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Prognostic value of blood-biomarkers related to hypoxia, inflammation, immune response and tumour load in non-small cell lung cancer – A survival model with external validation

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ABSTRACT

Aim: Improve the prognostic prediction of clinical variables for non-small cell lung cancer (NSCLC), by selecting from blood-biomarkers, non-invasively describing hypoxia, inflammation and tumour load.
Methods: Model development and validation included 182 and 181 inoperable stage I-IIIB NSCLC patients treated radically with radiotherapy (55.2%) or chemo-radiotherapy (44.8%). Least absolute shrinkage and selection operator (LASSO), selected from blood-biomarkers related to hypoxia [osteopontin (OPN) and carbonic anhydrase IX (CA-IX)], inflammation [interleukin-6 (IL-6), IL-8, and C-reactive protein (CRP)], and tumour load [carcinoembryonic antigen (CEA), and cytokeratin fragment 21-1 (Cyfra 21-1)]. Sequent model extension selected from alpha-2-macroglobulin (α2M), serum interleukin-2 receptor (sIL2r), toll-like receptor 4 (TLR4), and vascular endothelial growth factor (VEGF). Discrimination was reported by concordance-index.
Results: OPN and Cyfra 21-1 (hazard ratios of 3.3 and 1.7) significantly improved a clinical model comprising gender, World Health Organization performance-status, forced expiratory volume in 1 s, number of positive lymph node stations, and gross tumour volume, from a concordance-index of 0.66 to 0.70 (validation = 0.62 and 0.66). Extension of the validated model yielded a concordance-index of 0.67, including α2M, sIL2r and VEGF (hazard ratios of 4.6, 3.1, and 1.4).
Conclusion: Improvement of a clinical model including hypoxia and tumour load blood-biomarkers was validated. New immunological markers were associated with overall survival. Data and models can be found at www.cancerdata.org (http://dx.doi.org/10.17195/candat.2016.04.1) and www.predictcancer.org.

In the last three decades, lung cancer has been the leading cause of cancer deaths [1]. To increase the survival of lung cancer patients, we have witnessed an improvement of radiotherapy techniques and more effective (chemo)radiotherapy schemes (i.e., introduction of concurrent treatment) [2–4]. Attempts have been made to develop more accurate risk stratification for non-small cell lung cancer (NSCLC) patients, which would lead to more tailored, individualized and personalized care, avoiding over or under-treatment, by means of a radiation oncology based on multifactorial Decision Support Systems [5,6]. Therefore, the investigation of new prognostic parameters derived from, but not limited to, anatomic, molecular and functional imaging, genomics, and proteomics is warranted [7–9].

The analysis of biomarkers, including proteins, is a fast-developing, promising and challenging area of research, permitting the prediction or description of the evolution of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [10]. Oncoproteins are produced by tumour cells or in response to their presence, and may be released into the bloodstream of cancer patients. As tissue sampling is often not possible in lung cancer patients, blood sample collection by venipuncture is an attractive alternative, which is safe and easy to implement [10]. Blood-biomarkers reflect dissimilarities of the tumour microenvironment, are linked to disease prognosis and

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response to treatment. Blood-biomarkers, that can be measured in daily clinical practice and have been shown to be associated with treatment outcome were first identified in studies comprising large datasets. Based on this criterion, those which biological functions are related to processes of hypoxia [osteopontin (OPN) and carbonic anhydrase IX (CA-IX)]; inflammation [interleukin 6 (IL-6), IL-8, and C-reactive protein (CRP)], and tumour load [carcinoembryonic antigen (CEA) and cytokeratin fragment (CYFRA 21-1)], were analysed and externally validated [11–25]. As an exploratory step we investigated additional blood-biomarkers, including those related to immunological response, which could therefore be incorporated into immunotherapy assessment studies: alpha-2-macroglobulin (α2M), serum IL-2 receptor (sIL2R), toll-like receptor 4 (TLR4), and vascular endothelial growth factor (VEGF) [14,26–35].

