Prognostic value of tumor-associated regulatory T-cells as a biomarker in non-small cell lung cancer: a systematic review and meta-analysis

Dr. Kapil Khambholja (kapil.khambholja@genproresearch.com)
Genpro Research Inc., USA https://orcid.org/0000-0002-7450-459X
Dr Manish Gehani
Genpro Research Pvt. Ltd., India https://orcid.org/0000-0002-5894-4106
Dr Rushabh Kothari
Narayana Multispecialty Hospital, India https://orcid.org/0000-0002-3910-5649
Dr Sachin Marulkar
Genpro Research Pvt. Ltd., India

Systematic Review

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Abstract

Background

Despite continuous improvement, tumor, nodes, and metastases (TNM) staging has been deficient in prognosticating in patients suffering from non-small cell lung cancer (NSCLC). To supplement TNM staging, this systematic review and meta-analysis aimed to evaluate the prognostic value of the regulatory T cells (Treg).

Methods

A keyword search was conducted in the MEDLINE database through PubMed for full-text original human studies from any region published in English during the last 10 years. Eligible for inclusion were studies evaluating the prognostic value of the number of Treg cells and pre-specified biomarkers in NSCLC. Case studies, case series, systematic reviews, and meta-analyses were excluded. Two reviewers independently screened the studies and assessed risk-of-bias using the Quality in Prognosis Studies (QUIPS) tool. One reviewer used an automation tool for screening, which was also used to facilitate data extraction. Meta-analysis was done for studies reporting significant multivariate hazards ratio (HR).

Results

Out of 258 retrievals, 19 studies were included in the final review. The low number of Treg cells was found significantly associated with improved overall survival (pooled log OR: 1.626; 95% CI: 1.324, 1.928; p (2-tailed) < .001; SE: 0.1174), improved recurrence-free survival (HR: 1.99; 95% CI: 1.15, 3.46; p = .01), and worse disease-free survival (pooled log OR: 0.992; 95% CI: 0.820, 1.163; p (2-tailed) .009; SE: 0.0135), especially when identified by forkhead box P3 (FOXP3), in any stage or non-metastatic NSCLC.

Conclusion

A low number of Treg cells indicated better survival, suggesting its potential use as a prognostic biomarker in NSCLC.

Systematic review registration

The protocol of this review was prospectively registered on PROSPERO on August 28, 2021, and was assigned the registration number CRD42021270598. The protocol can be accessed from PROSPERO website.

1 Introduction

An estimated 2.1 million new cases and 1.8 million deaths occur annually due to lung cancer around the world [2]. Almost half of the patients die within one year of diagnosis and less than 18% survive beyond five years [3]. Further, as per data from the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute, around two-thirds of patients with non-small cell lung cancer (NSCLC) do not survive beyond two years [4]. The dismal prognosis is exacerbated by a lack of methods for early diagnosis and prognostication and limited access to opportune standard treatment. Historically, the clinical fraternity has relied upon the tumor, nodes, and metastases (TNM) staging as the gold standard prognostic tool for lung cancer [5], although, several researchers have raised concerns about various editions of the TNM classification. The fifth edition of TNM classification was unable to distinguish the prognosis between patients with pathologic (p) stage IIIA and IIIB disease [6]. A real-world validation of the seventh edition using the International Association for the Study of Lung Cancer (IASLC) database could not find a significant difference between survival rates of patients with pT1bN0M0 and pT2aN0M0 tumors, nor between survival rates of patients with pT4N0M0 and pT3N0M0 tumors [7]. Besides, there was no difference in the prediction of survival by the sixth and seven editions [7]. The current eighth edition of TNM classification is not flawless as well. Hattori et al. reported inconsistency between radiological solid component size and pathological invasive size in part-solid lung adenocarcinomas and difficulty in measuring the solid component size due to the presence of multiple, complicated, or scattered solid areas rather than a single focus [8].

At the same time, several studies have reported a profound prognostic impact of tumor-infiltrating lymphocytes (TILs) in malignant tumors [9–16]. Studies have further shown that TILs are associated with a positive clinical outcome in several cancers including lung cancer [17]. Immune scoring based on TILs or their ratios for differentiating prognosis within each tumor, node, and metastasis have been used to enhance the prognostic value of TNM staging [18–21].

TILs present in the immune infiltrate called the tumor microenvironment (TME) include macrophages, neutrophil granulocytes, dendritic cells, mast cells, natural killer (NK) cells, naive and memory lymphocytes, B cells, and effector T cells. Effector T cells in turn can be T helper cells (1, 2, and 17), regulatory T (Treg) cells, T follicular helper cells, and cytotoxic T cells [17, 22, 23]. These cells may be localized in the tumor parenchyma, invasive margin, or adjacent tumor stroma. The interaction of TILs with tumor cells can be seen as the three phases of immuno-editing [24]. During the elimination phase, the immune cells detect and destroy early tumors even before they become overt. In the equilibrium phase, the immune system cannot eliminate the tumor cells which resist immune recognition and go into dormancy. During the escape phase, tumor cells cause immune suppression through the production of cytokines and growth factors and facilitate the recruitment of immunosuppressive cells like Tregs [25–27].

