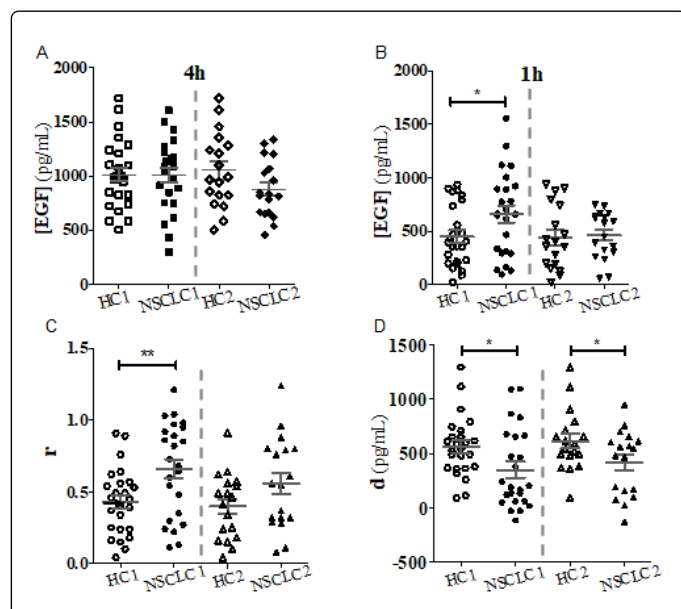
Figure 2: EGF and EGF-combined variables: HC vs. NSCLC patients before/after chemoradiotherapy (NSCLC1/NSCLC2). The picture shows that: patients and controls have on average the same stock of EGF (A); patients have the EGF more accessible to circulation at diagnosis (B); and differences in EGF accessibility are more evident when combined variables are compared (C and D). Mean values ± standard mean errors are represented in gray.

Serum EGF levels and platelets count in NSCLC patients

Figure 3A shows extremely significant differences between the count of platelets in controls and patients at diagnosis, with thrombocytosis[1]

present in 36% (9/25) of cases, which is in agreement with other reported incidences [36-38]. Moreover, Figure 3A also shows that chemoradiotherapy reduced platelets count (p=0.0072) and thrombocytosis (11% (2/18)) although not significantly (Chi-Square p=0.0650, odds ratio=4.5). Such decreases were expected, as thrombocytopenia is a well-known complication of chemotherapy [39] and can also be induced by radiation [40].

A positive linear correlation between [EGF] and platelets count has been reported in humans [14,41], evidencing that platelets are the main source of EGF in human blood. Therefore, we also studied this correlation in our patients (Figures 3B and 3C). Surprisingly, at diagnosis (To) there was no significant correlation between $[EGF]_{1h}$ or $[EGF]_{4h}$ and platelets count (Figure 3B). After first-line therapy (T1), however, there was no correlation for $[EGF]_{1h}$, but a weak correlation was observed for $[EGF]_{4h}$ (Figure 3C). Altogether, these results show that in NSCLC patients exists a different relationship between EGF and platelets, as compared to that observed in healthy controls. Interestingly, first-line chemoradiotherapy reduces serum EGF concentrations and platelets counts, but also tends to partially recover the correlation between them.

To further study the relation between [EGF] and platelets counts in NSCLC patients, some normalized variables were evaluated (Figures 3D-3F). Figure 3D shows that the average total stocks of EGF per platelet ($[EGF]_{4h}$/platelets/L) were equal in healthy controls and patients before/after chemoradiotherapy. Conversely, the EGF per platelet accessible to circulation ($[EGF]_{1h}$/platelets/L) was significantly higher in patients, also before/after chemoradiotherapy (Figure 3E), despite the reduction of [EGF] by therapy. Consequently, the EGF stored per platelet (d/platelets/L) was on average significantly lower in patients at diagnosis vs. healthy controls, and slightly lower after therapy (p>0.05, Figure 3F). Therefore, the comparison of cohorts through the normalized variables makes more evident the differences between them, further suggesting an altered relationship between EGF and platelets in NSCLC patients, as contrasted with healthy controls.

