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THE UTILITY OF KI-67 AS A PROGNOSTIC BIOMARKER IN PULMONARY NEUROENDOCRINE NEOPLASMS: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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ABSTRACT

Objectives: Ki-67, a marker of cellular proliferation, is associated with prognosis across a wide range of tumours including gastroenteropancreatic neuroendocrine neoplasms, lymphoma, urothelial tumours and breast carcinomas. Its omission from the classification system of pulmonary neuroendocrine neoplasms is controversial. This systematic review sought to assess whether Ki-67 is a prognostic biomarker in lung neuroendocrine neoplasms (NENs) and if feasible, proceed to a meta-analysis.

Research Design and Methods: Medline (Ovid), Embase, Scopus and the Cochrane library were searched for studies published prior to 28 February 2019 and investigating the role of Ki-67 in lung NENs. Eligible studies were those that included more than 20 patients and provided details of survival outcomes, namely hazard ratios with confidence intervals according to Ki-67 percentage. Studies not available as a full text or without an English manuscript were excluded. This study was prospectively registered with PROSPERO, number CRD42018093389.

Results: Of 11814 records identified, 7 studies met the inclusion criteria. These retrospective studies provided data for 1268 patients (693 TC, 281 AC, 94 LCNEC and 190 SCLC) and a meta-analysis was carried out to estimate a pooled effect. Random effects analyses demonstrated an association between a high Ki-67 index and poorer overall survival (HR of 2.02, 95% CI 1.16 – 3.52) and recurrence free survival (HR 1.42; 95% CI 1.01-2.00).

Conclusion: This meta-analysis provides evidence that high Ki-67 labelling indices correlate with poor clinical outcomes for patients diagnosed with pulmonary NENs. This study is subject to inherent limitations, but it does provide valuable insights regarding the use of the biomarker Ki-67, in a rare tumour.

Prospero registration: CRD42018093389

Strengths and Limitations of this study

This systematic review and meta-analysis provides a comprehensive synopsis of the literature published up to February 2020.

The protocol adheres to PRISMA guidelines, and was published in the *BMJ Open* ensuring transparency.

Heterogeneity in methodologies, diverse cohort sizes and types and variety of endpoints considered may limit comparison across studies.

INTRODUCTION

Bronchopulmonary neuroendocrine neoplasms (NENs) encompass a rare group of malignancies which exhibit considerable diversity and behave in an extremely heterogenous manner. Derived from pulmonary enterochromaffin (peptide and amine producing) neuroendocrine cells, lung NENs are classified through a combination of morphological neuroendocrine characteristics together with additional histological parameters by the 2015 World Health Organisation (WHO) classification.(1) This classification separates pulmonary NENs into four distinct groups ranging from typical and atypical carcinoids to large cell neuroendocrine carcinomas (LCNECs) and small cell lung carcinomas (SCLCs). Typical carcinoids (TC) are well differentiated, slow growing, indolent tumours which rarely metastasize. By way of contrast, SCLCs are aggressive, poorly differentiated tumours which have frequently metastasized at the point of presentation. Clinical outcomes are also markedly different; the 10-year survival for TCs is reported to be 82-87%, whilst the prognosis for untreated metastatic small cell lung cancer is 6-12 weeks.(2–4)

Originally identified in the 1980s by Gerdes *et al*, the DNA binding nuclear protein, Ki-67, is expressed during all phases of the cell cycle barring the rest phase (G_0).(5) *MKI67,* the gene which encodes the Ki-67 protein is located on chromosome 10q26.(6) Whilst a number of studies have implicated Ki-67 in ribosomal RNA synthesis, its exact function remains elusive. Nevertheless, in the setting of malignancy, Ki-67 has become established as a robust biomarker of cellular proliferation given its characteristic property of being rapid degradation during anaphase and telophase with a short half life of 1 to 1.5 hours.

Ki-67 is most frequently evaluated immunohistochemically on paraffin sections using the MIB-1 antibody. Scoring is generally formulated by the percentage of tumour cells stained positively to the antigen (also known as the labelling index). Several methods are available to evaluate the Ki-67 labelling index (LI), including digital image analysis, eyeball estimation and manual counting. The method currently considered 'gold standard' is to evaluate the area with the most dense Ki-67 staining (i.e. histological 'hotspots') and to subsequently manually count a minimum of 500 cells, with best practice being to count 2000 cells or $2mm^2$.(7,8) Manual counting is subject to limitations - not only can it become tedious, but it is time-consuming as counting 2000 cells can take approximately 40 minutes to complete. Utilising camera captured printed images reduces issues with inter-observer variability, although the issue of intra-tumoural heterogeneity remains as selecting which tumour area will be subjected to counting can be difficult to establish with consistency.(9) Therefore, some pathologists resort to eyeball estimations, resulting in poor reproducibility and inter-observer variability relating to the pathologists experience.(10) Digital image analysis has been heralded as a means of deriving uniformity, but it is not currently widely employed as a result of a number of obstacles including technical

issues (e.g. overcounting unwanted cells and underestimating negative cells) as well as its current lack of worldwide availability.