Tregs are a highly immune-suppressive subset of clusters of differentiation (CD) 4^+ T cells [25–27], which play an important role in the preservation of self-tolerance and modulation of the overall immune responses against tumor cells [28]. They exhibit their suppressive activity by numerous cellular and humoral mechanisms such as suppression of antigen-presenting cells via cytotoxic T lymphocyte-

associated antigen-4 (CTLA-4), secretion of inhibitory cytokines including interleukin (IL) 10, transforming growth factor (TGF) β, and IL-35, expression of granzyme B or perforin or lymphocyte activation gene (LAG) 3, consumption of IL-2, depletion of extracellular adenosine triphosphate (ATP), and “stripping” of co-stimulatory molecules [28, 29]. The effector Tregs express fork-head lineage-specific transcription factor forkhead box P3 (FOXP3) protein and cytokines such as CTLA-4, programmed cell death protein 1 (PD-1), T cell immunoglobulin and mucin-domain containing (TIM) 3, and C-C Motif Chemokine Receptor 4 (CCR4), while natural or thymic Tregs (nTregs) reportedly express high levels of Helios (a member of the Ikaros transcription factor family) and Neuropilin-1 (a type-1 transmembrane protein) [28].

Studies have shown that the composition of Tregs is altered in the TME, where the effector Treg numbers are increased as compared to healthy individuals [30]. TGF-β1 and IL-2 are the two crucial pro-inflammatory cytokines present at a high level in tumor tissues of NSCLC patients, which are involved in the differentiation of naïve T-cells into Tregs [30]. Moreover, due to self-antigens released in the TME by dying cancer cells, nTregs are converted into effector Tregs by expressing a higher level of activation biomarkers like CTLA-4, T-cell immunoreceptor with immunoglobulin (Ig) and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), TIM-3, inducible T cell co-stimulator (ICOS), OX40, 4-1BB and CD39 [30]. Tregs have been reported to increase in NSCLC patients as compared to healthy controls [31]. In a study, Erfani et al. reported that the NSCLC patients had almost twice the percentage of Treg cells than the healthy controls [32]. Further, they reported that the metastatic stages had three times more Treg cells than the healthy controls and almost two times more Treg cells than the non-metastatic stages [32]. This makes Tregs an ideal therapeutic target and candidate for prognostication of lung cancer, especially NSCLC, which comprises about 85% of all lung cancer cases [33]. While the therapeutic targeting of Treg by pathways like the blockade of immune checkpoint molecules has been studied by many authors [28], the prognostic value of measurement and localization of Treg cells has not been fully evaluated in all study populations, with various prognostic factor variables, causing individual studies to report piecemeal and mixed results.

This systematic review and meta-analysis aimed to evaluate the prognostic value of the number of Treg cells in predicting the survival of NSCLC patients based on the available evidence in various study populations, considering varied prognostic factor variables and survival outcomes. The findings of the review may inform clinical decision-makers on patient-centric NSCLC management by supplementing the TNM staging by a process like immuno-scoring and stratification based on the “number of Treg cells” in future.

2 Materials And Methods

The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The protocol of this review was prospectively registered on PROSPERO on August 28, 2021, and was assigned the registration number CRD42021270598.

2.1 Design and setting

The current study was designed as a systematic review and meta-analysis. An online electronic database search was conducted from July 19 to 25, 2022. Retrieved studies underwent screening, quality assessment, and extraction of the required information. The systematic review process was facilitated by an automation tool “MaiA”, an artificial intelligence-based proprietary platform developed in-house by Genpro Research Pvt Ltd.

2.2 Search strategy

The search was conducted in the MEDLINE database through PubMed. The keywords used were- ("non-small cell lung cancer" OR "non-small cell lung neoplasm" OR "non-small cell lung carcinoma") AND ("Treg" OR "regulatory T cells" OR "regulatory T lymphocytes" OR "Tumor-infiltrating regulatory T cells" OR "Foxp3" OR "Helios") AND (recurrence OR mortality OR metastasis OR survival OR "progression-free survival" OR "disease-free survival" OR "recurrence-free survival" OR "overall survival" OR prognosis OR prognostic)

The same broad set of keywords was used by both the manual reviewer and the reviewer using the automation tool, to run a comprehensive search for the relevant published studies describing the prognostic value of Treg in patients with NSCLC. Free full-text studies presenting original research in humans, published in English in any region during the last 10 years were included in the review process. Protocols or design papers, opinion papers, education literature, meeting reports, and position papers were excluded. Brief communications and letters to the editor were eligible for inclusion only if they described original research with its results. Although the review studies were not eligible for inclusion, cross-references from them were reviewed to ensure that no eligible article was missed. The search was not rerun before the final analysis and no unpublished study was sought. The last search was done on July 25, 2022.