Thrombocytosis is a hallmark in NSCLC at diagnosis, therefore, we attempted to understand the relationship between EGF and platelets in this specific context. For this aim, we divided naive patients into two strata, with and without thrombocytosis (platelets counts above 400 × 10⁹ platelets/L). We compared in these subgroups the average values of the study variables $[EGF]_{1h}$, $[EGF]_{1h}$/platelets/L and d/platelets/L (Figures 3G-3I). Interestingly, the average EGF stored per platelet (d/platelet/L) and the concentration of free EGF ($[EGF]_{1h}$) were the same in patients with/without thrombocytosis. However, the EGF in circulation per platelet ($[EGF]_{1h}$/platelets/L) was on average reduced in thrombocytotic patients (p=0.0239, Figure 3H). Moreover, when the stratification cut-off was established at 350 × 10⁹ platelets/L, the most common used threshold of thrombocytosis [21], this difference was extremely significant (p=0.0005, data not shown). Overall, these results further suggest the existence of an aberrant relationship between EGF and platelets in NSCLC patients. In thrombocytotic patients there is an increase in total platelets count. These platelets store an amount of EGF similar to that found in patients without thrombocytosis. But, the free

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[1]Note that HC3 cohort, employed as control in comparisons by platelets count, could not be properly balanced by age and gender (Supplementary Table S2). Nevertheless, the differences are expected not to be significant, given the report of Biino et al. [24], declaring a 9 × 10⁹/L decrease in platelets count per each 10-years of increase in age. This drop is about two orders lower than the difference we found between the compared cohorts. Additionally, gender-related differences in adults are much lower than those related to aging. Moreover, despite the mentioned imbalance, HC3 does not show differences by [EGF], as compared to the properly balanced controls HC1 and HC2 (Supplementary Table S2).

EGF is slightly lowered in thrombocytotic *vs.* non-thrombocytotic patients, although is still higher than that in healthy controls.
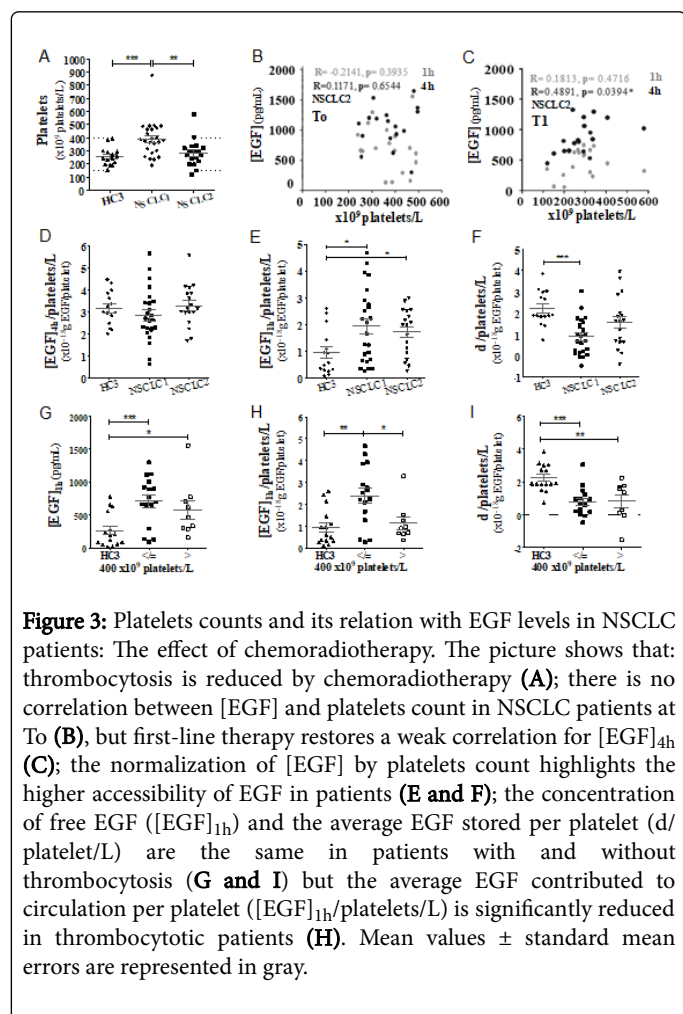
Figure 3: Platelets counts and its relation with EGF levels in NSCLC patients: The effect of chemoradiotherapy. The picture shows that: thrombocytosis is reduced by chemoradiotherapy **(A)**; there is no correlation between [EGF] and platelets count in NSCLC patients at To **(B)**, but first-line therapy restores a weak correlation for $[EGF]_{4h}$ **(C)**; the normalization of [EGF] by platelets count highlights the higher accessibility of EGF in patients **(E and F)**; the concentration of free EGF ($[EGF]_{1h}$) and the average EGF stored per platelet (d/platelet/L) are the same in patients with and without thrombocytosis **(G and I)** but the average EGF contributed to circulation per platelet ($[EGF]_{1h}$/platelets/L) is significantly reduced in thrombocytotic patients **(H)**. Mean values ± standard mean errors are represented in gray.

