



Early View

Original article

Validation of Lung EpiCheck®, a novel methylation-based blood assay, for the detection of lung cancer in European and Chinese high-risk individuals

Mina Gaga, Joanna Chorostowska-Wynimko, Ildikó Horváth, Martin C Tammemagi, David Shitrit, Vered H Eisenberg, Hao Liang, David Stav, Dan Levy Faber, Maarten Jansen, Yael Raviv, Vasileios Panagoulas, Piotr Rudzinski, Gabriel Izbicki, Ohad Ronen, Adiv Goldhaber, Rawia Moalem, Nadir Arber, Ilana Haas, Qinghau Zhou

Please cite this article as: Gaga M, Chorostowska-Wynimko J, Horváth Ió, *et al.* Validation of Lung EpiCheck®, a novel methylation-based blood assay, for the detection of lung cancer in European and Chinese high-risk individuals. *Eur Respir J* 2020; in press (<https://doi.org/10.1183/13993003.02682-2020>).

This manuscript has recently been accepted for publication in the *European Respiratory Journal*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJ online.

Validation of Lung EpiCheck®, a novel methylation-based blood assay, for the detection of lung cancer in European and Chinese high-risk individuals

Gaga Mina¹, Chorostowska-Wynimko Joanna², Horváth Ildikó³, Tammemagi Martin C⁴, Shitrit David⁵, Eisenberg Vered H⁶, Liang Hao⁷, Stav David⁸, Levy Faber Dan⁹, Jansen Maarten¹⁰, Raviv Yael¹¹, Panagoulas Vasileios¹², Rudzinski Piotr², Izbicki Gabriel¹³, Ronen Ohad¹⁴, Goldhaber Adiv¹⁵, Moalem Rawia¹⁶, Arber Nadir¹⁷, Haas Ilana¹⁸, Zhou QingHau⁷

¹ 7th Respiratory Medicine Department, Athens Chest Hospital, Athens, Greece

² National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland

³ National Korányi Institute of Pulmonology, Budapest, Hungary

⁴ Brock University, St. Catharines, ON, Canada.

⁵ Pulmonary Department, Meir Medical Center, Kfar Saba, Israel.

⁶ Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

⁷ Lung Cancer Center/Lung Cancer Institute, West China Hospital, Sichuan University, Chengdu, China

⁸ Lung Institute, Maccabi Health Services Hashalom, Tel-Aviv, Israel

⁹ Department of Cardiothoracic surgery, Lady Davis Carmel Medical Center, Haifa, Israel; Ruth and Bruch Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel

¹⁰ Pulmonary Department, Ziekenhuisgroep Twente, Hengelo, The Netherlands

¹¹ Department of Medicine, Pulmonology Institute, Soroka Medical Center, Ben-Gurion University, Beer-Sheva, Israel

¹² 2nd Respiratory Medicine Department, Athens Chest Hospital, Athens, Greece

¹³ Pulmonary Institute, Shaare Zedek Medical Center, Jerusalem, Israel.

¹⁴ Department of Otolaryngology - Head and Neck Surgery, Galilee Medical Center affiliated with Azrieli Faculty of Medicine, Safed, Israel

¹⁵ Family Physician, Clalit Health Services, Raanana, Israel

¹⁶ Gastroenterology Institute, The Holy Family Hospital, Nazareth, Israel

¹⁷ Integrated Cancer Prevention Center, Tel Aviv Sourasky Medical Centre, Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, Israel

¹⁸ Breast unit, Meir Medical Center, Kfar Saba, Israel

Corresponding author:

Mina Gaga MD, PhD

Director 7th Resp. Med. Dept and Asthma Center

152 Mesogion Ave Athens 11527, Greece

Tel + 30 210 7781720, + 30 210 7763667

Fax +30 210 7781911

minagaga@yahoo.com

Acknowledgements

This study was funded by Nucleix LTD. Rehovot, Israel

The results published here are in whole or part based upon data generated by The Cancer Genome Atlas managed by the NCI and NHGRI. Information about TCGA can be found at <http://cancergenome.nih.gov>

This study was partly supported by the grant from the National Key Research and Development Plan of China (No. 2016YEE0103400)

Take home message: Lung EpiCheck, a simple blood test, detected 85% of early stage lung cancers with specificity of 64% in high-risk population, reaching AUC of 0.942 when combined with risk factors. This could improve efficiency of implementing lung cancer screening.

Abstract

Aim: Lung cancer screening reduces mortality. We aim to validate the performance of Lung EpiCheck®, a 6-marker panel methylation-based plasma test, in the detection of lung cancer in European and Chinese samples.

Methods: A case-control European training set (102 lung cancer cases/265 controls) was used to define the panel and algorithm. Two cut-offs were selected, low cut-off (LCO) for high sensitivity and high cut-off (HCO) for high specificity. The performance was validated in case-control European and Chinese validation sets (cases/controls: 179/137 and 30/15).

Results: The European and Chinese validation sets achieved AUCs of 0.882 and 0.899, respectively. The respective sensitivity/specificity with LCO were 87.2%/64.2% and 76.7%/93.3% and with HCO were 74.3%/90.5% and 56.7%/100.0%, respectively. Stage I NSCLC sensitivity in European and Chinese samples with LCO was 78.4% and 70.0% and with HCO was 62.2% and 30.0%, respectively. SCLC was represented only in the European set and sensitivities with LCO and HCO were 100.0% and 93.3%. In multivariable analyses of the European validation set, the assay's ability to predict lung cancer was independent of established risk factors (age, smoking, COPD), and overall AUC was 0.942.

Conclusions: Lung EpiCheck demonstrated strong performance in lung cancer prediction in case-control European and Chinese samples, detecting high proportions of early stage NSCLC and SCLC and significantly improving predictive accuracy when added to established risk factors. Prospective studies are required to confirm these findings. Utilizing such a simple and inexpensive blood test has the potential to improve compliance and broaden access to screening for at-risk populations.

BACKGROUND

Lung cancer is the leading cause of death from cancer with 1.76 million deaths worldwide in 2018¹. Risk factors include age, smoking, family history and occupational/asbestos exposure. Five-year survival rate for lung cancer is only 18.6% mainly due to diagnosis at late stages². Screening with low-dose CT (LDCT) has been proven to reduce lung cancer mortality in high-risk population^{3,4}. However, LDCT has a significant rate of false positives and overdiagnosis, involves radiation hazard, is reader dependent and requires substantial infrastructure. In the USA, up to 14% of the eligible population undergo lung cancer screening (LCS)⁵. Current barriers are infrastructure and knowledge and awareness gaps among referring physicians and the public. Importantly, LCS is targeting a very high-risk population, representing merely a quarter of lung cancer patients⁶.

Several types of tumor-derived biomarkers have been assessed for lung cancer detection, including circulating tumor cells, exosomes, mutations and methylation changes in cell-free DNA (cfDNA), microRNA, and proteins^{7,8}. Genome-wide hypomethylation and hypermethylation changes are found in lung cancer and could potentially serve as markers⁹.

EpiCheck is a simple ultrasensitive PCR-based assay that detects cancer-associated hypermethylation changes in a selected panel of markers from any body fluid or tissue. The urine-based Bladder EpiCheck[®] demonstrated sensitivity of 92% for high-grade urothelial carcinoma with specificity of 88% in bladder cancer patients undergoing surveillance¹⁰.

The purpose of this study is to validate the performance of Lung EpiCheck, a 6 methylation markers blood test, in lung cancer detection.

METHODS

Study samples

Training set samples were used to select the markers for the panel using Nucleix's proprietary bioinformatics techniques. Six markers were selected based on their synergistic information and an algorithm calculating the EpiScore (ES) was developed and locked down (supplemental Figure S1). Two cut-offs were defined to allow for different clinical scenarios, a low cut-off (LCO) of $ES \geq 60$ favoring high sensitivity and a high cut-off (HCO) of $ES \geq 70$ favoring high specificity. The European validation set was a new set of samples used to validate the performance of the assay using the pre-defined algorithm and cut-offs.

The training and the European validation sets were obtained by applying a single protocol: a case-control study performed on samples from sequential recruitment in 18 departments and clinics in 16 healthcare organizations, and 3 biobanks in Europe and Israel (Supplemental Table S2). Samples were collected from July 2016 to March 2018. The initial series of cases and controls were used for training and the subsequent series was used for validation. Cases were recruited from pulmonology, thoracic surgery and oncology departments and clinics in Europe and Israel. Present and past smokers, serving as controls, were recruited from blood collection stations in primary care clinics and from general surgery departments in Israel. Potential participants were randomly approached as they came to perform a blood test for any reason (Table 1). Sample processing was performed at Nucleix laboratory, Rehovot, Israel. Disease staging of the cases was according to AJCC 7 and AJCC 8. Adenocarcinomas were included only if classified as invasive adenocarcinomas according to IASLC/ATS/ERS classification¹¹.