Patients and methods

Development dataset

The development cohort included 195 stage I-IIIb NSCLC patients treated with (chemo)radiotherapy between October 2003 and October 2008. Clinical data and blood samples were prospectively collected to ensure standardization. Exclusion criteria included surgery or palliative treatment, and insufficient material to perform blood measurements (OPN, CA-IX, IL-6, IL-8, CRP, CEA, and CYFRA 21-1). All patients participated in the Biobank project (Clinical trials.gov identifiers: NCT00181519, NCT00573040, and NCT00572325) launched in 2003, and provided written informed consent. One hundred and eighty-two patients were treated according to dissimilar radiotherapy (RT) regimens, with a minimum dose of 50 Gy:

1. Forty-nine patients (26.8%) received the standard external beam radiation therapy (EBRT) protocol used until August 2005, of either 70 Gy (Stage I–II) or 60 Gy after induction chemotherapy (Stage III) in once-daily fractions of 2 Gy.
2. One hundred and one patients (55.2%) were treated with EBRT only according to the protocol as of August 2005, with an individualized dose delivered in fractions of 1.8 Gy twice daily, until normal tissue dose constraints were met (e.g., mean lung dose, or maximum dose to the spinal cord) [36].
3. Thirty-three patients (18%) received concurrent (chemo)radiotherapy with a total dose of 45 Gy, delivered in fractions of 1.5 Gy twice daily, followed by an individualized dose of 8 to 24 Gy delivered in fractions of 2.0 Gy once daily, again limited by the dose to surrounding organs at risk [37].

Validation dataset

The validation cohort consisted of 200 NSCLC patients with same characteristics as the development cohort, treated between March 2007 and September 2013. Measurements included the above mentioned blood-biomarkers plus VEGF, α2 M, TLR4 and sIL2R (Clinicaltrials.gov identifier: NCT01936571). One hundred and eighty-one patients received a minimum dose of 50 Gy and were treated as follows:

1. Sixty-eight patients (37.6%) received radiotherapy alone according to the protocol as of August 2005, with an individualized total dose delivered in fractions of 1.8 Gy twice daily, limited by the mean lung dose or the spinal cord dose [36].
2. One hundred and one patients (55.8%) received concurrent chemo-radiotherapy scheme for a prescribed dose of 45 Gy, followed by an individualized dose ranging from 8 to 24 Gy, delivered in fractions of 2.0 Gy once daily, again limited by the dose to surrounding organs at risk [37].
3. Twelve patients (6.6%) followed the Phase II Positron Emission Tomography (PET) boost trial (clinicaltrals.gov identifier NCT01024829), in which a dose escalation protocol was based on the Fluorine-18-Fluorodeoxyglucose distribution of the PET scans [38].

Radiation treatment

Patients were irradiated in accordance with local protocols and stage of the disease. No elective nodal irradiation was performed and irradiation was delivered 5 days a week [39]. Radiotherapy planning was performed on a XiO system (Computerised Medical Systems) until July 2012, using a convolution–superposition algorithm with inhomogeneity corrections and according to International Commission on Radiation Units & Measurements 50 guidelines. As of July 2012, radiotherapy planning was performed using RapidArc (Eclipse version 11.0), with a type B dose calculation algorithm (AcurosXB-10.0).

Endpoint

Study endpoint was overall survival (OS) calculated from start of RT until the date of death or last follow-up. Survival information was retrieved from "Gemeentelijke Basis Administratie" (GBA), the decentralized population registration system in the Netherlands. A patient who was alive at the end of the study was considered right-censored.

Blood-biomarker measurement

Blood-biomarkers measurements of the development dataset can be found elsewhere [40]. Measurements of the validation cohort were performed in a certified laboratory, using commercially available kits, in order to easily translate the results into clinical practice. For each patient, 3 aliquots of 0.5 ml of serum and 3 aliquots of 1.5 ml of plasma were available, which had been collected before the first fraction of radiotherapy, processed using standard protocols and finally stored in the institutional biobank. Measurements in plasma were performed using enzyme-linked immunosorbent assays for OPN (Quantikine Human Osteopontin Immuno assay; R&D Systems, Minneapolis, MN), CA-IX (Nuclea Diagnostics, Cambridge, MA), VEGF (R&D Systems), and TLR4 (MyBioSource, San Diego, CA). Measurements in serum for IL-6 and IL-8 were determined on Immulite XPi 2000 with a solid phase, enzyme labelled, chemoluminescence sequential immunometric assay (Siemens Medical Solutions Diagnostics, LA), for CRP on Cobas 8000 using an immunoturbimetric assay (Roche Diagnostics, Mannheim, Germany), for CEA on Immulite XPi using a solid-phase, two-site sequential chemoluminescent immunometric assay (Siemens Medical Solutions Diagnostics), for CYFRA 21-1 on Kryptor with a sandwich immuno-fluorescent assay (Brahms, ThermoFisher, Hennigsdorf, Germany), for α2M on BN ProSpec using immunonephelometric assays (Siemens Medical Solutions Diagnostics, LA, USA), and for sIL2R using an enzyme-linked immunosorbent assay (Diaclone, Basancon Cedex, France).