2.3 Selection of studies

The two reviewers screened the unblinded titles, abstracts, and full text of the studies independently in a standardized manner. One reviewer performed screening manually, while the other used “MaiA”. The same predefined eligibility criteria based on the “Population, Prognostic Factor, Outcome” (PFO) approach were used by both reviewers. Both the reviewers were blinded to each other’s decisions. The disagreements were resolved by consultation with other co-authors and resolutions were recorded in an Excel sheet. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Flow Diagram was created based on the retrieved literature, screened records, excluded studies, and included studies (Fig. 1).

2.3.1 Inclusion criteria

Eligible for inclusion were the studies concerning both male and female participants, suffering from NSCLC, irrespective of TNM staging, with or without prior surgery or therapy, or those undergoing surgery

or therapy at the time of the respective original study.

Studies evaluating the prognostic factor as presence, degree, measurement, or localization of Tregs, in tumor tissue or body fluids through histological or cytological procedures like immunohistochemistry, flow cytometry, or quantitative real-time-polymerase chain reaction, and assessing either Treg alone or in combination with a biomarker out of $CD4^+$, $CD25^+$, $CD127^-$, $Helios^+$, and/or $FOXP3^+$ were included.

Studies describing primary outcomes of overall survival (OS), progression-free survival (PFS), disease-free survival (DFS), or recurrence-free survival (RFS), with point estimates as risk and ratios such as relative risk (RR), odds ratio (OR), or hazards ratio (HR), were included in the review. OS was defined as the time between the first diagnosis of NSCLC and the date of death regardless of the cause. PFS, DFS, and RFS were defined as the time from the date of diagnosis to the date of progression, recurrent symptoms, and first relapse respectively. Both prospective and retrospective studies were included in the review.

2.3.2 Exclusion criteria

Studies involving patients suffering from other lung cancers or cancers of other body parts, and not NSCLC, were excluded. Studies not concerned with the prognostic or predictive value of Tregs were excluded. Studies designed as case studies, case series, systematic reviews, or meta-analyses were also excluded.

2.4 Assessment of risk of bias and quality of studies

Cochrane's Quality in Prognosis Studies (QUIPS) tool was used for the quality assessment of the studies included in the review after screening. The assessment was done at the study level by both reviewers manually and independently. The disagreements were resolved by consultation with other co-authors and resolutions were recorded in an Excel sheet. Studies were rated based on a three-grade scale (high, moderate, or low) of risk of bias. Out of the six bias domains of the QUIPS tool, each prompting item was rated as yes or no based on the fulfillment of the criteria provided by the respective prompting item. If ≤ 33% prompting items of a bias domain were rated as "No", the risk of bias for that domain was rated as low. Similarly, for 34 to 50% of prompting items rated as no, the risk of bias for that domain was rated as moderate, and for > 50%, the risk of bias was rated as high. Studies with low risk of bias for "study participation", "prognostic factor measurement", and "outcome measurement" domains, while low to moderate risk of bias for "study attrition", "study confounding", and "statistical analysis and reporting" domains were included for extraction of data for the final narrative synthesis and quantitative analysis.

2.5 Data extraction

The data was extracted from the full text of the studies in a pre-piloted Excel-based data extraction form to record and analyze the required information from each of the identified studies. Data extraction was done by the first reviewer and verified by a second reviewer. The data extracted from the included studies consisted of- report characteristics including type of article, period of publication, and region of publication; study characteristics related to study design: sample size, comparator; population:

participant demographics, staging, grading, the subtype of NSCLC, prior treatment or surgery; prognostic factor: samples collected and procedures performed for measurement and localization of Treg cells, biomarkers evaluated; outcomes: results related to measurement and localization of Tregs, statistical test performed for the primary outcome, univariate and/or multivariate HRs with confidence interval and p-value for OS, PFS, DFS, or RFS, values of other estimates such as correlation coefficient, and data related to recurrence and metastasis. The automation tool “MaiA” was used to facilitate the extraction of data.

2.6 Statistical analysis

The primary outcome measured by this review was how the number of Treg cells affected the survival of the patients with NSCLC in terms of OS, PFS, DFS, or RFS. The secondary outcome of the study was to quantify recurrence and metastasis. All results that were compatible with each outcome domain in each study were sought.

The data were pooled for quantitative synthesis from the individual studies through meta-analysis using Statistical Package for the Social Sciences (SPSS). As per the Cochrane Consumers and Communication Group review for meta-analysis, a minimum of two studies with the same type of survival outcome were required to subject them to quantitative synthesis. In case a sufficient number of studies were not available for certain survival outcomes, findings from the individual studies were summarized in the narrative.

The survival analysis was reflected by the individual studies as HR, 95% confidence interval (CI), and p-value. Studies with a significant p-value (< .05) for multivariate HR were considered for meta-analysis to avoid bias due to confounding by co-factors. Studies having a low number of Tregs as the reference category for HR were eventually included in the meta-analysis.