The Chinese validation set was a small feasibility study assessing the applicability of Lung EpiCheck for lung cancer detection in a Chinese population. This was a blinded, case-control, single center, study performed in the Lung Cancer Institute/Center, West China Hospital, Sichuan University, Chengdu. Samples were collected from January 2018 to November 2018. Patients suspected or confirmed to have lung cancer arriving for lung surgery were enrolled. Healthy volunteers were sequentially enrolled as controls (Table 1). Sample processing was performed on site. Disease staging for the cases was according to AJCC 8.

Relevant medical, smoking and family history data were collected prior to study related procedures. The study was approved by the ethics committees of the various institutions involved, and all subjects signed an informed consent. The study registration is NCT02373917.

Lung EpiCheck Testing

Lung EpiCheck (Nucleix LTD, Rehovot, Israel) is a blood test that detects lung cancer-associated hypermethylation in 6 markers in cfDNA. Plasma is separated from a 10 ml EDTA tube within 4 hours of blood draw by two consecutive centrifugations at 1500g for 10 minutes and stored at -20°C to -80°C until DNA extraction. Lung EpiCheck's reagents and methylation-sensitive enzymes are used for DNA extraction, digestion, and amplification in real-time PCR (ABI 7500 Fast Dx, Applied Biosystems). 3 PCR wells are amplified for the markers and 1 for an internal control to verify the quality of plasma separation by detecting leukocyte-derived DNA. Lung EpiCheck software analyzes the PCR output calculating an ES, a numerical score 0-100 reflecting the overall methylation level in the assay's markers.

Statistical analysis

The groups' baseline characteristics were compared using chi-square test for categorical parameters and Wilcoxon Rank-Sum test for continuous parameters. Sensitivity and specificity were calculated for the entire sample and for different subgroups of interest along with 95% exact binomial confidence intervals. The predictive ability of the continuous ES was evaluated via logistic regression and the corresponding area under the receiver operator characteristic curve (AUC) was calculated. Positive likelihood ratio (LR+) and negative likelihood ratio (LR-) were calculated for the entire sample and for different subgroups of interest along with 95% exact binomial confidence intervals [LR+ = Sensitivity / (1-Specificity), LR- = (1-Sensitivity) / Specificity]. A multivariable logistic regression was used to examine the relationship between the true patient status (lung-cancer case or control) and their ES result. The contribution of the ES result was examined adjusting for the patient's personal characteristics and known risk factors for lung cancer. An additional multivariable logistic regression analysis was done to examine whether the ES outcome is affected by a patient's personal characteristics or known risk factors for lung cancer. Both analyses used the subset of patients for whom all relevant information was available. The contribution of each predictor in the model was evaluated via odds ratio (OR) and the overall prediction ability of the model was evaluated via AUC.

RESULTS

The training set included 367 subjects (102 cases and 265 controls). Cases were significantly older with a higher number of pack years compared to controls, while sex and number of years since quitting smoking were similar (Table 2). Smoking status was also significantly different, as half of the cases were

missing this information. A balanced distribution of histological subtypes and stages was achieved with 28% of NSCLC patients having stage I disease (Table 3). The AUC was 0.890 [95%CI 0.848;0.932] (Figure 1a), the sensitivity/specificity combinations were 84.3%[75.8%;90.8%]/77.7%[72.2%;82.6%] with LCO and 73.5%[63.9%;81.8%]/93.6%[89.9%;96.2%] with HCO (Table 3). Likelihood ratios are reported in supplemental table S5.

The European validation set included 363 subjects, out of which 316 subjects were eligible and had ES results (179 cases and 137 controls, supplemental Figure S3a). Cases were heavier smokers (median pack years 41 vs. 20), slightly older (median age 65 vs. 63), and were more likely to be male compared to controls (74% vs. 63%), all statistically significant (Table 2). Despite similar rates of current smokers (42%), smoking status was significantly different, as 1/3 of the cases were missing this information. Years since quitting smoking were similar between the groups (18y vs 17y). Adenocarcinoma was the most common histological subtype (46%), followed by squamous cell carcinoma (41%) (Table 3). SCLC was underrepresented compared to the incidence reported in the literature (8% vs. 13%¹²). A balanced distribution of NSCLC stages was achieved and 26% of NSCLC patients had stage I disease (Table 3). The AUC was 0.882[0.846;0.918] (Figure 1b) overall and 0.797[0.704;0.889], 0.830[0.764;0.895] and 0.862[0.813;0.910] in Stage I, Stages I&II, and early stages (stages I, II & IIIA), respectively (supplemental Figure S4). Overall sensitivity/specificity combinations were 87.2%[81.3%;91.7%]/64.2%[55.6%;72.2%] with LCO and 74.3%[67.2%;80.5%]/90.5%[84.3%;94.9%] with HCO. Applying the LCO/HCO cut-offs, Lung EpiCheck detected 85.1%[76.7%;91.4%]/70.3%[60.4%;79.0%] of early stage NSCLC, 78.4%[61.8%;90.2%]/62.2%[44.8%;77.5%] of stage I NSCLC and 57.1%[18.4%;90.1%]/42.9%[9.9%;81.6%] of stage I NSCLC ≤ 20 mm. Lung EpiCheck demonstrated high sensitivity of 100.0%[54.1%;100.0%]/100.0%[54.1%;100.0%] in limited and 100.0%[66.4%;100.0%]/88.9%[51.8%;99.7%] in extensive SCLC with LCO/HCO, however the numbers of SCLC were small. Sensitivities were similar ($p > 0.05$) across histological subtypes, NSCLC stages, NSCLC early/late stage groups and limited/extensive SCLC for each cutoff. The only factor significantly impacting sensitivity in univariate analyses were tumor size ($p < 0.001$ / $p < 0.0001$ in LCO/HCO) and tumor size of stage I ($p = 0.003$ / $p = 0.020$).

In a multivariable analysis of patients with smoking information ($n = 242$), established risk factors for lung cancer (age, smoking status, pack years and quit years) and sex did not influence having a positive Lung EpiCheck result in either cut-off (Figure 2). Presence of COPD significantly decreased the chance of having a positive Lung EpiCheck result at LCO. A trend of higher ESs in patients without COPD vs. patients with the condition was maintained when looking at various statistical measures of ES (mean, median, 1st and 3rd quartile) of cases and in controls separately, but when combining the two groups, this trend was reversed (data not shown). The only factor driving a positive result was the group (case/control) with odds ratio of 18.2 [7.2;45.7], $p < 0.0001$ with LCO and 23.7 [10.1;55.5], $p < 0.0001$ with HCO. Likelihood ratios are reported in supplemental table S5.

A multivariable analysis was performed to assess the accuracy of lung cancer prediction based on risk factors alone, or in combination with ES (Figure 3). In our data, age, sex, smoking status, quit years, pack years and COPD together yielded an AUC of 0.852 [0.805;0.900] Adding ES significantly increased the AUC to 0.942 [0.913;0.971], $p < 0.0001$. This analysis was performed on a subset of 242 patients with full smoking history, and the Lung EpiCheck AUC was similar to that of the entire cohort (0.881[0.843;0.918] vs. 0.882[0.846-0.918]), which suggests that this subsample is representative of the entire set, however these results should be interpreted with caution.

Ten (3.1%) of the tests in the validation sets failed to yield a result, 2 due to insufficient amount of DNA in the sample, and 8 due to failed plasma separation.

The Chinese validation set enrolled 92 sequential cases and 15 controls. Forty-one cases were eligible, out of which 30 were selected to ensure good representation of all stages (10 stage I, 6 stage II, 11 stage III and 3 stage IV) (supplemental Figure S3b). As expected, age and F/M ratio were not comparable between cases and controls, as the latter were healthy volunteers (Table 2). The AUC was 0.899[0.809;0.989] (Figure 1c), the sensitivity/specificity combinations were 76.7%[59.1%;88.2%]/93.3%[68.0%;99.9%] with LCO and 56.7%[39.2%;72.6%]/100.0%[78.1%;100.0%] with HCO (Table 3). Lung EpiCheck with LCO/HCO detected 70.8%[48.9%;87.4%]/50%[29.1%;70.9%] of early stage cancers, 70%[34.8%;93.3%]/30%[6.7%;65.2%] of stage I cancers and 60%[14.7%;94.7%]/20%[0.5%;71.6%] of stage I NSCLC ≤ 20 mm. Likelihood ratios are reported in supplemental table S5.