The analytes OPN, CA-IX, VEGF and TLR4 were assayed in plasma in duplicate using a Victor multilabel counter (Perkinelmer, Turku, Finland), while all other biomarkers were measured in singletons.

Descriptive statistics

Comparison of the development and validation datasets distributions was performed using a χ^2 test for categorical variables and a Student *t*-test for the continuous ones. Prior to this a variable transformation on the gross tumour volume (GTV) and blood

biomarker measurements were performed, using a logarithmic approach. Missing data imputation was performed by Multivariate Imputation by Chained Equations (MICE). Correlation analysis was performed between clinical and blood-biomarker variables under analysis for both datasets.

Model development

A Cox proportional hazards model was developed and included validated clinical variables: gender, World Health Organization performance status (WHO-PS), forced expiratory volume in one second (FEV1s), number of positive lymph node stations identified in the diagnostic PET scans (lymph nodes) and GTV, defined as the sum of the GTV of the primary tumour and metastatic lymph nodes [41]. A least absolute shrinkage and selection operator (LASSO) method was applied for feature selection of the blood-biomarkers, entered as continuous variables into the model. LASSO selects variables correlated to the measured outcome by shrinking coefficients weights, down to zero for the ones not correlated to outcome [42]. Regression coefficients were estimated, using the whole dataset, hazard ratios (HR) and confidence intervals (CI) were calculated. Schoenfeld residuals were used to check the proportional hazards assumption. Discrimination, reflecting a correct ordering of the relative predictions with respect to true outcomes (i.e. overall survival) for individuals, and model's ability to distinguish between individuals who experience the outcome from those who remained event free, was determined by the Harrell's concordance-index (c-index). This ranges from 0.5, no discrimination (no greater than the chance expectation) to 1 indicating perfect discrimination.

External model validation

Calibration of the model, which refers to the agreement between observed and predicted probabilities, was visually assessed (further details in the appendix). Discrimination ability of the model was also determined by the Harrell's c-index in an independent dataset.

Model updating

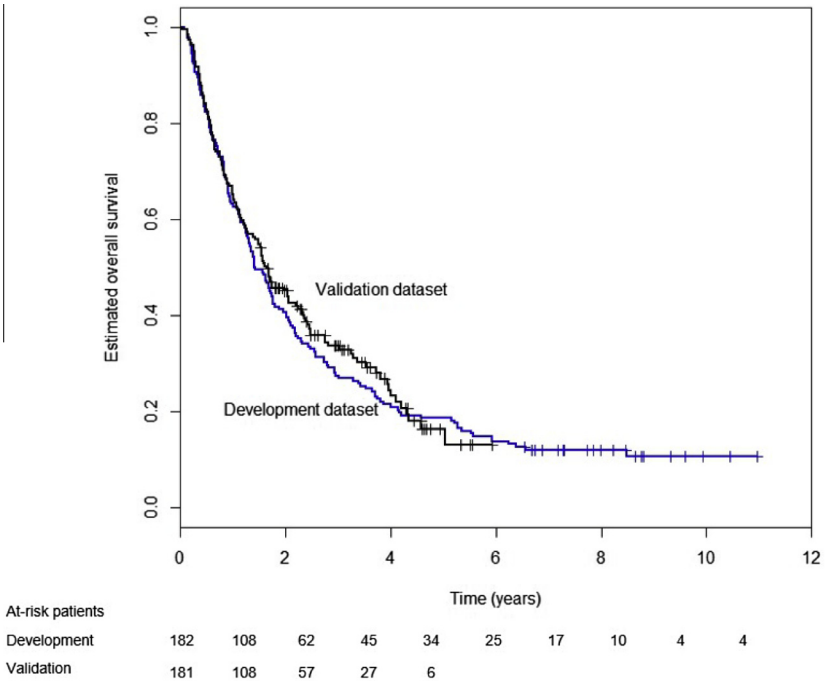
The prognostic index (PI) was computed by multiplying the covariates with the estimated coefficients on the validation dataset: $PI = \sum_i \beta_i x_i$. The validated model was extended by inputting the PI (recalibrated if necessary) as a fixed variable and further selecting among new proposed blood-biomarkers. Performance was evaluated by means of an internal 10-fold CV c-index (further details in the appendix). A log likelihood test of model fit was conducted between the validated and extended model.