Across multiple tumour sites, numerous studies have determined an association between the Ki-67 LI and patient survival.(11–15) Furthermore, evidence in other solid tumours suggests that Ki-67 is also a useful predictive biomarker, predicting response to treatment such as chemotherapy; in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) it assists oncologists to determine how best to sequence treatments for patients.

Pulmonary NENs are classified on the basis of morphological characteristics including mitotic activity and the presence or absence of necrosis (2015 WHO classification). As outlined above, they are stratified into the well differentiated NETs (TC and atypical carcinoids [ACs]) and the poorly differentiated NECs (LCNECs and SCLCs). Despite each of these subtypes being endowed with behavioural heterogeneity, these tumours are not further sub-categorised according to tumour grade.(16) This places pulmonary NENs at odds with GEP-NENs where the Ki-67 index together with the mitotic rate and necrosis are important considerations when determining the grade of disease and also significantly influences how therapies are sequenced. The updated 2017 WHO classification of pancreatic NENs has progressed further, by formally recognising the heterogeneity of well differentiated NETs - a well-differentiated grade 3 NET group has been included for the first time.(17)

Whilst a number of studies have been conducted to examine the prognostic utility of Ki-67 in pulmonary NENs, its omission from the pulmonary NEN classification system remains controversial. No consensus has been established for the routine use of Ki-67 in pulmonary NENs. Nevertheless, oncologists continue to request this in the belief that this marker is predictive and/or prognostic.(18) Therefore, the primary aim of this systematic review and meta-analysis is to determine whether existing evidence supports or refutes the use of Ki-67 as a prognostic biomarker in pulmonary NENs.

METHODS

This study was prospectively registered with the International Prospective Register of Systematic Reviews (PROSPERO) website (registration number CRD42018093389) following the production of a protocol in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A copy of the PRISMA protocol is also available via the *BMJ Open*.(19)

Search strategy and selection criteria

A systematic review was conducted evaluating the prognostic relevance of the Ki-67 LI in patients with bronchopulmonary NENs. MEDLINE Ovid, Embase, the Cochrane Library and Scopus were searched to look for relevant studies published from the inception of each database to 28 February 2019. The following search terms were employed: “Ki-67”, “mib-1”, “neuroendocrine tumor”, “carcinoid” and “small cell lung carcinoma”. References of articles included in the analysis were also screened to ensure a complete dataset was available for review.

To be eligible, studies had to provide details of prognostic outcomes (hazard ratios with confidence intervals or 5-year overall survival) in more than 20 subjects with pulmonary NENs according to Ki-67 LI. Studies which did not provide sufficient prognostic details for the pulmonary NEN cohort, studies not published in English, or not available as a full manuscript were excluded. Articles which contained only predictive outcomes were also excluded.

Two independent reviewers (SN and CH) screened the title and abstracts against the pre-defined eligibility criteria independently of each other. Where discrepancies arose, a third reviewer (GP) served as arbitrator and a collective decision was then reached. Data from the studies was extracted (SN) and reviewed (GP).

Data analysis

For each study included in the meta-analysis, the following study characteristics were extracted wherever possible: first author, year of publication, country where the study was carried out, study design, number of patients, histological subtypes, mean age, disease stage, gender distribution, length of follow-up and methodology for calculating Ki-67. Hazard ratios (HR) with 95% confidence intervals (CIs) were sought as the primary outcome measure from each study in terms of overall survival (OS), disease free survival (DFS) and recurrence free survival (RFS). Secondary outcomes for each study were five year survival rates.

The Newcastle-Ottawa Scale (as recommended by the Cochrane Non-Randomised Studies Methods Working Group) was utilised to appraise the quality of studies eligible for meta-analysis.(20) This involved appraising the selection, comparability and outcome of each study with scores ranging from 0 to 9. Scores of 0-3 indicate a low quality study, 4-5 and 6-9 are considered medium and high quality respectively. Only medium and high quality studies were considered for inclusion in the meta-analysis.