For pooling HRs, a meta-analysis for binary outcomes with pre-calculated effect sizes was run in SPSS. Confidence intervals were converted to variance. Since the variables related to the study population, prognostic factor, outcome, and study design were not completely similar among the included studies, the random effect model was applied. Inverse-variance method was used to determine the weight of the studies. DerSimonian-Laird was used as the estimation method. Standard error adjustment was done using the Knapp-Hartung adjustment method. The low number of Treg became the control category for the pooled OR. A sensitivity analysis for the standard method was also run but eventually, the significant results obtained from the Knapp-Hartung adjustment method were reported in the results. The summary effect estimate (log OR) and its confidence interval was determined by generating forest plots using the HRs and associated variance for survival outcomes. The I^2 statistic was calculated for determining heterogeneity where an I^2 value lower than 30% was considered indicative of homogeneity. The symmetry of funnel plots generated using effect size and variance was used to assess publication bias visually. No subgroup analysis was performed. The data generated during screening, risk-of-bias assessment, and extraction process was uploaded in the online Harvard Dataverse Repository [1].

3 Results

3.1 The flow of the studies through the review process and characteristics of the included studies

Out of a total of 258 retrievals through automation, manual search, and cross-references, 152 studies were filtered, 76 studies were screened, two studies were not available in full text, and nine studies could not clear quality assessment. Eventually, 19 studies (16 from manual search and three from cross-references) were included in the review (Fig. 1) [34, 35, 44–52, 36–43].

Most studies had the study population as non-metastatic NSCLC (n = 5) or any NSCLC (n = 4). Most of the studies included all sub-types of NSCLC (n = 16), except two studies that included adenocarcinoma and squamous cell carcinoma, and one study which included only adenocarcinoma. Six studies included patients who underwent surgery without any neo-adjuvant therapy, while two studies included patients who did not receive any adjuvant therapy. The majority of studies performed tests on Formalin-fixed paraffin-embedded (FFPE) sections or preserved tissue-microarray blocks, either alone or with other types of samples (n = 14), commonly using immuno-histochemistry method (n = 13). FOXP3 (n = 7) was the commonest biomarker used. Most studies reported OS (n = 13) either as the only survival outcome or along with other outcomes. Sixteen studies (84.2%) were designed as an analytical cross-sectional study with or without a comparison group. Out of the seven studies with a comparison group (Cross-sectional: 5, Cohort: 1, Controlled before-after: 1), six used healthy volunteers (three used age-matched healthy volunteers, one used both age- and sex-matched healthy volunteers, and two studies used unmatched healthy volunteers). Thirteen studies were retrospective in nature, while five were prospective, and one study was both prospective and retrospective. Report characteristics and study characteristics of the included studies are detailed in Table 1. The study-wise details of report characteristics, population under study, prognostic factor, outcome, and study design are provided in supplementary table in Audio Resource 1.

Table 1
Characteristics of the included studies

Report Characteristics	n	Study Characteristics	n	Study Characteristics	n
Year of publication (N = 19)		Population under study (N = 19)		Flow cytometry and antibody staining	1
2014	1	Any NSCLC	4	Immuno-histochemistry with FOXP3 staining	1
2015	1	Any stage NSCLC	1	*Biomarkers used for Treg (N = 19)*	
2016	5	Chemotherapy-naïve any stage NSCLC	1	FOXP3+	7
2017	3	Newly diagnosed any stage NSCLC	1	CD4 + CD25+/++ CD127-/dim	4
2018	1	Stage 1 NSCLC	1	CD4 + FOXP3+	2
2019	2	Non-metastatic NSCLC	5	CD4+	1
2020	3	Chemotherapy-naïve non-metastatic NSCLC	1	Helios + FOXP3+	1
2021	3	Metastatic NSCLC	1	CD3 + FOXP3+	1
Language (N = 19)		First diagnosis and relapsed NSCLC	1	CD8 + FOXP3+	1
English	19	NSCLC with KPS > 80%	1	CD3 + CD45RO + FOXP3+	1
Region (N = 19)		Resected NSCLC without neo-adjuvant chemo-radiotherapy	1	No biomarker	1
China	7	Solitary lesion in localized NSCLC	1	Outcomes (N = 19)	
Japan	3	Prognostic factor (N = 19)		Overall survival	6
Brazil	2	*Samples for Treg assessment*		Disease-free survival	3
Germany	2	FFPE sections or preserved tissue-microarray blocks	11	Progression-free survival	3
France	1	Peripheral blood	4	Recurrence-free survival	1
Greece	1	Both FFPE and peripheral blood	2	Both overall survival and disease-free survival	2

CD, clusters of differentiation; FFPE, formalin-fixed paraffin-embedded; FOXP3, forkhead box P3; KPS, Karnofsky Performance Status; NSCLC, non-small cell lung cancer; Treg, regulatory T cell.

Report Characteristics	n	Study Characteristics	n	Study Characteristics	n
Australia	1	Fresh surgical specimens	1	Both overall survival and progression-free survival	1
Spain	1	FFPE and fresh tumor biopsies	1	Both overall survival and recurrence-free survival	3
United States of America	1	*Tests conducted on samples*		Study design (N = 19)	
Species (N = 19)		Immuno-histochemistry	8	Analytical cross-sectional design	11
Humans	19	Flow cytometry	3	Analytical cross-sectional design with comparison group	5
Article type (N = 19)		Flow cytometry and immuno-histochemistry	3	Longitudinal	1
Original research article	18	Multiplex immuno-fluorescence staining	2	Controlled before after study	1
Letter to the editor	1	Immuno-histochemistry and immuno-fluorescence staining	1	Cohort study	1

CD, clusters of differentiation; FFPE, formalin-fixed paraffin-embedded; FOXP3, forkhead box P3; KPS, Karnofsky Performance Status; NSCLC, non-small cell lung cancer; Treg, regulatory T cell.