Discriminatory capacity of different variables

Table 2 summarizes the ROC analysis for discrimination between NSCLC patients and healthy controls, for all variables that showed some relevant differences between these cohorts (Figures 2 and 3). With the obtained sample sizes and the ratios healthy/patients in ROC analysis, statistical powers of 0.8-0.9 were achieved in the detection of clinically important differences, at α=0.05, according to Obuchowski [42]. Several variables achieved a successful discrimination at diagnosis (To) and after chemoradiotherapy (T1). At To the variables d=$[EGF]_{4h}$-$[EGF]_{1h}$ and $[EGF]_{1h}$/platelets/L discriminated just fairly (AUC 0.70-0.80), $[EGF]_{1h}$, r and platelets/L had good discriminatory capacities (AUC 0.80-0.90), while the variable d/platelets/L reached the best discrimination with an AUC=0.8875. At T1, however, only the normalized variables had enough accuracy for a fair discrimination.

Figure 4 shows the ROC curves for those variables with higher discriminatory capacities at To and T1, respectively. The curves cross each other and none of pairwise comparisons were statistically significant. This suggests that all selected variables have a roughly equivalent discriminatory capacity (at least with this relatively small sample size). However, the simultaneous discrimination obtained at To and T1 with the normalized variables ($[EGF]_{1h}$/platelets/L, d/

platelets/L) might favor them as potential candidates for patient's stratification. Actually, d/platelets/L had the highest pAUC in the range of clinical interest (Se ≥ 0.7, Sp ≥ 0.8), among all variables, at To and T1. This result, along with the absence of bias for this variable under thrombocytosis, might support its choice for stratification purposes before/after first-line therapy.

Inference of EGF-dependency in NSCLC

EGF levels in circulation, EGFR mutation status and platelets counts have documented implications in prognosis of NSCLC and its response to therapy. The latter fact supports the existence of NSCLC variants with different underlying biology of the EGF/EGFR system. Particularly, the seminal results of Rodriguez et al. [10] suggest the existence of NSCLC patients with different levels of dependency on the availability of EGF in serum. NSCLC patients with high [EGF] have a poor prognosis and respond better to therapy with CIMAvax-EGF® vaccine, which induces a deprivation of free EGF in the blood of treated patients. Inspired in these findings, we tried to further infer the dependency on EGF in different NSCLC patients, using the studied above EGF-related variables, for stratification purposes. We reasoned that those variables with a higher capacity for discrimination between patients and healthy controls might better capture the aberrant EGF biology in cancer patients. Therefore, these variables might be better for the identification of those patients probably more sensitive to therapies attempting to normalize EGF/EGFR interactions.