Statistical analysis

The statistical analyses were performed using the RevMan 5.3 software (Cochrane Collaboration, Copenhagen, Denmark). The generic inverse variance model was employed to pool and weight hazard

ratios. In order to assess the heterogeneity of results across studies, a pooled hazard ratio was ascertained using Higgins I^2 statistic. Where there was evidence of high levels of heterogeneity (i.e. I^2 > 50%), a random effects model was utilised. It was intended to assess the risk of bias using funnel-plot visual inspections together with Begg's and Egger's test.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

The database searches identified 11814 publications. Following the exclusion of duplicates, 8057 studies remained. 8008 articles were excluded following initial screening of titles and abstracts. The remaining 49 articles were retrieved for full text review. 42 further articles were excluded, with the main reason for exclusion being insufficient prognostic data to facilitate a meta-analysis. A flowchart of the study selection process is shown in Figure 1. Although the planned protocol had intended to also capture 5 year survival data, due to the heterogeneity of Ki-67 cut-offs utilised and data presentation via Kaplan Meier curves, it was not possible to present this in a meaningful way.

Study characteristics and quality evaluation

Seven papers, published between 2013 and 2018 including 1268 patients (693 TC, 281 AC, 94 LCNEC and 190 SCLC) fulfilled the inclusion criteria for meta-analysis.(21–27) All included studies were retrospective and observational in nature, with no prospective studies identified. The cohort sizes varied between 82 and 399 subjects. Only one study (Rindi *et al*) was inclusive of the full range of pulmonary NENs with most studies only including the well-differentiated NETs (typical and atypical carcinoids). Four of the studies included Italian cohorts, with France, Brazil, Finland and the UK each contributing a single study. The majority of studies used the MIB-1 antibody (4 of 7), although not all studies provided this information.

The majority (76.8%) of the patients had well-differentiated tumours (either in the form TC or AC) with only a minority (23.1%) having poorly differentiated NECs. 51% of the participants were female. The age range of participants varied between 15 and 83 years with one study failing to provide this information. Three studies did not report data for tumour stage. Across the remaining four studies, the majority of participants were noted to have early stage disease (54.3% of patients had stage I disease,

15.5% stage II, 14.2% stage III, 13.8% stage IV, 4.2% stage X). Median length of follow-up ranged between 9.6 and 70 months. The population characteristics of studies included in the meta-analysis are summarised in Table 1.

Quality evaluation revealed that the studies included in the meta-analysis were of an overall good quality. The median Newcastle-Ottawa Scale score was seven, with three papers scoring seven and eight each and one scoring six [Table 2]. Five and three studies made hazard ratio and confidence interval data available for OS and RFS respectively.

Meta-analysis of overall survival

In the meta-analysis of overall survival, five studies were included (Cusumano *et al* published a death HR, whilst Vesterinen *et al* offered a HR for disease specific mortality – both were deemed to be surrogate markers of OS).(21,22) Hazard ratios derived from univariate analyses were considered for meta-analysis over their multivariate counterparts in an effort to limit the heterogeneity resulting from how hazard ratios are derivation. The pooled HR for Ki-67 was 2.02 (95% CI 1.16 – 3.52) with a p-value of 0.01 [Figure 2]. The heterogeneity was high: I^2 = 69%. This necessitated use of a random effects model.

Meta-analysis of recurrence free survival

In the meta-analysis of recurrence free survival (RFS), three studies were available (in one recurrence HR was available whilst a second study provided a time to progression HR – both were considered to be surrogate markers of RFS). The pooled HR was 1.42 (95% CI 1.01-2.00; p 0.04) [Figure 3]. Once again, the heterogeneity was high (I^2 = 89%) and therefore a random effects model was appropriate.

Risk of bias

Despite the intention to assess the risk of bias using funnel-plot visual inspections, Begg's and Egger's test, this was not feasible due to the low number of studies included in the meta-analysis.

DISCUSSION

Prognostic biomarkers and tools play an important role in oncological management and decision making processes. In pulmonary NENs the dearth of prognostic biomarkers is notable and therefore oncologists often request Ki-67 indices in order to assist in therapeutic decisions despite the fact this has not been

formally adopted. The primary aim of this study was to evaluate whether existing Ki-67 LI is associated with prognosis in pulmonary NENs as has been demonstrated in numerous other tumour types (e.g. urothelial carcinomas, breast cancer, lymphoma and lung cancer).