3.2 Quantitative synthesis of the study results

Out of the 19 included studies, only ten studies, which reported significant ($p \leq .05$) multivariate analysis, were included in the meta-analysis. Among these studies, eight studies reported ten OS values, of which one study reported OS value for two different biomarkers ($FOXP3^+$ and $CD8^+$), while another study reported separate OS for naïve and terminal effector Tregs. To reinforce the *a priori* inclusion criteria of reporting pre-specified biomarkers, one OS value related to $CD8^+$ was excluded from the analysis. Further, to ensure similarity between the study population, prognostic factor, outcome, and design, for applying the random effect model, three OS values were excluded from the final meta-analysis. The excluded OS values included one study reporting OS for FOXP3 positive/negative instead of low/high number of Tregs; one study reporting results concerning cell density and reporting log HR instead of HR; and another study reporting one out of the total two values of OS with a high number of Treg as the reference category. Eventually, six OS values from six studies were included in the meta-analysis. The population under study for the included studies were non-metastatic NSCLC (n = 2), any stage NSCLC (n = 2), naïve NSCLC (n = 1), and NSCLC with Karnofsky Performance Status (KPS) > 80% (n = 1). Four studies measured Treg in FFPE sections by immunohistochemistry, while two studies measured the same in peripheral blood using flow cytometry. Four studies measured FOXP3 as the biomarker for Treg and two

studies measured multiple biomarkers. All six studies ran Cox proportional hazards model for survival analysis. All of them were analytical cross-sectional in design. Two of these studies were prospective and four studies were retrospective.

Among the ten studies reporting significant multivariate analysis, two studies reported three values for DFS, including one study reporting DFS for cell number as well as cell density. For applying the random effect model, the DFS value for cell density was excluded from the final meta-analysis. Eventually, two studies reporting two values for DFS were included in the meta-analysis. Similarly, three studies reported values for PFS. One of these studies had the reference category as high Treg and was excluded from the meta-analysis. Eventually, two studies reporting two values of PFS were included in the meta-analysis. Only one study reported RFS among the ten studies reporting significant multivariate analysis. Since a minimum of two studies were required for performing a meta-analysis, the same could not be done for RFS.

Out of the final 19 studies included in the review, one study evaluated the $FOXP3^+$ category and the FOXP3- category in non-metastatic NSCLC, while the remaining 18 studies assessed a high and low number of Tregs. The low Treg was defined differently in different studies. In the study including only stage 1 NSCLC patients, low Treg was considered as a low cell count of less than 45 per high power field (HPF). Among the studies involving non-metastatic NSCLC patients, one study considered low Treg as less than 10% of the total lymphocyte count; another study described it as a low area under the curve than a cut-off; another study defined it as lower than the median; another study specified it as below the optimal cutoff point according to the built-in risk scoring formula in X-tile; while another study did not specify the criteria or cut-off for labeling low number of Tregs, although its text mentions the comparative frequency of Tregs in patients and healthy controls. In the study including only metastatic NSCLC patients, a low level of Tregs was defined as lower than the median. Among the studies including NSCLC patients irrespective of the stage, two studies defined low Tregs as lower than the median; two studies as lower than mean; another two studies as lower than a cut-off concerning percent of CD4 count; one study considered it as less than 95% of controls; and one study defined it as a low concentration in tumor tissue than a pre-specified level. Another such study used the criteria of high (score 2–3) and low (score 0–1) on a scale of 0–3, based on the percentage of positively stained cells, as a proportion of the total nucleated cells. Yet another study mentioned low Treg as below 25 in number, but no further details were specified.

3.3 Summary estimates from the meta-analysis

The six studies analyzed for OS had a Q value less than the degree of freedom (K-1), hence I^2 was zero and the included studies had low heterogeneity. The summary log OR was 1.626; 95% CI: 1.324, 1.928; p (2-tailed) < .001; standard error (SE): 0.1174 (Fig. 2a). The two studies pooled for DFS had low heterogeneity ($I^2 = 0$), and the summary log OR was 0.992; 95% CI: 0.820, 1.163; p (2-tailed) .009; SE: 0.0135 (Fig. 2b). The two studies pooled for PFS had low heterogeneity ($I^2 = 0$), and the summary log OR as 2.128; 95% CI: -7.983, 12.239; p (2-tailed) = .228; SE: 0.7958 (Fig. 2c). The one study which evaluated

RFS reported multivariate HR as 1.99; 95% CI: 1.15, 3.46; p = .01. The funnel plots for OS, DFS, and PFS did not show any publication bias as depicted in supplementary figure (Audio Resource 2).