Stratification of patients with study variables

Patients were stratified using for each study variable the optimal cut-off value $C^{(>/<)}$, obtained in ROC analysis as explained in Materials and Methods. Patients were predicted as highly EGF-dependent for values of the study variable above $C^>$/below $C^<$, or vice versa, in each specific case. To compare alternative stratifications, its percentages of overlapping in predictions were calculated by pairs of variables (Table 3). In this analysis each equal classification of a given patient (either as dependent or as independent of EGF) by two different study variables, directly increases its overlapping percentage.

For the sake of comparison, we also included the stratification method reported by Rodriguez et al. [10], for the identification of patients more benefitted from CIMAvax-EGF® vaccine. In Rodriguez´s method, patients with [EGF] above the median of the studied population appear to carry tumors apparently more EGF-dependent. To apply this stratification method to our data, the cut-off values ($C^>$) were set to the medians of either the [EGF] at 1 h and 4 h. Note that in Rodriguez´s report the time of sera separation was not controlled, although it was likely close to our 4 h processing, since the median [EGF] reported (873 pg/ml) is close to our value for $[EGF]_{4h}$ (829 pg/ml) in the T1 sample.

Table 3 summarizes the comparison of stratifications achieved with the different variables in our study. At the time of diagnosis (To) the variable $[EGF]_{1h}$/platelets/L appears to classify patients quite similarly to variables d/platelets/L and $[EGF]_{1h}$, with 83% and 92% of coincidence between the overall selections, respectively. Interestingly, the classifications by the medians of [EGF] ($[EGF]_{1h,4h}{}^b$ in Table 3) were quite different to those obtained with the normalized variables at To, with the maximal overlapping of about 70% between $[EGF]_{1h}{}^b$ and $[EGF]_{1h}$/platelets/L.

Table 3 also shows a low overlapping between the classifications achieved by the EGF-related variables and the variable platelets/L. This

result suggests that both could determine the bad prognosis in patients through different pathways. This is not contradictory, considering that, in addition to EGF, platelets possess an armory of other pro-angiogenic proteins, which can be released under its activation [43]. Moreover, platelets also contain several anti-angiogenic proteins, which are delivered following specific stimuli [44]. Therefore, the classification of patients by platelets might go no via EGF pathway, but through other of these several factors and/or its interactions.

After first-line chemoradiotherapy (T1), the normalized variables showed a remarkably high (94%) coincidence in patient´s classification, and moderate overlappings with $[EGF]_{1h}{}^b$ (82%-88%).

However, the classification by the median of $[EGF]_{4h}{}^b$ showed a very low overlapping with the selections of any other study variable, including $[EGF]_{1h}{}^b$ (53%).

Overall, our results suggest that normalized variables $[EGF]_{1h}$/platelets/L and d/platelets/L are quite complementary and therefore will classify patients similarly. However, they could provide classifications different to those obtained using the medians of $[EGF]_{1h,4h}{}^b$, as proposed by Rodriguez et al. [10], especially when using $[EGF]_{4h}$. This result was expected, as we know from our data that $[EGF]_{4h}$ are unable to discriminate cases from controls.

Variable	To					T1				
	AUC	p	$C^{a\,(>/<)}$	Se (%)[b]	Sp (%)[b]	AUC	p	$C^{a\,(>/<)}$	Se (%)[b]	Sp (%)[b]
Platelets/L	0.8181	0.0009553	302 (>)	75	87	--	--	--	--	--
$[EGF]_{1h}$	0.8250	0.0007371	291 (>)	83	73	--	--	--	--	--
r	0.8278	0.0006635	0.68 (>)	54	100	--	--	--	--	--
d	0.7056	0.0178	239 (<)	58	100	--	--	--	--	--
$[EGF]_{1h}$/platelets/L	0.7389	0.01308	0.63 (>)	83	53	0.7431	0.0071	1.62 (>)	65	80
d/platelets/L	0.8875	<0.0001	1.80 (<)	92	80	0.7059	0.0349	1.84 (<)	71	80