This meta-analysis provides tentative evidence demonstrating that high Ki-67 indices are associated with a 40% greater risk of recurrence amongst patients diagnosed with pulmonary NENs. This risk appears to be further exaggerated when considering overall survival where patients with a high Ki-67 have double the risk of death in comparison with patients with a lower Ki-67 LI. The strength of the association between Ki-67 LI and prognosis was only evaluated in studies that calculated hazard ratios using univariate analyses. As a result, no attempt has been made to account for confounding factors (such as stage, grade, and mitotic index).

One of the major pitfalls of including Ki-67 in the classification of pulmonary NENs has in establishing the most appropriate thresholds or cut-offs that should be utilised when grading tumours. In the main, Ki-67 has not been used as a linear biomarker within the whole pulmonary NEN cohort, instead focusing on its utility within each categorical histological subtype. Whilst categorising NENs by grade is helpful in establishing management plans, it is likely that proliferative markers (such as Ki-67 and mitotic index) are continuous rather than categorical variables. Therefore, there may not be a single or absolute optimal cut off value to categorise tumours into distinct entities and a pragmatic approach is likely to be needed. In order to facilitate clinical clarity, it would be preferable to use the same thresholds as are utilised in GEP-NETs and any future studies should attempt to clarify this further. However, it is unclear whether attempting to implement a similar grading system in pulmonary NENs as GEP NENs does a disservice to the fundamental biological diversity between the two different tumour sites.

As with all studies, this meta-analysis is also subject to inherent limitations. None of the studies included in the meta-analyses were prospective in design; retrospective analyses are prone to error through issues with selection bias and reporting. Secondly, studies with a variety of endpoints (e.g. RFS analyses included studies where the endpoint was DFS and time to progression analyses etc.), diverse cohort sizes, differences in the dilution of the primary antibody as well as variable Ki-67 cut-offs have all been amalgamated. Whilst some degree of heterogeneity is always to be expected, it diminishes the validity of the combined data-set and subsequent results. This is reflected in the I^2 statistics noted across both meta-analyses.

This study also preferentially utilised univariate analyses. Whilst multivariate analyses can be significantly distorted by differing in their approach to modelling or prognostic factors, univariate analyses fail to account for confounding variables. Furthermore, given the small number of studies identified as suitable for inclusion in this meta-analysis, it is clear that future international multi-centre

efforts are needed to develop studies which are prospective with large cohorts to clarify whether Ki-67 labelling index is truly a prognostic biomarker in the setting of bronchopulmonary neuroendocrine neoplasms.

CONCLUSIONS

Although it is difficult to draw definitive conclusions, this meta-analysis of over 1250 patients with pulmonary NENs indicates that a high Ki-67 LI correlates with an adverse prognosis. Whilst these findings are subject to a number of limitations, they provide a valuable insight into a rare tumour and should be considered when producing new guidelines regarding the use of Ki-67 in pulmonary NENs.

Conflict of interest statement: The authors have declared no competing interests.

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Patient and public involvement: Patients and the public were not involved in the development of this systematic review or meta-analysis.

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Author (Year)	Trial Design (study centres)	Number of Subjects	Histological subtypes	Age Range	Gender M:F	Stage	Antibody	Methodology for calculating Ki-67	Median length of follow-up (months)	Ki-67 cut-off thresholds (%)	Outcome measure	Hazard Ratio (95% CI) from univariate analyses
Cusumano et al (2017)	Retrospective multicentre study (France, Italy)	195	TC (159); AC (36)	52.94 (TC); 60.16 (AC) [mean values]	89:106	I = 163; II = 16; III = 16; IV = 0	NR	NR	75 (mean)	NR	OS (Death HR) DFS	1.07 (0.97-1.17) 0.97 (1.01–1.2)
Rego et al (2017)	Retrospective, multicentre study (Brazil)	82	SCLC (82)	35-81 (mean 59)	48:34	I = 0; II = 0; III = 6; IV = 76	MIB-1 (1:1000)	Hotspot method; otherwise not specified	10.3	55	OS	1.15 (0.70-1.89)
Marchio et al (2017)	Retrospective multicentre study (Italy)	239	TC (171); AC (68)	NR	100:139	NR	NR	Manual counting of >1000 cells	NR	4	OS TTP	4.31 (1.624-11.45) 3.994 (1.58-10)
Filosso et al (2013)	Retrospective, single centre study (Italy)	126 [NB 110 included in Ki-67 analysis]	TC (83); AC (43). [In Ki-67 analysis TC (79); AC (31)]	15-82 (mean 60)	52:74	I = 90; II = 18; III = 16; IV = 2; X = 1	anti–Ki-67 antibody (DAKO) not further specified	NR	60	6	OS	2.08 (1.02-4.27)
Rindi et al (2014)	Retrospective, multicentre study (Italy)	399	TC (113); AC (84); LCNEC (94); SCLC (108)	63.26 (median)	245:154	I = 183; II = 90; III = 76; IV = 17; X = 33	MIB-1 antibody	Computer assisted manual count method 500-2000 cells	70.7	<4 vs 4-20	OS	1.26 (0.84-1.89)
Clay et al (2017)	Retrospective, single centre study (UK)	94 [NB survival analysis performed on 84]	TC (75); AC (19) [NB survival analysis performed 67 TC, 17 AC patients]	21-83 (median 60.5)	39:55	NR	MIB-1 antibody (1:50)	Manual count method 500-2000 cells in hot spot	35	NR	RFS	1.47 (1.25-1.74)
Vesterinen et al (2018)	Retrospective, single centre study (Finland)	133 (129 included in Ki-67 analysis)	TC (100); AC (33)		47:86	NR	MIB-1 antibody (1:100)	Manual and automated counting of 2000 cells	9.6	2.5	Disease specific mortality	10.51 (2.12-52.13)