3.4 Other results reported by the studies included in the review

3.4.1 Quantification

Four studies reported an increase in Tregs in patients with NSCLC as compared to healthy volunteers, while one study further elaborated that the patients with relapsed NSCLC had an even higher number of Tregs. One study also reported that the density of Tregs increased with age (Audio Resource 1).

3.4.2 Localization

One study reported that the presence of low Tregs in tumor stroma was significantly associated (p = .024) with worse DFS than high Tregs. Another study reported significantly worse OS (p = .029) due to high Treg ($FOXP3^+$) infiltration of the stroma. Another study reported that the increased interaction between tumor cells and Tregs in the core of the tumor (p = .003) (unlike the periphery or margin of the tumor) was associated with worse OS (Audio Resource 1).

3.4.3 Recurrence and metastasis

One study reported that a higher number of Tregs were present in TME in recurrence cases than in non-recurrence cases. Another study reported the presence of a significantly higher (p = .046) mean Treg cells in patients with local recurrence than in patients with distant metastasis. Further, one study reported that the most common sites for distant metastasis were the brain (19.6%), bone (5.4%), pleura (5.4%), lung (4.5%), and liver (2.7%). Another study reported that almost all the patients with distant metastases also had lymph node metastases and that the overall local recurrence and distant metastasis occurred more with adenocarcinoma (53%) than with squamous cell carcinoma (40%) or large cell carcinoma (7%).

3.4.4 Correlation of PD-L1 with the level of Tregs

Total of six studies evaluated or mentioned the correlation of PD-L1 with the level of Tregs. One study reported that FOXP3 positive status was strongly correlated with increasing PD-L1 expression (p = 0.021), while another study reported that the presence of $FOXP3^+$ Treg cells was significantly more likely in PD-L1 positive NSCLC tissues (p < 0.001). One study did not find any significant correlation between PD-L1 and Treg number in the stroma (correlation coefficient 0.021, p = 1.00E + 00), and in the tumor (correlation coefficient − 0.003, p = 1.00E + 00), while another study did not report any significant correlation between FOXP3 high status and PD-L1 status (p = 0.059). Another study reported that $FOXP3^+$ cell density was not significantly associated with PD-L1 expression (p = 0.089). Yet another study did not evaluate the correlation between TILs and PD-L1 expression because they considered that it may be induced by TILs or cancer cells intrinsically, because their specificities in immunohistochemistry have been unstable, and

established evaluation of their expression has not been developed yet. In this way, the results were equivocal regarding the correlation of PD-L1 with the level of Tregs.

4 Discussion

The results of the review indicate that the number of Treg cells was significantly associated with OS ($p < .001$) and DFS ($p = .009$) in NSCLC. The pooled log OR of more than one indicates that the reference category as a low number of Treg was associated with improved OS. On the contrary, DFS was better with the high number of Tregs, although the analysis pooled only two studies and both of them used different biomarkers and study populations. The low number of Tregs also showed clinically important higher PFS, although the same was not statistically significant ($p = .228$). Based on one study that reported significant HR for RFS, a low number of Treg had significantly better RFS ($p = .01$). The log OR generated by the forest plot for OS was significant for the study population “any stage NSCLC” and FOXP3 as the biomarker, while the one generated for DFS was significant for the study population “any stage NSCLC” and “non-metastatic NSCLC” and biomarker as FOXP3 and $CD4^{+}FOXP3$. The study reporting significant HR for RFS also evaluated FOXP3 in any stage NSCLC. Overall, the meta-analysis reflected that a low number of Treg cells indicated better survival, especially in any stage NSCLC or non-metastatic NSCLC, while using FOXP3 as the biomarker.

The majority of the past studies reported findings similar to our study, although few studies contradicted our conclusion. Peng et al. concluded that a low number of $CD4^{+}FOXP3^{+}$ Tregs had significantly poor DFS [35]. Similarly, Kotsakis et al. found that a high number of terminal effector Tregs ($CD25^{high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$) was associated with significantly ($p = .044$) better OS and PFS [44]. On univariate Cox regression, Muto et al. reported significantly ($p = .04$) better OS [47], while Ameratunga et al. reported significantly ($p = .038$) better DFS in patients with a high number of Treg cells [38]. A recent literature review published by Liang et al. summarized the published evidence regarding the importance of Tregs as a prognostic factor for NSCLC and presented the current understanding of the mechanisms involved in this regard [53]. Fantini et al. reviewed the literature related to colorectal cancer, and postulated that Tregs activity may be spatially and functionally compartmentalized [29]. None of the published reviews on this topic was designed as a systematic review and meta-analysis. None of the previous studies could highlight the role of Tregs in different study populations, considering different prognostic factor variables, outcomes, and study designs. On the contrary, our review was designed as a systematic review and meta-analysis to generate robust evidence. Additionally, our review was comprehensive and included data concerning varied study populations, prognostic factor variables, outcomes, and several other aspects. This enabled us to reflect on the state of the art regarding the prognostic value of the number of Treg cells in NSCLC.