No tests failed in the Chinese set.

DISCUSSION

Our data demonstrates that Lung EpiCheck achieved performance characteristics suggesting that, if prospectively validated, may be suitable for clinical use in early detection of lung cancer. The predictive performance of Lung EpiCheck in the European validation data was very high with AUC of 0.882. While maximizing sensitivity (87.2%) with the LCO, the specificity remained good (64.2%), and while maximizing specificity (90.5%) using the HCO, the sensitivity remained high (74.3%). In the Chinese set the AUC of 0.899 yielded high specificity in both-cut-offs (LCO 93.3% and HCO 100.0%), with good sensitivity with LCO (76.7%). Differences between the validation sets are probably due to including young non-smoking controls and surgical patients with small resectable tumors in the Chinese cohort. Detection at early stages is the key performance factor for Lung EpiCheck to ensure patients are detected in time for curative treatment. In the European set, the AUC's were consistently high in stage I NSCLC (0.797), stages I&II NSCLC (0.830) and early stage (stages I,II & IIIA) NSCLC (0.862). In the validation sets, Lung EpiCheck detected 70%-80% of stage I NSCLC with LCO, detecting tumors as small as 8mm (adenocarcinoma in the Chinese set). With HCO, European results remained strong with stage I NSCLC sensitivity of 62.2%, but Chinese performance deteriorated to 30.0%. Early stages performance should be interpreted with caution, as the controls were not scanned or followed-up to ensure no asymptomatic cancer existed. These results compare favorably with published results of other blood-tests for lung cancer detection, reporting stage I sensitivity around 40% (22%-71%), many of which are from training sets^{13,14,15,16,17}. Sensitivity of NSCLC tumors was significantly impacted by size, but even in stage I NSCLC ≤ 20 mm, there is a good signal of effectiveness detecting $\geq 50\%$ of tumors with LCO (4/7 and 2/4 in the European and Chinese validations sets, respectively) with LCO. With HCO this stage I NSCLC ≤ 20 mm sensitivity was similar in the European set (3/7), but all were missed in the Chinese cohort. Likelihood ratios can be used to simply and quickly estimate the post-test probability, however, there is no established gold-standard threshold for determining an acceptable likelihood ratio in the developing field of biomarkers for lung cancer screening. Regardless, we believe that the likelihood ratios achieved by Lung EpiCheck appear to be in a good range.

Both CMS¹⁸ and USPSTF¹⁹ recommend LCS with LDCT for high-risk populations, but national screening rates are very low, up to 14%⁵. Obstacles to LCS uptake are likely due to patient and primary care provider concerns about costs, inconvenience and possible risks associated with radiation and false-positive results. Additional limiting factors are absence of efficient proven programs or lack of program infrastructure. Offering a simple blood test to non-compliant eligible patients, as a tool to motivate them to get LDCT, could help overcome some of these barriers. Ease and safety of a blood test could encourage patients to be tested, and a positive blood test result could potentially convince patients to participate in LDCT screening programs. If performance is confirmed in a prospective clinical study, prioritizing eligible patients for LDCT based on such a test could alleviate systems restraints by reducing the number of unnecessary procedures and providing effective patient selection. Reducing the number of LDCTs could also indirectly impact on the number of false positive findings, and their adverse outcomes and costs.

Alternatively, such a test can be used to better identify high-risk people who should undergo LDCT. Currently, high-risk populations are defined by demographic and exposure factors (age and smoking history) with very limited discrimination of 0.6-0.7²⁰. Subsequently, in the USA, merely 2.51 cancers are detected per 1,000 LDCT scans²¹ and more than 50% of lung cancer patients will not be considered eligible⁷. More elaborate risk models, such as PLCOm2012, report better performance (AUC=0.7-0.8) as they include other surrogate markers such as COPD and history of cancer, but they are more cumbersome and harder to implement in the clinical routine. In our analysis, relationship of cases and positive Lung EpiCheck did not vary substantially by value of other risk factors (age, pack years, quit years, sex, smoking status and COPD). Presence of these risk factors did not change or impact the strong relationship between lung cancer and the test result. Moreover, combining Lung EpiCheck with risk factors achieved a very high discrimination of 94.2%, allowing for optimal selection of high-risk populations for LCS. Further validation is required to confirm Lung EpiCheck predictive performance and to define the best way to combine Lung EpiCheck and risk factors.

Similar to published evidence, showing correlation of cfDNA levels in the blood with tumor burden from NSCLC²² and other solid tumors^{23,24,25,26}, Lung EpiCheck sensitivity correlated with tumor size. Two studies found cfDNA signal to inversely correlate with survival in patients with newly diagnosed lung cancer^{27,28}, suggesting that it is also a prognostic marker for aggressiveness of the tumor. Further investigations are needed to inform whether there is a lower size limit of detectability by cfDNA, and if lack of cfDNA signal is an independent prognostic factor, or potentially a sign of overdiagnosis. Either way, a liquid biopsy for early detection must be very sensitive, in order to pick up the signal in the blood of early curable cancers. Lung EpiCheck's good preliminary results in early cancer classification can be explained by an analytical sensitivity of 1:200,000²⁹ which is 20 to 200-fold higher than other liquid biopsy products available^{30,31,32}.

Cost-effectiveness is an important consideration in the applicability of screening tests. In a recently published model, in order to maintain the cost-effectiveness threshold of \$50,000 per life-year gained, a marker added to risk factors to improve selection of patients for LCS could cost up to \$300³³. Therefore, the NGS-based liquid biopsy tests common in advanced settings, are irrelevant for this field, as their running costs alone are currently much higher at \$1,000-\$2,000 per test. Similarly, other available lung cancer biomarker tests have sensitivity and specificity below screening requirements, lowering further the price level they can charge to be cost-effective. In contrast, Lung EpiCheck, with its high preliminary performance and its simple PCR-based technology, appear to be potentially well situated to be cost effective and commercially viable.

Mutations are established key factors of cancer development (e.g., driver mutations, acquired therapy resistance) as well as important targets for treatment, and could be potential markers for lung cancer detection. However, mutations in genes such as p53³⁴ drive clonal hematopoiesis³⁵ in up to 21% of healthy elderly people. This can pose as a serious confounder and can generate false positive results when using these mutations in blood tests for early detection of cancer. Alternatively, and unhampered by such problems, methylation changes have recently emerged as promising markers for cancer detection^{16,36}.

LIMITATIONS

Case-control studies are prone to selection bias, as cases and controls do not necessarily come from the same population and are not truly comparable. This is reflected in our study by the significant difference between the groups in parameters such as age, sex, smoking status and pack years. Cases were patients diagnosed with lung cancer due to any reason (symptoms, incidental finding, screening), and do not reflect a screening population. Controls did not receive LDCT screening, nor were they followed-up after blood draw, therefore, it is unknown whether lung cancer cases were among them and were missed. Therefore, a prospective study in high-risk individuals undergoing LDCT with follow-up for lung cancer incidence is essential to confirm the current study findings. Staging was performed locally according to local standard of care in each site, so in the European validation there was a mix between AJCC 7 and AJCC 8. This probably translates to a potential overlap between large stage I to small stage II cancers. Collection of some data were limited in biobank samples, leading to missing smoking histories in 19% of the European validation cohort, limiting the multivariable analyses to a subpopulation of that set. Personal history of cancer is a known risk factor for lung cancer³⁷, however, to ensure that the signal emerges from lung cancer, such patients were excluded. The Chinese set was a small single center study that included surgical patients only, therefore not representative of the Chinese lung cancer population, additionally, the controls were healthy young and mostly non-smokers, not representative of patients at risk. A larger prospective study in China is warranted to confirm the performance of Lung EpiCheck in this population.

Our current findings need to be validated in prospective trials.

CONCLUSIONS

Lung EpiCheck demonstrated strong suggestive performance in lung cancer prediction in case-control European and Chinese samples, detecting up to 78% of stage I tumors, up to 100% of SCLC and significantly improving predictive accuracy when added to established risk factors. Prospective studies are required to confirm these findings. Utilizing such a simple and inexpensive blood test to select people for LCS has the potential to improve compliance and broaden access to screening for high-risk populations.