Table 1: Main Characteristics of all studies included in the meta-analysis.

NR = not recorded, OS = overall survival, RFS = recurrence free survival, DFS = disease free survival, TTP = time to progression.

Author (Year)	Representativeness of cohort (1 point)	Adequate definition of cases (1 point)	Assessment of exposure (1 point)	Outcome of interest not present at start of study (1 point)	Comparibility on the basis of the design or analysis (2 points)	Assessment of Outcome (death or recurrence) (1 point)	Adequacy of median follow-up for outcome (>2 years) (1 point)	Adequacy of follow-up of cases (<20% or reported) (1 point)	Total Quality Score
Cusumano et al (2017)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Rego et al (2017)	☆	☆	☆	-	☆	☆	☆	☆	6
Marchio et al (2017)	☆	☆	☆	-	☆☆	☆	☆	☆	7
Filosso et al (2013)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Rindi et al (2014)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Clay et al (2017)	☆	☆	☆	-	☆	☆	☆	☆	7
Vesterinen et al (2018)	☆	☆	☆	-	☆☆	☆		☆	7

Table 2: Quality assessment of included studies utilising the (modified) Newcastle-Ottawa Scale.

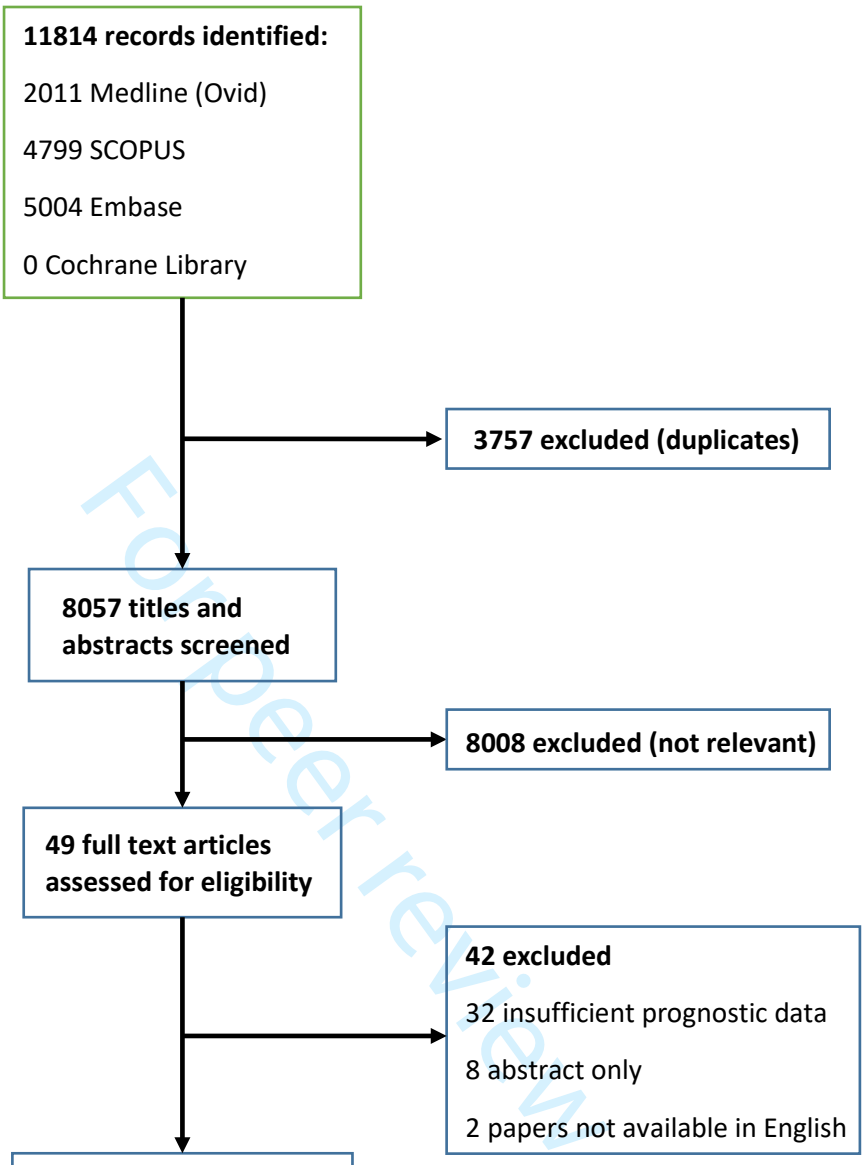

Figure 1: Study Selection

Study or Subgroup	log[Hazard Ratio]	SE	Total	Total	Weight	Hazard Ratio IV, Random, 95% CI	Year
Filosso 2013	0.7324	0.3636	0	0	21.2%	2.08 [1.02, 4.24]	2013
Rindi 2014	0.2311	0.2069	0	0	27.8%	1.26 [0.84, 1.89]	2014
Rego 2017	0.1398	0.2533	0	0	25.9%	1.15 [0.70, 1.89]	2017
Marchio 2017	1.4609	0.498	0	0	16.3%	4.31 [1.62, 11.44]	2017
Vesterinen 2018	2.3523	0.8168	0	0	8.8%	10.51 [2.12, 52.10]	2018
Total (95% CI)			**0**	**0**	**100.0%**	**2.02 [1.16, 3.52]**	

Heterogeneity: Tau² = 0.25; Chi² = 12.75, df = 4 (P = 0.01); I² = 69%

Test for overall effect: Z = 2.47 (P = 0.01)

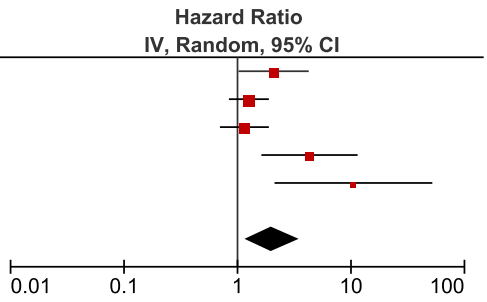

Figure 2: Forest plot of studies evaluating the association between Ki-67 expression and overall survival in pulmonary neuroendocrine neoplasms.

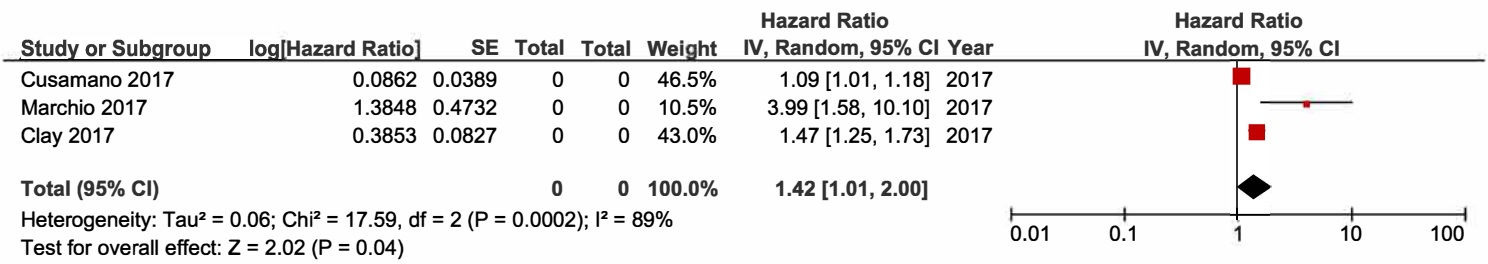

Figure 3: Forest plot of studies evaluating the association between Ki-67 expression and disease free survival in pulmonary neuroendocrine neoplasms.