All statistical methods were performed in R (version 2.15.2), using the libraries *survcomp*, *survival*, *rms*, *glmnet* and *corrplot* [43].

Results

Upon analysis, 161 and 132 patients in the derivation and validation cohorts, respectively, had died, and median follow-up time was 8.2 (95% CI: 7.3–9.3) and 3.6 years (95% CI: 3.2–4.6). Kaplan Meier plots of the overall survival for both datasets are shown in Fig. 1. Patient characteristics and blood biomarker measurements are shown in Tables 1 and 2, including the missing measurements count. Homogeneity tests on the blood-biomarkers measures reflect the small differences that could be observed between development and validation datasets, for which a non-significant p-value could be demonstrated. The exception to this lies on IL-8, that was significantly higher in the validation dataset, with, however, a non-drastically higher median. The highest Pearson correlation was 0.61 between IL-6 and CRP in the development dataset ($r = 0.72$ in the validation dataset). Correlation between clinical features and blood-biomarkers was lower than 0.5 for all comparisons, demonstrating that these variables provide Complementary information (Fig. 2). Radiotherapy administered doses Radiotherapy regimen up to August 2005 had an HR = 0.17 ($p < 0.01$), while the one afterwards had an HR = 0.90 ($p = 0.52$). Concurrent scheme presented with an HR = 0.62 ($p = 0.03$). Administered dose had a risk of 0.98 ($p = 0.03$).

The model including solely clinical parameters achieved a performance with a c-index of 0.66 ($p < 0.01$). The automatic feature



Curve di Kaplan Meier dei due dataset (sviluppo e validazione), è apprezzabile un andamento simile, a indicare probabilmente che la popolazione obiettivo (il dataset di validazione) è corretta.

Fig. 1. Kaplan–Meier plots for overall survival for development and validation datasets.

Table 1
Demographic information of the study population. Comparison between datasets distribution were performed after missing data imputation, with a $\chi 2$ test for categorical variables and a Student *t*-test for the continuous ones (*).

	Derivation dataset (n = 182)	Validation dataset (n = 181)	p-Value*
Age			0.40
Range (median)	42–87 (69)	44–88 (68)	
Mean ± SD	67.2 ± 10.5	68 ± 9.3	
Gender			0.09
Male	139 (76.4%)	123 (68%)	
Female	43 (23.6%)	58 (32%)	
Stage			0.06
I	33 (18.1%)	20 (11%)	
II	14 (7.7%)	25 (13.8%)	
IIIa	48 (26.4%)	49 (27.1%)	
IIIb	87 (47.8%)	85 (47%)	
Unknown (M0)		2 (1.1%)	
Histology			<0.01
Adenocarcinoma	26 (14.3%)	39 (21.5%)	
Squamous cell	49 (26.9%)	68 (37.6%)	
NOS	91 (50%)	66 (36.5%)	
Unknown	16 (8.8%)	8 (4.4%)	
WHO-PS			0.43
0	47 (25.8%)	48 (26.5%)	
1	81 (44.5%)	93 (51.4%)	
⩾2	17 (9.3%)	28 (15.5%)	
Unknown	37	12 (6.6%)	
FEV 1s			0.90
Range (median)	33–124 (76)	25–136 (76)	
Mean ± SD	74 ± 23.3	74 ± 24	
Unknown	14		
Lymph nodes			0.02
0	73 (40.1%)	45 (24.9%)	
1	26 (14.3%)	28 (15.5%)	
2	32 (17.6%)	34 (18.8%)	
3	20 (11%)	25 (13.8%)	
⩾4	31 (17%)	49 (27%)	
GTV (cm^3)			0.12
Range (median)	0.84–674.4 (63.5)	6.15–1076 (104)	
Mean ± SD	89.8 ± 97.7	128 ± 142	
Unknown	3	8	
Radiotherapy (Range (mean ± SD), in Gy)			
Standard protocol			
Before August 2005	58–70 (62.4 ± 4.3)	–	
After August 2005	50.40–79.2 (64.2 ± 9.8)	52.2–79.2 (68.9 ± 11.9)	<0.01
Concurrent scheme			
First Dose	45–51 (46.9 ± 3.5)	45 (45.9 ± 6.1)	
Second Dose	8–24 Gy (13.3 ± 8.8)	8–24 (22.0 ± 7.2)	
PET Boost	–	46.8 – 95.5 (71.9 ± 11.7)	