TABLES AND FIGURES

Table 1. Eligibility criteria

	Inclusion criteria	Exclusion criteria
Cases	<ul style="list-style-type: none"> Subjects with pathologically proven primary lung cancer (NSCLC and SCLC) <i>or</i> Subjects suspected for primary lung cancer undergoing a diagnostic procedure. Patients enrolled with suspicion for lung cancer were included in the analysis if diagnosis was pathologically confirmed primary lung cancer (SCLC and NSCLC); Treatment naïve - no chemotherapy, radiotherapy, immunotherapy, surgery or any type of ablation, etc. 	1) Subjects with cancer, other than lung cancer; (2) Subjects with history of cancer from any kind (except for fully-resected non-melanoma skin cancer)
Controls	European sets: <ul style="list-style-type: none"> Age ≥50 years Current or former smoker Chinese set: <ul style="list-style-type: none"> Healthy individuals willing to donate blood for the study 	Subjects with current diagnosis or history of cancer from any kind (except for fully-resected non-melanoma skin cancer)

Table 2. Patient demographics

	Training set		European validation set		Chinese validation set	
	Cases	Controls	Cases	Control	Cases	Control
Number	102	265	179	137	30	15
Age	p<0.0001		p=0.0130		p=<0.0001	
Median (Range)	67 (51-83)	62 (49-82)	65 (23-89)	63 (50-87)	64 (40-79)	33 (23-51)
Sex # (%)	p=0.182		p=0.037		p=0.053	
Male	70 (68.6)	162 (61.1)	132 (73.7)	86 (62.8)	21 (70.0)	6 (40.0)
Female	32 (31.4)	103 (38.9)	47 (26.3)	51 (37.2)	9 (30.0)	9 (60.0)
Smoking status	p<0.0001 ^a		p<0.0001 ^a		p<0.001 ^a	
Current smoker #(%)[‡]	36 (35.3)	117 (44.2)	75 (41.9)	57 (41.6)	11 (36.7)	0 (0.0)
Former smoker #(%)	7 (6.9)	148 (55.8)	35 (19.6)	80 (58.4)	4 (13.3)	1 (6.7)
Never smoker #(%)	6 (5.9)	-	9 (5.0)	-	14 (46.7)	14 (93.3)
Unknown	53 (52.0)	-	60 (33.5)	-	1 (3.3)	0 (0.0)
Pack years	p=0.010		p=<0.0001		°	
Median (range)*	40 (2-129)	20 (1-138)	41 (4-182)	20 (1-120)	30 (15-80)	10 (NA)
Year since quitting	p=0.267		p=0.394		°	
Median (range)^	13 (2-41)	17 (1-50)	18 (1-42)	17 (1-58)	7 (3-10)	10 (NA)

^a this analysis included only patients in the categories of current and former smoker;

[‡] smokers reporting quitting within one year prior to study inclusion were considered current smokers;

*pack years are reported in smokers only. This information was missing for 5 cases in the training set;

[^] former smokers only. This information was missing for 5 controls in the training set, and in 5 patients (4 cases, 1 controls) in the European validation set;

[°] p value was not calculated as there was only 1 control with history of smoking in this set

Table 3. Lung EpiCheck performance by sensitivity and specificity

	Training set				European validation set				Chinese validation set			
Number of cases/ controls	102/265				179/137				30/15			
AUC [95% CI]	0.890 [0.848;0.932]				0.882 [0.846;0.918]				0.899 [0.809;0.989]			
	Low cut-off EpiScore=60		High cut-off EpiScore=70		Low cut-off EpiScore=60		High cut-off EpiScore=70		Low cut-off EpiScore=60		High cut-off EpiScore=70	
	n/N	% [95% CI]	n/N	% [95% CI]	n/N	% [95% CI]	n/N	% [95% CI]	n/N	% [95% CI]	n/N	% [95% CI]
Overall sensitivity	86/102	84.3% [75.8%;90.8]	75/102	73.5% [63.9%;81.8%]	156/179	87.2% [81.3%;91.7%]	133/179	74.3% [67.2%;80.5%]	23/30	76.7% [59.1%;88.2%]	17/30	56.7% [39.2%;72.6%]
Overall specificity	206/265	77.7% [72.2%;82.6%]	248/265	93.6% [89.9%;96.2%]	88/137	64.2% [55.6%;72.2%]	124/137	90.5% [84.3%;94.9%]	14/15	93.3% [68.0%;99.9%]	15/15	100.0% [78.1%;100.0%]
Sensitivity by histological subtype		Overall* P=0.075 NSCLC vs. SCLC p=0.213		Overall* P=0.051 NSCLC vs. SCLC p=0.278		Overall* P=0.250 NSCLC vs. SCLC p=0.224		Overall* P=0.311 NSCLC vs. SCLC p=0.120		P=0.029		P<0.001
Adenocarcinoma	34/45	75.6% [60.5%;87.1%]	27/45	60.0% [44.3%;74.3%]	73/82	89.0% [80.2%;94.9%]	59/82	72.0% [60.9%;81.3%]	12/19	63.2% [38.4%;83.7%]	6/19	31.6% [12.6%;56.6%]
Squamous Cell Carcinoma [^]	35/38	92.1% [78.6%;98.3%]	32/38	84.2% [68.7%;94.0]	61/74	82.4% [71.8%;90.3%]	53/74	71.6% [59.9%;81.5%]	11/11	100.0% [71.5%;100.0%]	11/11	100.0% [71.5%;100.0%]
Other NSCLC	2/3	66.7% [9.4%;99.2%]	2/3	66.7% [9.4%;99.2%]	5/6	83.3% [35.9%;99.6%]	5/6	83.3% [35.9%;99.6%]	-	-	-	-
All NSCLC	71/86	82.6% [72.9%;89.9%]	61/86	70.9% [60.1%;80.2%]	139/162	85.8% [79.5%;90.8%]	117/162	72.2% [64.7%;79.0%]	23/30	76.7% [57.7%;90.1%]	17/30	56.7% [37.4%;74.5%]
Small cell carcinoma	10/10	100.0% [69.2%;100.0%]	9/10	90.0% [55.5%;99.7%]	15/15	100.0% [78.2%;100.0%]	14/15	93.3% [68.1%;99.8%]	-	-	-	-
Other/ Unknown	5/6	83.3% [35.9%;99.6%]	5/6	83.3% [35.9%;99.6%]	2/2	100.0% [15.8%;100.0%]	2/2	100.0% [15.8%;100.0%]	-	-	-	-
Sensitivity by NSCLC stage		P=0.075		P=0.012		P=0.089		P=0.187		P=0.193		P=0.018
Stage I	18/26	69.2% [48.2%;85.7%]	13/26	50.0% [29.9%;70.1]	29/37	78.4% [61.8%;90.2%]	23/37	62.2% [44.8%;77.5%]	7/10	70.0% [34.8%;93.3%]	3/10	30.0% [6.7%;65.2%]
Stage II	17/21	81.0% [58.1%;94.6]	14/21	66.7% [43.0%;85.4%]	24/28	85.7% [67.3%;96.0%]	20/28	71.4% [51.3%;86.8%]	3/6	50.0% [11.8%;88.2%]	2/6	33.3% [4.3%;77.7%]
Stage III	17/20	85.0% [62.1%;96.8%]	16/20	80.0% [56.3%;94.3%]	53/59	89.8% [79.2%;96.2%]	44/59	74.6% [61.6%;85.0%]	10/11	90.9% [58.7%;99.8%]	10/11	90.9% [58.7%;99.8%]
Stage IV	17/17	100.0% [80.5%;100.0%]	16/17	94.1% [71.3%;99.9%]	33/37	89.2% [74.6%;97.0%]	30/37	81.1% [64.8%;92.0%]	3/3	100.0% [29.2%;100.0%]	2/3	66.7% [9.4%;99.2%]
Unstaged	2/2	100.0% [15.8%;100.0%]	2/2	100.0% [15.8%;100.0%]	0/1	0.0% [0.0%;97.5%]	0/1	0.0% [0.0%;97.5%]	-	-	-	-
Sensitivity by tumor size (largest diameter), NSCLC only						P<0.001 [†]		P<0.0001 [†]		P=0.584		P=0.043
≤20mm	-	-	-	-	11/16	68.8% [41.3%;89.0%]	7/16	43.8% [19.8%;70.1%]	3/5	60.0% [14.7%;94.7%]	1/5	20.0% [0.5%;71.6%]
21-30mm	-	-	-	-	14/22	63.6% [40.7%;82.8%]	10/22	45.5% [24.4%;67.8%]	4/6	66.7% [22.3%;95.7%]	2/6	33.3% [4.3%;77.7%]
31-50mm	-	-	-	-	46/49	93.9% [83.1%;98.7%]	38/49	77.6% [63.4%;88.2%]	11/14	78.6% [49.2%;95.3%]	9/14	64.3% [35.1%;87.2%]
>50mm	-	-	-	-	47/50	94.0% [83.5%;98.7%]	44/50	88.0% [75.7%;95.5%]	5/5	100.0% [47.8%;100.0%]	5/5	100.0% [47.8%;100.0%]
Unknown [‡]	-	-	-	-	23/27	85.2% [66.3%;95.8%]	20/27	74.1% [53.7%;88.9%]	-	-	-	-
Sensitivity by tumor size (largest diameter), stage I NSCLC only						P=0.003		P=0.020		P=0.700		P=0.200
≤20mm	-	-	-	-	4/7	57.1% [18.4%;90.1%]	3/7	42.9% [9.9%;81.6%]	2/4	50.0% [6.8%;93.2%]	0/4	0.0% [0.0%;60.2%]
21-30mm	-	-	-	-	6/11	54.5% [23.4%;83.3%]	4/11	36.4% [10.9%;69.2%]	3/3	100.0% [29.2%;100.0%]	1/3	33.3% [0.8%;90.6%]
>30mm	-	-	-	-	19/19	100.0% [82.4%;100.0%]	16/19	84.2% [60.4%;96.6%]	2/3	66.7% [9.4%;99.2%]	2/3	66.7% [9.4%;99.2%]
Sensitivity by stage group, NSCLC only		P=0.058		P=0.008		P=0.641		P=0.465		P=0.290		P=0.196
Early stages (I,II & IIIA)	45/59	76.3% [63.4%;86.4%]	36/59	61.0% [47.4%;73.5%]	86/101	85.1% [76.7%;91.4%]	71/101	70.3% [60.4%;79.0%]	17/24	70.8% [48.9%;87.4%]	12/24	50.0% [29.1%;70.9%]
Advanced stages (IIIB & IV) [‡]	24/25	96.0% [79.6%;99.9%]	23/25	92.0% [74.0%;99.0%]	53/60	88.3% [77.4%;95.2%]	46/60	76.7% [64.0%;86.6%]	6/6	100.0% [54.0%;100.0%]	5/6	83.3% [35.9%;99.6%]
Sensitivity by SCLC stage		P=1.000		P=0.300		P=1.000		P=1.000				
Limited	3/3	100.0% [29.2%;100.0%]	2/3	66.7% [9.4%;99.2%]	6/6	100.0% [54.1%;100.0%]	6/6	100.0% [54.1%;100.0%]	-	-	-	-
Extensive	7/7	100.0% [59.0%;100.0%]	7/7	100.0% [59.0%;100.0%]	9/9	100.0% [66.4%;100.0%]	8/9	88.9% [51.8%;99.7%]	-	-	-	-