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5-6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5-6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5-6

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5-6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5-6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6-7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	6-7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	7
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	7-8
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	7-8
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	6

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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THE UTILITY OF KI-67 AS A PROGNOSTIC BIOMARKER IN PULMONARY NEUROENDOCRINE NEOPLASMS: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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THE UTILITY OF KI-67 AS A PROGNOSTIC BIOMARKER IN PULMONARY NEUROENDOCRINE NEOPLASMS: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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Word Count: 2915

ABSTRACT

Objectives: Ki-67, a marker of cellular proliferation, is associated with prognosis across a wide range of tumours including gastroenteropancreatic neuroendocrine neoplasms, lymphoma, urothelial tumours and breast carcinomas. Its omission from the classification system of pulmonary neuroendocrine neoplasms is controversial. This systematic review sought to assess whether Ki-67 is a prognostic biomarker in lung neuroendocrine neoplasms (NENs) and if feasible, proceed to a meta-analysis.

Research Design and Methods: Medline (Ovid), Embase, Scopus and the Cochrane library were searched for studies published prior to 28 February 2019 and investigating the role of Ki-67 in lung NENs. Eligible studies were those that included more than 20 patients and provided details of survival outcomes, namely hazard ratios with confidence intervals according to Ki-67 percentage. Studies not available as a full text or without an English manuscript were excluded. This study was prospectively registered with PROSPERO, number CRD42018093389.

Results: Of 11814 records identified, 7 studies met the inclusion criteria. These retrospective studies provided data for 1268 patients (693 TC, 281 AC, 94 LCNEC and 190 SCLC) and a meta-analysis was carried out to estimate a pooled effect. Random effects analyses demonstrated an association between a high Ki-67 index and poorer overall survival (HR of 2.02, 95% CI 1.16 – 3.52) and recurrence free survival (HR 1.42; 95% CI 1.01-2.00).

Conclusion: This meta-analysis provides evidence that high Ki-67 labelling indices are associated with poor clinical outcomes for patients diagnosed with pulmonary NENs. This study is subject to inherent limitations, but it does provide valuable insights regarding the use of the biomarker Ki-67, in a rare tumour.

Prospero registration: CRD42018093389

Strengths and Limitations of this study

This systematic review and meta-analysis provides a comprehensive synopsis of the literature published up to February 2019.

The protocol adheres to PRISMA guidelines, and was published in the *BMJ Open* ensuring transparency.

Heterogeneity in methodologies, diverse cohort sizes and types and variety of endpoints considered may limit comparison across studies.

INTRODUCTION

Bronchopulmonary neuroendocrine neoplasms (NENs) encompass a rare group of malignancies which exhibit considerable diversity and behave in an extremely heterogenous manner. Pulmonary NENs are classified through a combination of morphological neuroendocrine characteristics together with additional histological parameters by the 2015 World Health Organisation (WHO) classification.(1) This classification separates pulmonary NENs into four distinct groups ranging from typical and atypical carcinoids to large cell neuroendocrine carcinomas (LCNECs) and small cell lung carcinomas (SCLCs). Typical carcinoids (TC) are well differentiated, slow growing, indolent tumours which rarely metastasize. By way of contrast, SCLCs are aggressive, poorly differentiated tumours which have frequently metastasized at the point of presentation. Clinical outcomes are also markedly different; the 10-year survival for TCs is reported to be 82-87%, whilst the prognosis for untreated metastatic small cell lung cancer is 6-12 weeks.(2–4)

Originally identified in the 1980s by Gerdes *et al*, the DNA binding nuclear protein, Ki-67, is expressed during all phases of the cell cycle barring the rest phase (G_0).(5) *MKI67,* the gene which encodes the Ki-67 protein is located on chromosome 10q26.(6) Whilst a number of studies initially implicated Ki-67 in ribosomal RNA synthesis, more recent evidence suggests its main role is as a biological surfactant to disperse mitotic chromosomes.(7) In the setting of malignancy, Ki-67 has become established as a robust biomarker of cellular proliferation given its characteristic property of being rapidly degraded during anaphase and telophase with a short half life of 1 to 1.5 hours. Across multiple tumour sites, numerous studies have determined an association between the Ki-67 LI and patient survival.(8–12) Furthermore, evidence in other solid tumours suggests that Ki-67 is also a useful predictive biomarker, predicting response to treatment such as chemotherapy; in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) Ki-67 LI is not only integral to grading and classification but subsequently also assists oncologists to determine how best to sequence treatments for patients.