The current study had certain limitations. Searching only one portal might have introduced bias, although the unavailability of filters appropriate for clinical studies in search portals like Scopus and Google Scholar discouraged the authors from including them in the search strategy. Since MEDLINE is the biggest database for clinical studies, the authors chose to conduct the review on the PubMed portal.

Another limitation was the low number of studies included in the final meta-analysis, due to which sub-group analysis could not be conducted for many variables such as study population and biomarkers. Moreover, variation in study populations, prognostic factor variables (such as types of Tregs, biomarkers, samples, and procedures of measurement), outcomes, study designs, analytical tests, reference categories, quantification, and localization of Tregs, among the included studies may have introduced imprecision in results and may be a source of bias.

Further, several factors influence the function of Tregs in the TME by acting on the development and maturation of Tregs, or by providing favorable conditions for the functioning of Tregs [53]. Some of these include Glucocorticoid-induced tumor necrosis factor receptors (TNFR)-related protein (GITR) (TNFRSF18), OX40 (TNFRSF4), TNFR2 (TNFRSF1B), insufficient glucose supply, increased intracellular glycolysis in cancer cells, TGF-β, ATP, and Indoleamine 2, 3-dioxygenase (IDO) [53–59]. Additionally, the secretions of tumor cells such as chemokines, “Chemokine (C-C motif) ligand 20” (CCL20), and Amphiregulin (AREG) protein also promote the regulatory function of Tregs [53, 60, 61]. Mutations like Kirsten rat sarcoma viral oncogene (*KRAS)* in lung cancer cells have been shown to promote the differentiation of $CD4^{+}$T cells into Tregs [53, 62]. Finally, multiple mechanisms of action of Tregs involving immuno-modulatory cytokines, molecules, and processes pose difficulty in explaining the exact mechanism contributing to the results of the individual studies [28, 29]. Since the current review did not assess any of these factors influencing the function of Tregs or the underlying mechanisms, they could have potentially confounded the results. Nevertheless, the present review applied meta-analysis to synthesize the best available evidence for use of the number of Treg cells as a prognostic indicator in NSCLC.

5 Conclusions

Based on the results of this meta-analysis, authors recommend using the number of Treg cells as a prognostic biomarker, especially in any stage NSCLC or non-metastatic NSCLC, while using FOXP3 expression as the specific marker, although further experimental studies are needed to confirm these findings. The evidence generated by this review can help the researchers design suitable studies concerning populations, biomarkers, outcomes, and designs. By highlighting the significance of Treg cells in the prognosis of NSCLC, this study can help in developing better prognostic tests and novel therapeutic approaches in cancer treatment.

Declarations

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Data transparency: The datasets generated during screening, risk-of-bias assessment, and extraction process and analyzed during the current study are available in the online Harvard Dataverse Repository,

https://doi.org/10.7910/DVN/JQOI9V [1].

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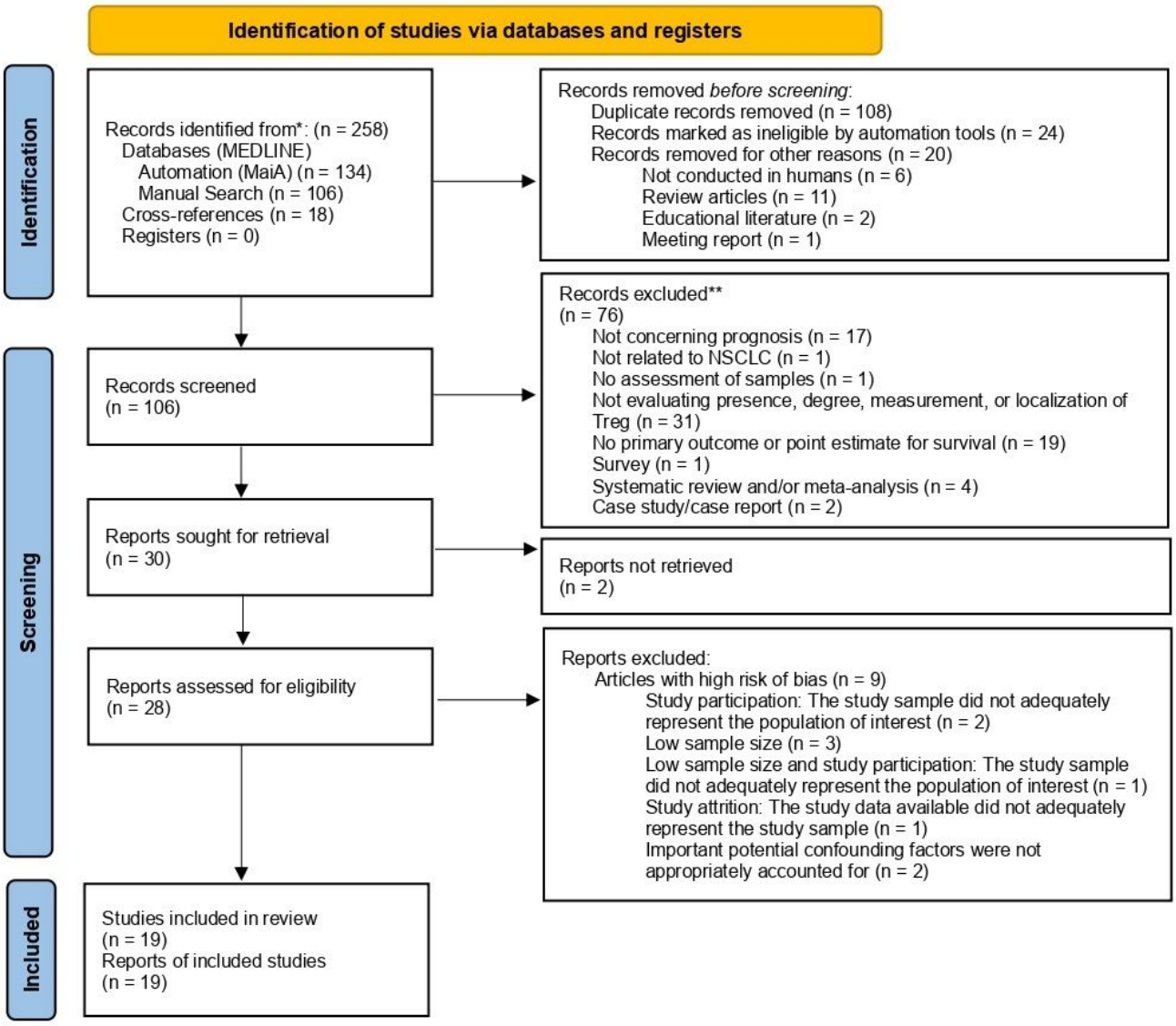
Figures