In the cases of complete or quasi-complete separation exact p-values were calculated;

* p value calculation for the comparison of histological subtypes did not include 'unknown' group;

[^] patients with adenosquamous carcinoma histology were grouped with the squamous cell carcinoma histology subtype;

[‡] tumor sizes were missing or unmeasurable for 3 stage II, 14 stage III and 10 stage IV;

[†] p-value calculation for the comparison by tumor size did not include 'Unknown' group;

! stage III tumors without stage IIIA/IIIB classification were included in the advanced stages group

Figure 1. ROC curves

- 1a. ROC curve training set
- 1b. ROC curve European validation set
- 1.c ROC curve Chinese validation set

Figure 2. Multivariable logistic regression of factors potentially impacting Lung EpiCheck positive result, by cut-off. This analysis included only patients with history of smoking and full smoking data, n=242 (106 cases, 136 controls). Risk factors included: Age, Pack years and Quit years as continuous measures. Sex (Female vs. Male), Smoking status (former vs. current Smoker), COPD (yes vs. no), Group (cases vs. controls). For current smokers, quit years were counted as zero.

Figure 3. Multivariable logistic regression analysis of predictive factors for lung cancer. This analysis included only patients with history of smoking and full smoking data, n=242 (106 cases, 136 controls). Risk factors: Age, Pack Years, Quit Years (continuous), Sex (male/female), Smoking Status (current/past), COPD (yes/no). For current smokers, quit years were counted as zero.

SUPPLEMENTARY MATERIALS

Supplemental Figure S1. Marker panel and algorithm development

Supplemental Table S2. List of sites, European sets

1	Athens Chest Hospital Sotiria, Athens, Greece
2	BioIVT (Seralab) LLC.
3	Carmel Medical Center, Haifa, Israel
4	Emek Medical Center, Afula, Israel
5	Holy Family Hospital, Nazareth, Israel
6	Indivumed GmbH
7	Maccabi Petach Tikva Clinic, Maccabi Health services, Petach Tikva, Israel
8	Maccabi Ramat Hasharon Clinic, Maccabi Health services, Ramat Hasharon, Israel
9	Meir Medical Center, Kfar Saba, Israel
10	National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland
11	National Korányi Institute of Pulmonology, Budapest, Hungary
12	Shaare Zedek Medical Center, Jerusalem, Israel
13	Shamir Medical Center (Assaf Harofeh), Zrifin, Israel
14	Shuali Ra'anana Clinic, Clalit Health Services, Ra'anana, Israel
15	Soroka Medical Center, Beer Sheva, Israel
16	Tel-Aviv Sourasky Medical Center (Ichilov), Tel-Aviv, Israel
17	Tissue solutions Ltd.
18	Wolfson Medical Center, Holon, Israel
19	Ziekenhuisgroep Twente (ZGT), Hengelo, the Netherlands
20	Ziv Medical Center, Safed, Israel