Acronyms: SD – Standard Deviation; NOS – not otherwise specified, also includes patients categorized as "large cell"; WHO-PS – world health organization performance status; FEV 1 s – forced expiratory volume in 1 s; Lymph nodes refer to the PET positive stations identified; GTV – gross tumour volume (GTV = volume of primary tumour + volume of PET positive lymph node stations).
* Significance of the homogeneity tests between datasets distributions.

selection algorithm used (LASSO), selected from the blood-biomarkers and extended the clinical model with OPN and Cyfra 21-1 (hazard ratios (HR) of 3.3 and 1.7), which led to a significant improvement in the performance up to 0.70 ($p < 0.01$; Table 3). Starting point for automatic feature selection methodology includes simultaneous consideration of all 8 biomarkers together with previously developed clinical model. Then, lower priority is assigned to features less associated with outcome, and therefore removed from the final model. Finally, features associated with survival outcome and not mutually correlated, were retained for the final model.

As a side remark, most of the analysed blood biomarkers presented with a univariable significance correlation to overall survival (results in the online appendix), with the exception of CA-IX and IL-8. In addition, features included in final model presented with the most prominent HR of 7.2 (OPN) and 2.38 (Cyfra 21-1).

External validation of the clinical model yielded a c-index of 0.62 while the extended one improved to 0.66. Despite the lower performance of the external validation, the addition of blood-biomarkers again showed an improvement in the performance. A nomogram based on this model is presented in the online appendix of this manuscript.

In an exploratory exercise, the validated model was extended with α2M, sIL2r, and VEGF, and presented a c-index of 0.67 ($p < 0.01$; 10-fold CV c-index = 0.66, Table 4). The validated model, as in Table 3, is represented by its prognostic index (PI) in a new automatic feature selection routine, that extends it to include the mentioned blood-biomarkers. Adding these biomarkers resulted in a better fitted model ($p = 0.01$; likelihood ratio test), and particularly α2M and sIL2r were significantly associated with survival with HR of 4.6 and 3.1. Further details on how this extension is performed are descripted in the appendix, including model's recalibration.

Discussion

Prognostic modelling of NSCLC is becoming an important element of the disease management. Several sources of information now available make it an emerging and constantly changing field, however the choice for non-invasive techniques is preferred in order to diminish the burden for patients. The use of blood-biomarker measurements appears to be a non-invasive, fast and promising source of extra information, reflecting dissimilarities of the tumour microenvironment that has been shown to be associated with disease prognosis and response to treatment [44,45].

We demonstrated and validated in two large cohorts of NSCLC patients the added value of blood-biomarkers related to hypoxia (OPN) and tumour load (Cyfra 21-1), reflected by a statistically significant improvement in the performance of a clinical model after their inclusion. *A priori* selection of blood-biomarkers was based on a literature search and evidence of their prognostic value for NSCLC [11–25]. Hypoxia affects malignant progression by increasing the tumour's metastatic ability and diminishing the apoptotic potential, and also the response to therapy, by decreasing the effects of anticancer therapies including (chemo)radiotherapy [44]. In addition, imaging studies with hypoxia-specific markers have shown that hypoxia is a prevalent effect in NSCLC [46]. The hypoxia marker OPN, has also been associated with tumour aggressiveness and metastatic potential, accompanying a poor prognosis in NSCLC, confirmed rather strongly in our study (HR = 3.3) [19,20]. Tumour load is often associated with disease development and prognosis [41,47]. Cyfra 21-1, a marker associated with tumour load, was previously identified as a prognostic factor for NSCLC, which was also confirmed in our analysis [48].