AUC: Area Under Curve; $C^{(>/<)}$: Optimized cut-off points (cases are above/below $C^>/C^<$, respectively); Se: Sensitivity; Sp: Specificity; [a]Expressed in the standard units of each variable; [b]Rounded values

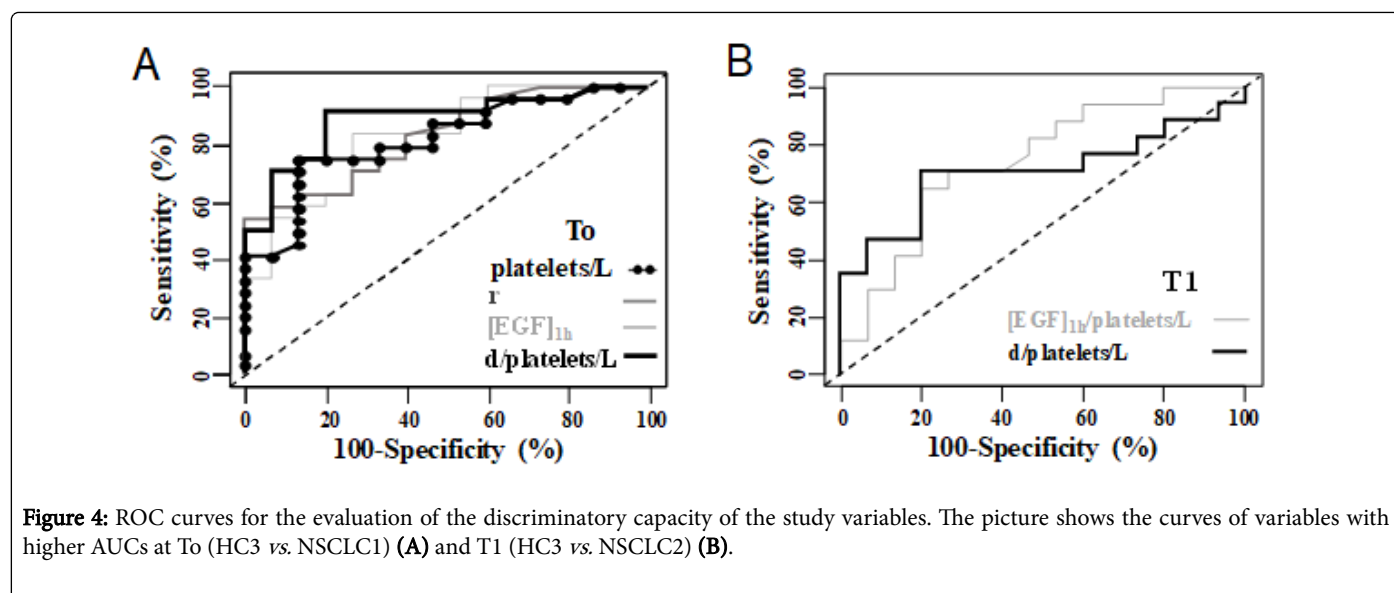**Table 2:** ROC analysis of discriminatory variables.

Figure 4: ROC curves for the evaluation of the discriminatory capacity of the study variables. The picture shows the curves of variables with higher AUCs at To (HC3 *vs.* NSCLC1) **(A)** and T1 (HC3 *vs.* NSCLC2) **(B)**.

Discussion

Overall, EGF concentrations in NSCLC patients were highly variable, just as reported before in healthy people. This natural variability could be mainly attributed to the single nucleotide polymorphism (SNP) at the promoter region of the EGF gene [45,46], which is functional and determines different total stocks of EGF in individuals. Environmental factors could additionally contribute according to Pantsulaia et al. [35].