Supplemental Figure S3. CONSORT diagrams

S3a. CONSORT diagram European validation set

S3.b. CONSORT diagram Chinese validation set

Supplemental Figure S4. ROC curves by extent of disease

Supplemental Table S5. Lung EpiCheck performance by likelihood ratio

Supplemental Table S5. Lung EpiCheck performance by likelihood ratio

	Training Set		European Validation Set		Chinese Validation Set	
	Low cut-off EpiScore=60	High cut-off EpiScore=70	Low cut-off EpiScore=60	High cut-off EpiScore=70	Low cut-off EpiScore=60	High cut-off EpiScore=70
Positive Likelihood Ratio [95% CI]	3.8 [3.0;4.8]	11.5 [7.1;18.4]	2.4 [1.9;3.1]	7.8 [4.6;13.2]	11.5 [1.7;77.2]	18.1 [1.2;281.4]
Negative Likelihood Ratio [95% CI]	0.2 [0.1;0.3]	0.3 [0.2;0.4]	0.2 [0.1;0.3]	0.3 [0.2;0.4]	0.2 [0.1;0.5]	0.4 [0.3;0.7]
Positive Likelihood Ratio by histological subtype [95% CI]						
Adenocarcinoma	3.4 [2.6;4.5]	9.4 [5.6;15.7]	2.5 [2.0;3.2]	7.6 [4.4;12.9]	9.5 [1.4;64.9]	10.4 [0.6;171.1]
Squamous Cell Carcinoma	4.1 [3.2;5.3]	13.1 [8.1;21.2]	2.3 [1.8;3.0]	7.5 [4.4;12.9]	15.0 [2.3;99.6]	30.7 [2.0;470.6]*
Other NSCLC	3.0 [1.3;6.9]	10.4 [4.1;26.2]	2.3 [1.5;3.6]	8.8 [4.7;16.5]		
All NSCLC	3.7 [2.9;4.7]	11.1 [6.8;17.9]	2.4 [1.9;3.0]	7.6 [4.5;12.9]	11.5 [1.7;77.2]	18.1 [1.2;281.4]
Small cell carcinoma	4.5 [3.6;5.6]	14.0 [8.5;23.2]	2.8 [2.2;3.5]	9.8 [5.8;16.8]		
Other/ Unknown	3.7 [2.5;5.7]	13.0 [7.3;23.3]	2.8 [2.2;3.5]	10.5 [6.3;17.7]		
Positive Likelihood Ratio by NSCLC stage						
I	3.1 [2.2;4.4]	7.8 [4.3;14.2]	2.2 [1.7;2.9]	6.6 [3.7;11.6]	10.5 [1.5;72.8]	10.2 [0.6;178.2]
II	3.6 [2.7;4.9]	10.4 [6.0;18.0]	2.4 [1.8;3.1]	7.5 [4.3;13.3]	7.5 [1.0;58.6]	11.4 [0.6;208.5]
III	3.8 [2.9;5.1]	12.5 [7.5;20.8]	2.5 [2.0;3.2]	7.9 [4.6;13.5]	13.6 [2.0;91.4]	28.0 [1.8;432.2]
IV	4.5 [3.6;5.6]	14.7 [9.1;23.6]	2.5 [1.9;3.2]	8.5 [5.0;14.7]	15.0 [2.3;99.6]	20.0 [1.2;339.5]
Unstaged	4.5 [3.6;5.6]	15.6 [9.8;24.7]	0.7 [0.1;7.8]	2.6 [0.2;30.7]		
Positive Likelihood Ratio by tumor size (largest diameter), NSCLC only						
≤20	1.8 [0.8;4.0]	4.7 [1.6;13.4]	1.9 [1.3;2.9]	4.6 [2.2;9.8]	9.0 [1.2;68.1]	8.0 [0.4;170.6]
21-30	3.1 [2.0;4.8]	7.2 [3.4;15.2]	1.8 [1.2;2.6]	4.8 [2.4;9.6]	10.0 [1.4;72.2]	11.4 [0.6;208.5]
31-50	3.9 [3.0;5.2]	12.3 [7.5;20.4]	2.6 [2.1;3.3]	8.2 [4.8;14.0]	11.8 [1.7;79.8]	20.3 [1.3;318.7]
>50	4.5 [3.6;5.6]	15.6 [9.8;24.7]	2.6 [2.1;3.3]	9.3 [5.5;15.7]	15.0 [2.3;99.6]	29.3 [1.9;453.8]*
Unknown	4.1 [3.2;5.2]	12.1 [7.3;19.8]	2.4 [1.8;3.1]	7.8 [4.4;13.7]		
Positive Likelihood Ratio by tumor size (largest diameter), stage I NSCLC only						
≤20	0.6 [0.1;4.0]	1.0 [0.1;14.8]	1.6 [0.8;3.2]	4.5 [1.7;12.3]	7.5 [0.9;63.2]	3.2 [0.1;141.5]**
21-30	3.5 [2.3;5.3]	6.9 [2.9;16.4]	1.5 [0.9;2.7]	3.8 [1.5;9.8]	15.0 [2.3;99.6]	12.0 [0.6;242.8]
>30	4.5 [3.6;5.6]	13.9 [8.3;23.2]	2.8 [2.2;3.5]	8.9 [5.1;15.4]	10.0 [1.3;78.1]	20.0 [1.2;339.5]
Positive Likelihood Ratio by stage group, NSCLC only						
Early stages (I,II & IIIA)	3.4 [2.6;4.5]	9.5 [5.8;15.7]	2.4 [1.9;3.0]	7.4 [4.3;12.6]	10.6 [1.6;71.8]	16.0 [1.0;251.8]
Advanced stages (IIIB, IIIC, IV, IVA)	4.3 [3.4;5.5]	14.3 [8.9;23.0]	2.5 [1.9;3.1]	8.1 [4.7;13.8]	15.0 [2.3;99.6]	25.1 [1.6;395.5]
Positive Likelihood Ratio by SCLC stage						
Extensive	4.5 [3.6;5.6]	15.6 [9.8;24.7]	2.8 [2.2;3.5]	9.5 [5.4;16.6]		
Limited	4.5 [3.6;5.6]	10.4 [4.1;26.2]	2.8 [2.2;3.5]	10.5 [6.3;17.7]		

For all tumor characteristics, only Positive Likelihood Ratio [95% CI] is presented. The denominator for these PLRs is the overall (1-specificity) of each set.

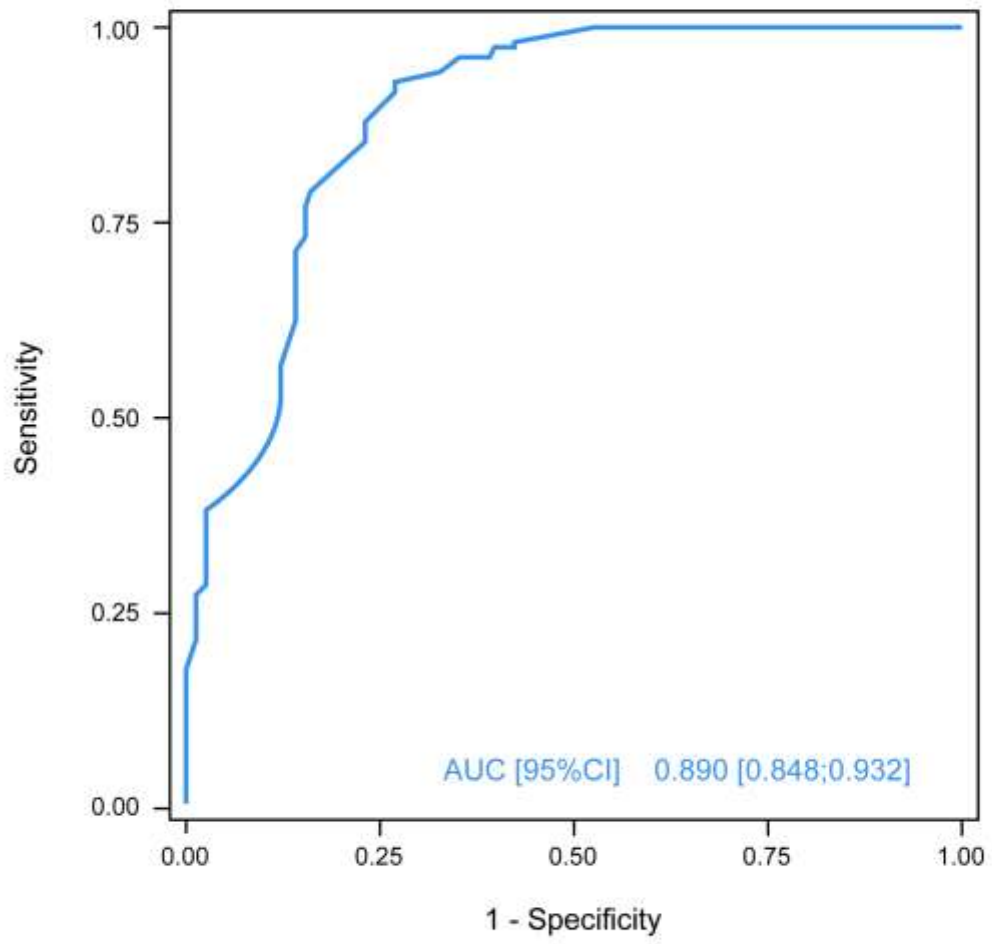
*Positive Likelihood Ratio was adjusted as sensitivity was 100%

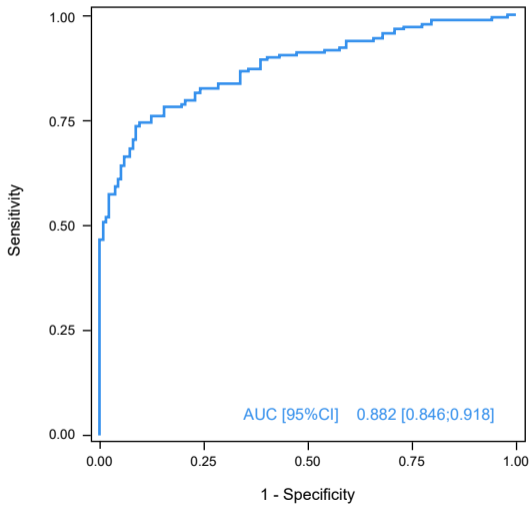
**Positive Likelihood Ratio was adjusted as sensitivity was 0%