We have already investigated the prognostic potential of blood-biomarkers using a support vector machine (SVM) for model development. We then extended a validated 2-year survival clinical model with blood-biomarkers related to inflammation (IL-6) and tumour load (CEA). Performance expressed as the area under the curve (AUC) improved from 0.72 (solely clinical parameters) to 0.81 [40,41]. However, the limited number of patients included in the study made it difficult to draw definitive conclusions. Moreover, SVM is less suited to a time-to-event analysis, as it requires dichotomous outcome. Therefore we re-analysed the data,

Table 2
Distribution of the blood-biomarkers after data imputation: range (median); mean ± standard deviation. Biomarkers were not measured in case there was not enough material. New biomarkers were available in the latest measurements solely performed in the validation dataset. Comparison of blood-biomarkers distribution in the two datasets was conducted with a Student *t*-test (*).

Blood biomarker	Development dataset (n = 182) Range (median) Mean ± SD	Not measured	Validation dataset (n = 181) Range (median) Mean ± SD	Not measured	p-Value*
Hypoxia					
Osteopontin (OPN)	40–304 (99) 111 ± 46	3	16–1802 (140) 128 ± 137	1	0.13
Carbonic Anhydrase IX (CA-IX)	59–2477 (221) 337 ± 346	2	48–8508 (254) 402 ± 696	1	0.26
Inflammation					
Interleukin 6 (IL-6)	1.1–86 (7.2) 10 ± 11	4	1.8–462 (6.4) 14 ± 39	0	0.26
Interleukin 8 (IL-8)	2.3–91 (11) 14 ± 13	5	4.7–316 (13) 20 ± 28	0	0.01
C-reactive protein (CRP)	1–315 (12) 26 ± 41	16	0.9–258 (8.8) 24 ± 39	0	0.55
Tumour load					
Carcinoembryonic antigen (CEA)	0.8–1806 (4.3) 32 ± 152	9	0.7–1068 (5.4) 33 ± 115	0	0.91
Cytokeratin fragment (CYFRA 21-1)	0.2–49 (1.7) 3.7 ± 6.4	5	0.1–119 (2.0) 6.2 ± 16	1	0.05
New biomarkers					
Alpha-2-Macroglobulin (α2M)			1.1–5.2 (2.3) 2.4 ± 0.8	5	
Serum IL-2 receptor (sIL2R)			1121–20000 (5926) 6129 ± 2907	0	
Toll-like receptor 4 (TLR4)			1.4–30 (6.4) 7.5 ± 4.6	1	
Vascular Endothelial Growth Factor (VEGF)			18–505 (84) 112 ± 94	1	

Acronyms: SD – Standard Deviation.
* Significance of the homogeneity tests between datasets distributions.