NSCLC, non-small cell lung cancer.

Figure 1

PRISMA Diagram: Flow of the studies through the review process

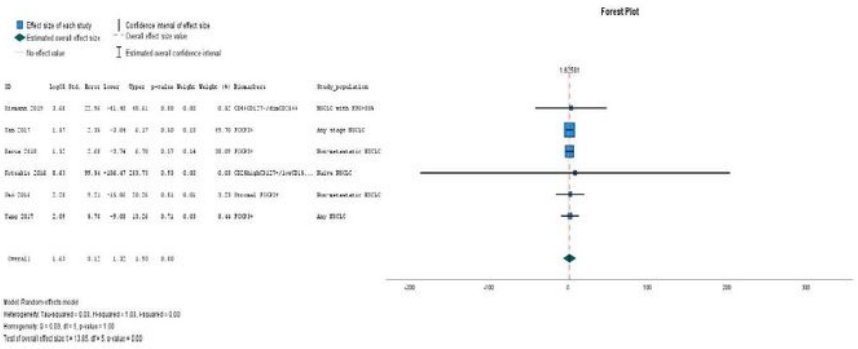


Fig.2a

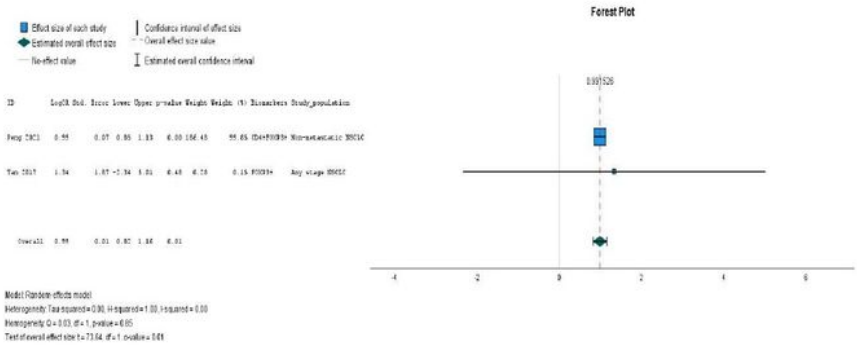


Fig.2b

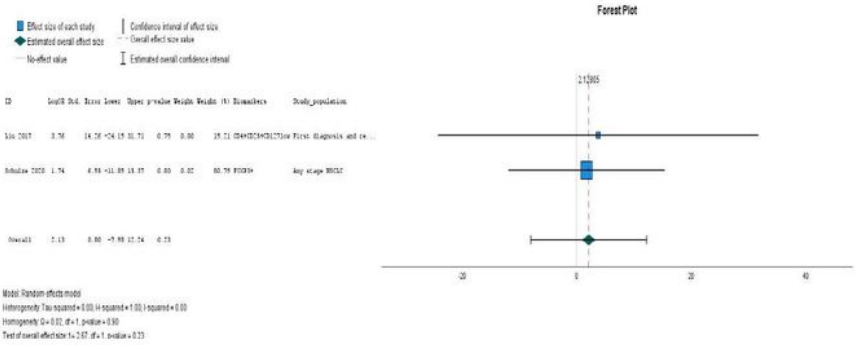


Fig.2c

Figure 2

Forest plots generated by meta-analysis

2a Forest plot for overall survival

2b Forest plot for disease-free survival

2c Forest plot for progression-free survival

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.StudywisePFOTable.xlsx
- SupplementaryFig1.docx
- PRISMA2020checklist.docx