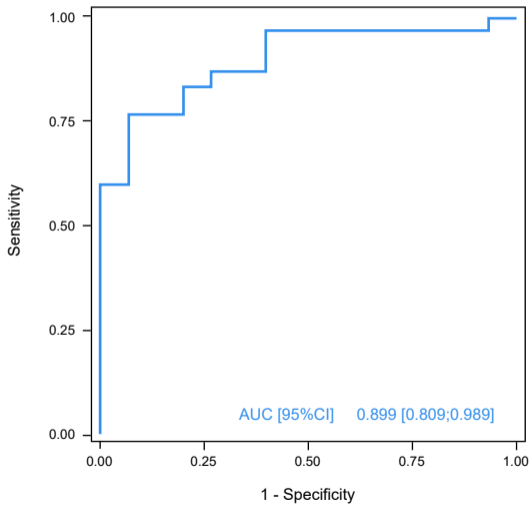
REFERENCES

- ¹ GLOBOCAN2018 Global Cancer Observatory <http://gco.iarc.fr>
- ² Lung cancer Stat Facts: Lung and Bronchus Cancer. <https://seer.cancer.gov/statfacts/html/lungb.html> entered December 2018.
- ³ National Lung Screening Trial Research Team, et al. Reduced Lung-Cancer Mortality with Low-Dose Computed Tomographic Screening NEJM 2011;365:395-409. doi: 10.1056/NEJMoa1102873
- ⁴ de Koning HJ, van der Aalst CM, de Jong PA, et al. Reduced Lung-Cancer Mortality with Volume CT Screening in a Randomized Trial. N Engl J Med. 2020;382(6):503-513. doi:10.1056/NEJMoa1911793
- ⁵ Zahnd WE, Eberth JM. Lung cancer screening utilization: A behavioral risk factor surveillance system analysis. *Am J Prev Med.* 2019;57:250-255. doi: 10.1016/j.amepre.2019.03.015
- ⁶ Pinsky PF, Berg CD. Applying the National Lung Screening Trial eligibility criteria to the US population: what percent of the population and of incident lung cancers would be covered?. *J Med Screen.* 2012;19(3):154-156. doi:10.1258/jms.2012.012010
- ⁷ Rijavec E, Coco S, Genova C, Rossi G, Longo L, Grossi F. Liquid Biopsy in Non-Small Cell Lung Cancer: Highlights and Challenges. *Cancers (Basel).* 2019;12(1):17. Published 2019 Dec 19. doi:10.3390/cancers12010017
- ⁸ Hanash SM, Ostrin EJ, Fahrman JF. Blood based biomarkers beyond genomics for lung cancer screening. *Transl Lung Cancer Res.* 2018;7(3):327-335. doi:10.21037/tlcr.2018.05.13
- ⁹ Li L, Fu K, Zhou W, et al. Applying circulating tumor DNA methylation in the diagnosis of lung cancer. *Precision Clinical Medicine* 2019;2(1):45–56. <https://doi.org/10.1093/pcmedi/pbz003>
- ¹⁰ Witjes JA, Morote J, Cornel EB, et al. Performance of the Bladder EpiCheck™ Methylation Test for Patients Under Surveillance for Non-muscle-invasive Bladder Cancer: Results of a Multicenter, Prospective, Blinded Clinical Trial. *Eur Urol Oncol.* 2018;1(4):307-313. doi:10.1016/j.euo.2018.06.011
- ¹¹ Travis WD, Brambilla E, Noguchi M et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma. *J Thorac Oncol* 2011;6(2):244-85. doi: 10.1097/JTO.0b013e318206a221.
- ¹² American Cancer Society Cancer Facts & Figures 2019 <https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2019/cancer-facts-and-figures-2019.pdf>
- ¹³ Liu MC, Jamshidi A, Venn O et al. Genome-wide Cell-free DNA (cfDNA) Methylation Signatures and Effect on Tissue of Origin (TOO) Performance. *J Clin Oncol* 2019;37(suppl; abstr 3049). Doi:10.1200/JCO.2019.37.15_suppl.3049
- ¹⁴ Weiss G, Schlegel A, Kottwitz D, König T, Tetzner R. Validation of the SHOX2/PTGER4 DNA Methylation Marker Panel for Plasma-Based Discrimination between Patients with Malignant and Nonmalignant Lung Disease. *J Thorac Oncol.* 2017;12(1):77-84. doi:10.1016/j.jtho.2016.08.123
- ¹⁵ Sullivan FM, Mair FS, Anderson W et al. Earlier diagnosis of lung cancer in a randomized trial of an autoantibody blood test followed by imaging. *Eur Respir J* 2020;2000670. doi: 10.1183/13993003.00670-2020. Online ahead of print.
- ¹⁶ Doseeva V, Colpitts T, Gao G, Woodcock J, Knezevic V. Performance of a multiplexed dual analyte immunoassay for the early detection of non-small cell lung cancer. *J Transl Med.* 2015;13:55. Published 2015 Feb 12. doi:10.1186/s12967-015-0419-y
- ¹⁷ Cohen JD, Li L, Wang Y, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science.* 2018;359(6378):926-930. doi:10.1126/science.aar3247
- ¹⁸ <https://www.medicare.gov/coverage/lung-cancer-screenings>
- ¹⁹ <https://www.uspreventiveservicestaskforce.org/Page/Document/UpdateSummaryFinal/lung-cancer-screening>
- ²⁰ Ten Haaf K, Jeon J, Tammemägi MC, et al. Risk prediction models for selection of lung cancer screening candidates: A retrospective validation study. *PLoS Med.* 2017;14(4):e1002277. Published 2017 Apr 4. doi:10.1371/journal.pmed.1002277
- ²¹ LCSR_100853 FacilityReport2019Q4 accessed September 23, 2020 at <https://nrdrsupport.acr.org/support/solutions/articles/11000039783-lcsr-available-reports>

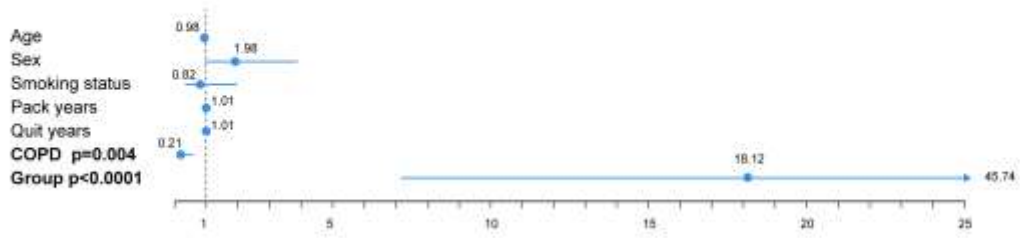
-
- ²² Mondaca S, Offin M, Borsu L, et al. Lessons learned from routine, targeted assessment of liquid biopsies for EGFR T790M resistance mutation in patients with EGFR mutant lung cancers. *Acta Oncol.* 2019;58(11):1634-1639. doi:10.1080/0284186X.2019.1645354
- ²³ Valpione S, Gremel G, Mundra P, et al. Plasma total cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for survival in metastatic melanoma patients. *Eur J Cancer.* 2018;88:1-9. doi:10.1016/j.ejca.2017.10.029
- ²⁴ Strijker M, Soer EC, de Pastena M, et al. Circulating tumor DNA quantity is related to tumor volume and both predict survival in metastatic pancreatic ductal adenocarcinoma. *Int J Cancer.* 2020;146(5):1445-1456. doi:10.1002/ijc.32586
- ²⁵ Egyud M, Tejani M, Pennathur A, et al. Detection of Circulating Tumor DNA in Plasma: A Potential Biomarker for Esophageal Adenocarcinoma. *Ann Thorac Surg.* 2019;108(2):343-349. doi:10.1016/j.athoracsur.2019.04.004
- ²⁶ Hamfjord J, Guren TK, Dajani O, et al. Total circulating cell-free DNA as a prognostic biomarker in metastatic colorectal cancer before first-line oxaliplatin-based chemotherapy. *Ann Oncol.* 2019;30(7):1088-1095. doi:10.1093/annonc/mdz139
- ²⁷ Chabon JJ, Hamilton EG, Kurtz DM, et al. Integrating genomic features for non-invasive early lung cancer detection. *Nature.* 2020;580(7802):245-251. doi:10.1038/s41586-020-2140-0
- ²⁸ Oxnard GR, Chen x, Fung et, et al. Prognostic Significance of Blood-based Cancer Detection in Plasma Cell-free DNA (cfDNA): Evaluating Risk of Overdiagnosis. *Journal of Clinical Oncology* 2019 37:15_suppl, 1545-1545. DOI: 10.1200/JCO.2019.37.15_suppl.1545
- ²⁹ Frumkin D, Shuali A, Savin O, et al. A new ultrasensitive assay for detection of hypermethylated tumor DNA in liquid biopsies. Poster p-21 at the 11th CNAPS (Circulating Nucleic Acids in Plasma and Serum) international symposium
- ³⁰ Zou H, Allawi H, Cao X, et al. Quantification of methylated markers with a multiplex methylation-specific technology. *Clin Chem.* 2012;58(2):375-383. doi:10.1373/clinchem.2011.171264.
- ³¹ Cottrell SE, Distler J, Goodman NS, et al. A real-time PCR assay for DNA-methylation using methylation-specific blockers. *Nucleic Acids Res.* 2004;32(1):e10. Published 2004 Jan 13. doi:10.1093/nar/gnh008
- ³² Kristensen LS, Hansen LL. PCR-based methods for detecting single-locus DNA methylation biomarkers in cancer diagnostics, prognostics, and response to treatment. *Clin Chem.* 2009;55(8):1471-1483. doi:10.1373/clinchem.2008.121962
- ³³ Larose TL, Meheus F, Brennan P, Johansson M, Robbins HA. Assessment of Biomarker Testing for Lung Cancer Screening Eligibility. *JAMA Netw Open.* 2020;3(3):e200409. Published 2020 Mar 2. doi:10.1001/jamanetworkopen.2020.0409
- ³⁴ Chen S, Wang Q, Yu H, et al. Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathway. *Nat Commun.* 2019;10(1):5649. Published 2019 Dec 11. doi:10.1038/s41467-019-13542-2
- ³⁵ Shlush LI. Age-related clonal hematopoiesis. *Blood.* 2018;131(5):496-504. doi:10.1182/blood-2017-07-746453
- ³⁶ Parikh AR, Van Seventer EE, Boland GM, et al. A plasma-only integrated genomic and epigenomic circulating tumor DNA (ctDNA) assay to inform recurrence risk in colorectal cancer (CRC). *Journal of Clinical Oncology* 37, no. 15_suppl (May 20, 2019) 3602-3602. DOI: 10.1200/JCO.2019.37.15_suppl.3602
- ³⁷ Tammemägi MC, Katki HA, Hocking WG, et al. Selection criteria for lung-cancer screening [published correction appears in *N Engl J Med.* 2013 Jul 25;369(4):394]. *N Engl J Med.* 2013;368(8):728-736. doi:10.1056/NEJMoa1211776