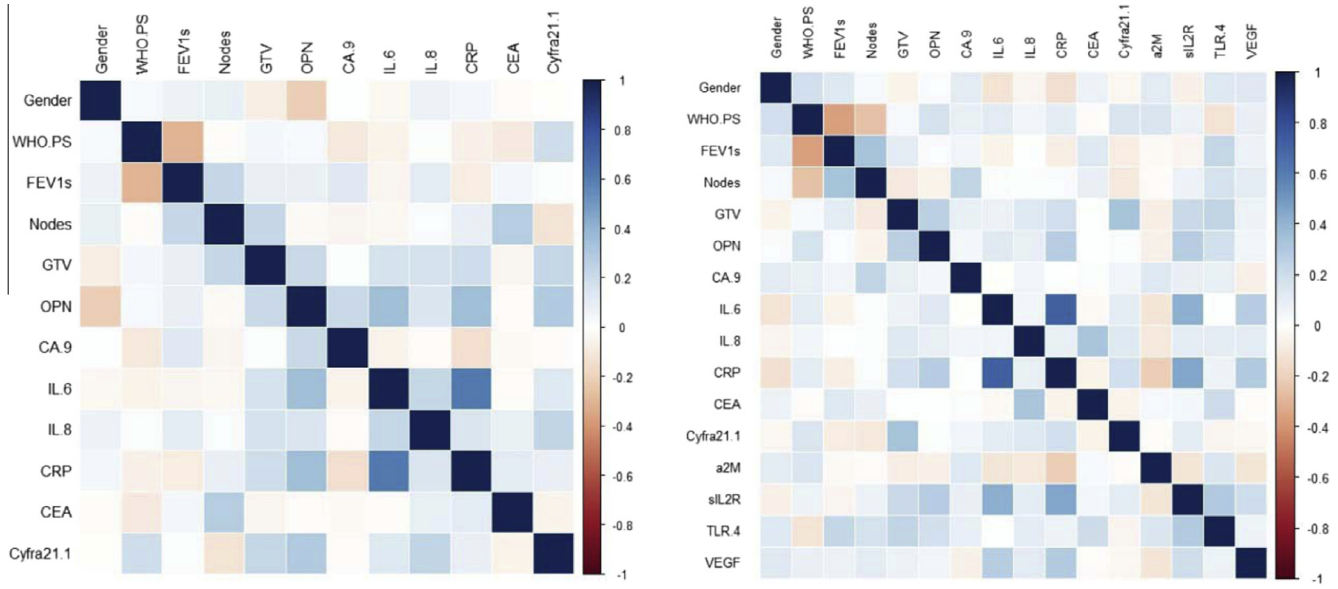


Fig. 2. Heat maps illustrating Pearson correlation between clinical features and blood biomarkers in the derivation (left) and validation (right) datasets.

applying Cox regression. In the newly fitted clinical model, moderately good performance (0.66, external = 0.62) could be achieved with female gender which is associated with a better prognosis, given the lower risk (HR = 0.50), while WHO-PS ⩾ 2 (HR = 2.65) and larger GTV (HR = 1.41) are worse prognostic factors.

The validated model was extended to include α2M, sIL2r, and VEGF. Despite the comparable c-index (0.67; 10-fold CV = 0.66), most importantly it showed that higher concentrations of these new markers are associated with a worse prognosis, particularly α2M and sIL2r with HRs of 4.6 and 3.1. Previous studies showed α2M as a top candidate for radiation pneumonitis [31]. We further confirmed its prognostic potential for NSCLC. IL-2 was already identified as an independent prognostic marker in patients with advanced NSCLC [49]. Its cell surface receptor, a soluble form of

Table 3
Multivariable Cox PH regression of the clinical variables and of clinical variables and blood-biomarkers fitted on the derivation dataset. Performance of the model expressed in terms of internal and external validation (*) by Harrell's c-index.

Feature	Hazard ratio	*p*-Value	95% CI HR	c-index	Hazard ratio	*p*-Value	95% CI HR	c-index
Gender				0.66				0.70
Male	Reference			0.62*	Reference			0.66*
Female	0.50	<0.01	0.33–0.75		0.54	<0.01	0.36–0.82	
WHO-PS								
0	Reference				Reference			
1	1.30	0.01	0.90–1.88		1.20	0.01	0.82–1.74	
⩾2	2.65		1.57–4.45		2.09		1.22–3.58	
FEV 1 s	1.00	0.88	0.99–1.01		1.00	0.45	0.99–1.00	
Lymph nodes								
0	Reference				Reference			
1	0.63	0.14	0.37–1.06		0.63	0.14	0.37–1.07	
2	1.03		0.63–1.66		1.12		0.69–1.83	
3	0.59		0.34–1.05		0.62		0.35–1.09	
4	0.91		0.57–1.46		1.10		0.68–1.80	
ln(GTV) (cm^3)	1.41	<0.01	1.21–1.64		1.28	<0.01	1.09–1.50	
OPN					3.31	0.01	1.31–8.38	
Cyfra 21-1					1.71	0.01	1.18–2.50	

A logarithmic transformation was performed on the blood-biomarkers measurements prior to analysis.
Acronyms: CI – Confidence Interval; HR – Hazard Ratio; WHO-PS – World Health Organization Performance Status; FEV 1 s – forced expiratory volume in 1 s; Lymph nodes refer to the PET positive stations identified; GTV – gross tumour volume (GTV = volume of primary tumour + volume of PET positive lymph node stations); OPN – Osteopontin; Cyfra 21-1 – cytokeratin fragment 21-1
* External validation.