The higher accessibility of EGF to circulation in NSCLC patients, as compared to healthy controls, suggests that this increase in free/accessible EGF in blood circulation might be relevant to the biology of NSCLC, most likely because it reflects as well a higher accessibility to this tumoral growth factor by the tumor. Moreover, it is known that platelets are often activated in patients [47,48], which elicits the release of several anti- and pro-angiogenic proteins by them [44], including EGF. Additionally, the circulating tumor cells can induce the degranulation of platelets [49], also provoking the EGF release in

patients, and its higher accessibility to serum as suggest our results. Previous findings, altogether with the proven efficacy of the EGF-targeted-immunotherapy CIMAvax-EGF®, support the involvement of EGF in the biology of cancer.

Our results further suggest the existence of an aberrant relationship between EGF and platelets in NSCLC patients. The correlation [EGF]-platelets/L, previously reported for normal individuals [14], is lost in naïve patients, probably due to the significant reduction of circulating EGF levels per platelet under thrombocytosis. Thus, thrombocytosis, an independent indicator of bad prognosis [36,38,48], and low survival and response to therapy in LC [50,51], modifies this correlation. Moreover, the better discrimination achieved by the normalized variables, also confirms the altered relationship EGF-platelets in patients. Finally, the good accuracy of the variable platelets/L in ROC analysis reveals the platelets count as a good surrogate marker of the tumor.

Variable		Platelets/L[a]	$[EGF]_{1h}$[a]	r[a]	d[a]	$[EGF]_{1h}$/platelets/L[a]	d/platelets/L[a]	$[EGF]_{1h}$[b]	$[EGF]_{4h}$[b]
T0	Platelets/L[a]	100	54	33	38	58	63	42	58
	$[EGF]_{1h}$[a]	--	100	71	67	92	79	67	63
	r[a]	--	--	100	88	71	63	92	50
	d[a]	--	--	--	100	63	67	92	46
	$[EGF]_{1h}$/platelets/L[a]	--	--	--	--	100	83	71	29
	d/platelets/L[a]	--	--	--	--	--	100	63	21
	$[EGF]_{1h}$[b]	--	--	--	--	--	--	100	58
	$[EGF]_{4h}$[b]	--	--	--	--	--	--		100
T1	$[EGF]_{1h}$/platelets/L[a]	--	--	--	--	100	94	88	41
	d/platelets/L[a]	--		--	--	--	100	82	47
	$[EGF]_{1h}$[b]	--	--	--	--	--	--	100	53
	$[EGF]_{4h}$[b]	--	--	--	--	--	--	--	100

[a]The selected cut-off points were $C^>/C^<$ from ROC analysis; [b]The selected cut-off points were the medians of [EGF]; [a,b]The intersections row-column show the percentages of patients that were equally classified by both variables, in rounded values

Table 3: Percentages of agreement between different classifications of patients.

Our study has also an added value to the efforts of finding an efficacy biomarker for CIMAvax-EGF® vaccine. This vaccine is approved as a second-line therapy, so the selection of patients for this treatment occurs after chemoradiotherapy. Overall, our results suggest that normalized variables $[EGF]_{1h}$/platelets/L and d/platelets/L are quite complementary and therefore will provide similar selections of patients for this treatment. However, they could provide a classification different to that obtained using the median [EGF] as cut-off, especially when using $[EGF]_{4h}$, a variable which was not able to discriminate cases from controls. Therefore, although in Rodriguez´s approach $[EGF]_{4h}$ could explain in some measure the prognosis of patients and the vaccine´s efficacy after chemoradiotherapy [10], we believe that the normalized variables, discriminatory at T1, might be more valuable for these purposes than $[EGF]_{4h}$.