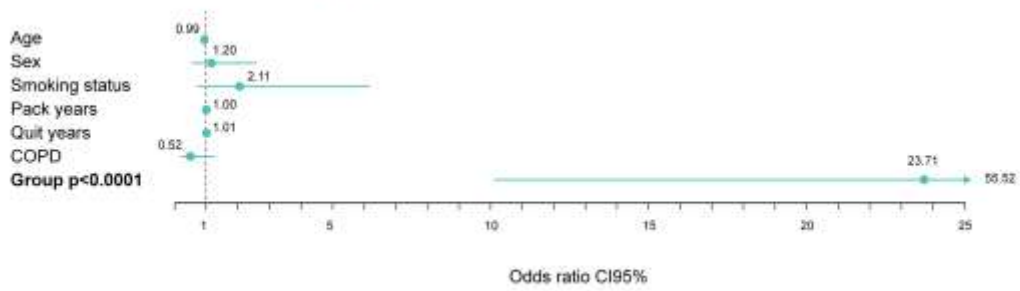


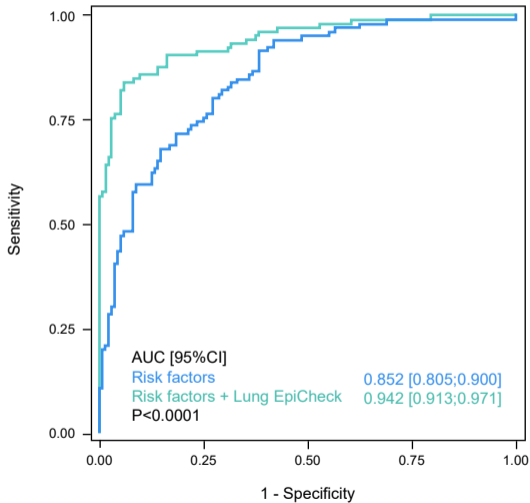


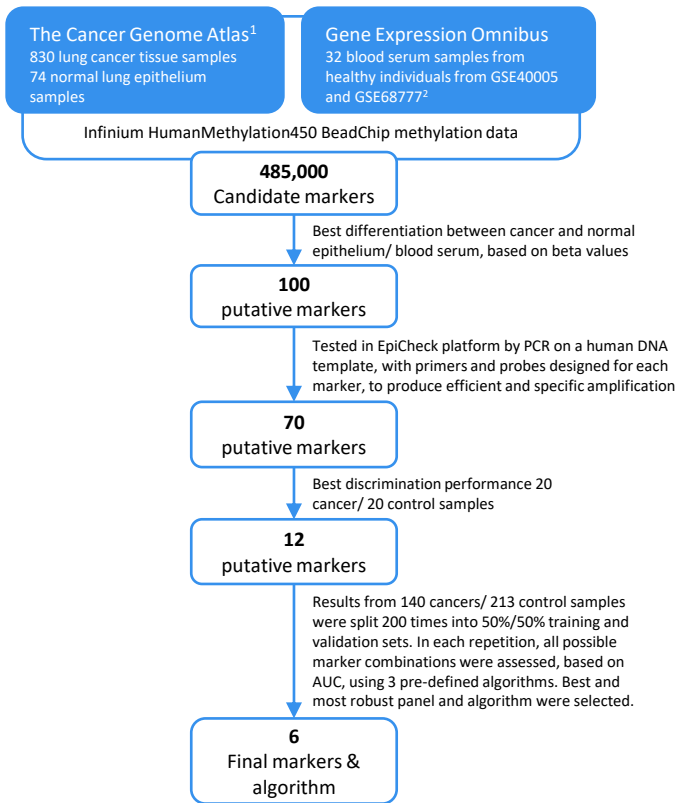
Risk factors impact on positive Episcore ≥ 60



Risk factors impact on positive Episcore ≥ 70







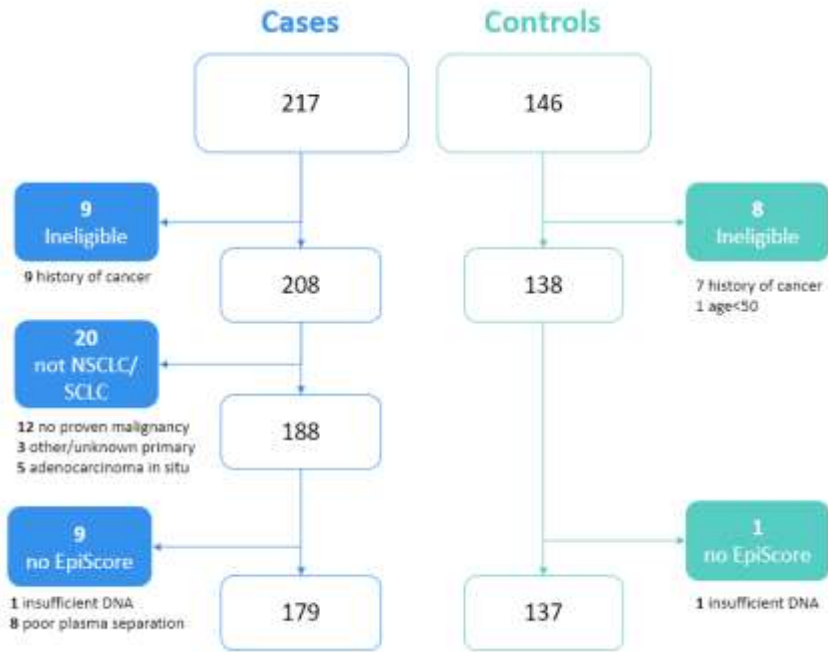
¹ Weisenberger. Characterizing DNA methylation alterations from The Cancer Genome Atlas. *J Clin Invest* 2014;124:17-23

² Sabuncuyan et al. Association of DNA Methylation with Acute Mania and Inflammatory Markers. *PLoS One* 2015;10(7):e0132001

List of sites and biobanks, European cohort

1	Athens Chest Hospital Sotiria, Athens, Greece
2	BioIVT (Seralab) LLC.
3	Carmel Medical Center, Haifa, Israel
4	Emek Medical Center, Afula, Israel
5	Holy Family Hospital, Nazareth, Israel
6	Indivumed GmbH
7	Maccabi Petach Tikva Clinic, Maccabi Health services, Petach Tikva, Israel
8	Maccabi Ramat Hasharon Clinic, Maccabi Health services, Ramat Hasharon, Israel
9	Meir Medical Center, Kfar Saba, Israel
10	National Korányi Institute of Pulmonology, Budapest, Hungary
11	Shaare Zedek Medical Center, Jerusalem, Israel
12	Shamir Medical Center (Assaf Harofeh), Zrifin, Israel
13	Shuali Ra'anana Clinic, Clalit Health Services, Ra'anana, Israel
14	Soroka Medical Center, Beer Sheva, Israel
15	Tel-Aviv Sourasky Medical Center (Ichilov), Tel-Aviv, Israel
16	Tissue solutions Ltd.
17	Wolfson Medical Center, Holon, Israel
18	Ziekenhuisgroep Twente (ZGT), Hengelo, the Netherlands
19	Ziv Medical Center, Safed, Israel

S3a. CONSORT diagram European validation set



S3.b. CONSORT diagram Chinese validation set