Table 4
Multivariable Cox PH regression of the clinical variables and blood-biomarkers fitted on the validation dataset, after a feature selection made by LASSO from newly analysed blood-biomarkers. Performance of the model is expressed in terms of internal c-index, corrected for optimism by a 10-fold CV (**).

Feature	Hazard ratio	*p*-Value	95% CI HR	c-index
Calibrated PI*	2.44	<0.01	1.69–3.51	0.67
α2M	4.62	<0.01	1.31–16.3	0.66**
sIL2R	3.15	<0.01	1.08–9.21	
VEGF	1.37	0.28	0.78–2.43	

A logarithmic transformation was performed on the blood-biomarkers measurements prior to analysis
Acronyms: α2M – alpha-2-macroglobulin; sIL2r – serum interleukin-2 receptor; VEGF – vascular endothelial growth factor.
* Validated model in Table 3 entered as a variable – the calibrated prognostic index (PI).
** Internal c-index corrected for optimism by a 10-fold CV.

IL-2 receptor (sIL2r) is released into the bloodstream and involved in the regulation of IL-2. High sIL2r levels were associated with shorter survival in an advanced stage [50,51]. Elevated levels of sIL2r may lead to a decreased cellular response to IL-2, reinforcing the importance of measuring this marker for patients receiving IL-2 immunotherapy [51–53]. VEGF, also included in the final model with a less expressive HR of 1.4, is a common angiogenesis factor for a variety of solid tumours, including NSCLC [54]. It has been shown previously to be an independent prognostic factor, which was not however confirmed by our data [14].

An advantage of our study is the large number of patients available, making the derived message a more solid one. Conversely, this study had some limitations. First, it was impossible to validate our latest findings using the newer biomarkers as these were only measured in the validation dataset. This should be thoroughly considered in the future, by gathering data from external institute(s). The heterogeneity of our dataset, including more recently treated patients following concurrent schemes in the validation dataset, the different types and scheduling of chemotherapy, and the different laboratories involved in the biomarkers measurements may provide us to a better understanding of the derived calibration slope, which is below 1 [55]. However, the c-index achieved in the context of a prognostic analysis must not be disregarded, particularly when compared with the limited capacity of humans to infer better prognosis from the same sources of information [7]. Also due to its great heterogeneity, it is not possible with this cohort to assess stage-specific treatment variables. We are however able to deliver a set of prognostic factors, independent of dissimilar types of treatment or disease stage, but spanning a larger cohort of heterogeneous patients. This model could be improved by including other sources of information, for example imaging (Radiomics), as the relevant prognostic value of sophisticated image analysis has been shown, to be investigated in the future [8,9]. One last point that we would like to emphasize is the increasing number of PET tracers currently available and the ability to obtain extra information, in particular relating to hypoxia (^{18}F-FMISO, ^{18}F-FAZA and ^{18}F-HX4), tumour proliferation (^{18}F-fluorothymidine), and assessment of epidermal growth factor receptor (EGFR) [56–58]. The potential use of blood-biomarkers to predict which imaging tracer is best suited to an individual patient, would convert prognostic markers into predictive information and increase the cost effectiveness of imaging procedures, while diminishing patient burden from extensive diagnostic imaging procedures.

In conclusion, we improved a clinical model by the inclusion of blood-biomarkers related to hypoxia and tumour load. This improvement was validated externally, which reinforces its potential relevance for shared decision-making. An extended model demonstrated that higher concentrations of the inflammation marker α2M and immunological marker sIL2r have strong negative prognostic value for NSCLC, which requires extra validation. Data are publicly available at www.cancerdata.org (http://dx.doi.org/10.17195/candat.2016.04.1) and the developed models can be found at www.predictcancer.org.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2016.04.024.

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