Finding´s scope

There are enough proofs supporting the role of EGF/EGFR axis in tumor progression and metastasis in several cancer types. In NSCLC specifically, the EGFR is overexpressed in 40-85% of cases [52,53]. Moreover, this overexpression has been implicated in the process of malignant transformation by promoting cell proliferation, cell survival and motility [54]. The EGF, a potent growth factor measured in numerous tumors [55-62] including LC [12,57], has been implicated in the process of invasion and metastasis [63] and correlated with disease stage, course and prognosis [64]. Additionally, some authors have evidenced the mutual interaction and regulation established between EGF and EGFR. Clark et al. reported that EGF regulates its own receptor [65] and an association between EGFR overexpression and an increased expression of its ligands has also been reported [66]. Consequently, EGF and EGFR are frequently synchronously expressed in gastric [56], lung [52] and other carcinomas. Similarly, minimally invasive colorectal resection is associated with a significant decrease in EGF levels early post operation [57]. All these facts sustain the usefulness of EGF/EGFR as potential biomarkers for several carcinomas.

The status of EGFR was not assessed in our study, which could be considered as a limitation of this work. Several reports have described the role of the expression level and mutational status of EGFR in predicting the efficacy of anti-EGFR therapies [67-71], but the association between the expression of EGFR mutations and response to anti-EGFR treatments in patients is still controversial. This responds to variations in the assessed expressions, caused by the use of different cut-off values for EGFR immunostaining [72], the use of antibodies that do not discriminate between the wild-type and mutated forms of the EGFR [73] and discordances between the expression of EGFR in the primary tumor and the metastatic sites [74,75]. Moreover, the interactions between the EGFR´s ligand(s) and the receptor (wild type or mutated), the molecular mechanism of EGFR activation and its

impact on patient´s clinical outcome, are not fully elucidated [73]. The existence of multiple routes for EGFR activation, including a ligand-independent untethering in wild type EGFR (wEGFR), sensitive to differential ligand concentrations, has also been suggested [73,76]. This alternative activation might explain the response to small molecules erlotinib and gefitinib [77], achieved in NSCLC patients with wEGFR (not harboring EGFRvIII). It could also explain the interaction between the overexpressed wEGFR [78,79] and the mAb806 antibody, originally raised against the EGFRvIII mutation [80].

On the other hand, little is known about the mechanisms of action of the anti-EGF antibodies elicited by vaccination with CIMAvax-EGF® or its differential activity in tumors with EGFR mutations or other genetic alterations. Nevertheless, this immunotherapy has proven its efficacy in a phase III clinical trial carried out on unselected NSCLC patients with advanced disease [10]. Recently, Rosell et al. [81] published that anti-EGF antibodies generated with this vaccine suppress the EGF-induced cell proliferation, the cycle progression and also inhibit downstream EGFR signaling, in EGFR-mutant NSCLC cell lines sensitive to different generations of EGFR TKIs. Finally, he concludes that patients with an EGFR-mutant can also derive benefit from immunization against EGF, particularly if combined with EGFR TKIs. In summary, Rosell´s findings prove the contribution of EGF to NSCLC progression also in patients with a mutant EGFR and hence the possible role of this ligand as a biomarker in this subset of patients too. Therefore, present work´s results, along with Rossell´s findings, heighten the usefulness of EGF-related variables as biomarkers of efficacy for CIMAvax-EGF® treatment, independently of the EGFR mutational status. Further studies including the assessment of mutations and expression of EGFR in NSCLC patients, will validate the applicability of these biomarkers in the context of other EGF/EGFR directed therapies.

Conclusion

Concluding, our study revealed that what differentiates NSCLC patients from healthy individuals is not the total stock of EGF, but its higher accessibility to serum. Additionally, a different relation between [EGF] and platelets count was observed in patients. Moreover, several EGF-related variables with enough accuracy for discrimination were identified. Particularly, those normalized by platelets count make more evident the differences between patients and controls, and might be potential biomarkers in NSCLC, and good candidate biomarkers of efficacy for CIMAvax-EGF® treatment, independently of the patient´s EGFR mutational status. Further studies are needed to evaluate the usefulness of these normalized variables on its predictive value to select good responders to treatment with therapies targeting the EGF/EGFR system, and also in prognosis, monitoring of therapy and evaluation of response, in NSCLC and other epithelial cancers.

Acknowledgements

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