Pulmonary NENs are classified on the basis of morphological characteristics including mitotic activity and the presence or absence of necrosis (2015 WHO classification). As outlined above, they are stratified into the well differentiated NETs (TC and atypical carcinoids [ACs]) and the poorly differentiated NECs (LCNECs and SCLCs). Despite each of these subtypes being endowed with behavioural heterogeneity, these tumours are not further sub-categorised according to tumour grade.(13) This places pulmonary NENs at odds with GEP-NENs where the Ki-67 index together with the mitotic rate are important considerations when determining the grade of disease and also significantly influences how therapies are sequenced. The updated 2019 WHO classification of digestiveNENs has progressed further, by formally recognising the heterogeneity of well differentiated NETs - a well-differentiated grade 3 NET group has been included for the first time.(14)

Whilst a number of studies have been conducted to examine the prognostic utility of Ki-67 in pulmonary NENs, its omission from the pulmonary NEN classification system remains controversial. No consensus has been established for the routine use of Ki-67 in pulmonary NENs. Nevertheless, oncologists continue to request this in the belief that this marker is predictive and/or prognostic.(15) Therefore, the primary aim of this systematic review and meta-analysis is to determine whether existing evidence supports or refutes the use of Ki-67 as a prognostic biomarker in pulmonary NENs.

METHODS

This study was prospectively registered with the International Prospective Register of Systematic Reviews (PROSPERO) website (registration number CRD42018093389) following the production of a protocol in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A copy of the PRISMA protocol is also available via the *BMJ Open*.(16)

Search strategy and selection criteria

A systematic review was conducted evaluating the prognostic relevance of the Ki-67 LI in patients with bronchopulmonary NENs. MEDLINE Ovid, Embase, the Cochrane Library and Scopus were searched to look for relevant studies published from the inception of each database to 28 February 2019. The following search terms were employed: “Ki-67”, “mib-1”, “neuroendocrine tumor”, “carcinoid” and “small cell lung carcinoma”. References of articles included in the analysis were also screened to ensure a complete dataset was available for review. An example of the full search strategy is available in Supplementary File 1.

To be eligible, studies had to provide details of prognostic outcomes (hazard ratios with confidence intervals or 5-year overall survival) in more than 20 subjects with pulmonary NENs according to Ki-67 LI. Studies which did not provide sufficient prognostic details for the pulmonary NEN cohort, studies not published in English, or not available as a full manuscript were excluded. Articles which contained only predictive outcomes were also excluded.

Two independent reviewers (SN and CH) screened the title and abstracts against the pre-defined eligibility criteria independently of each other. Where discrepancies arose, a third reviewer (GP) served as arbitrator and a collective decision was then reached. Data from the studies was extracted (SN) and reviewed (GP).

Data analysis

For each study included in the meta-analysis, the following study characteristics were extracted wherever possible: first author, year of publication, country where the study was carried out, study design, number of patients, histological subtypes, mean age, disease stage, gender distribution, length of follow-up and methodology for calculating Ki-67. Hazard ratios (HR) with 95% confidence intervals (CIs) were sought as the primary outcome measure from each study in terms of overall survival (OS), disease free survival (DFS) and recurrence free survival (RFS). Secondary outcomes for each study were five year survival rates. Disease free survival denotes the length of time between primary treatment and first relapse, whereas recurrence free survival refers to the time between primary treatment and local or regional relapse.

The Newcastle-Ottawa Scale (as recommended by the Cochrane Non-Randomised Studies Methods Working Group) was utilised to appraise the quality of studies eligible for meta-analysis.(17) This involved appraising the selection, comparability and outcome of each study with scores ranging from 0 to 9. Scores of 0-3 indicate a low quality study, 4-5 and 6-9 are considered medium and high quality respectively. Only medium and high quality studies were considered for inclusion in the meta-analysis.

Statistical analysis

The statistical analyses were performed using the RevMan 5.3 software (Cochrane Collaboration, Copenhagen, Denmark). The generic inverse variance model was employed to pool and weight hazard ratios. In order to assess the heterogeneity of results between studies, Higgins I^2 statistic was used. Where there was evidence of high levels of heterogeneity (i.e. $I^2 > 50\%$), a random effects model was utilised. It was intended to assess the risk of bias using funnel-plot visual inspections together with Begg's and Egger's test.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

The database searches identified 11814 publications. Following the exclusion of duplicates, 8057 studies remained. 8008 articles were excluded following initial screening of titles and abstracts. The remaining 49 articles were retrieved for full text review. 42 further articles were excluded, with the main

reason for exclusion being insufficient prognostic data to facilitate a meta-analysis. A flowchart of the study selection process is shown in Figure 1. Although the planned protocol had intended to also capture 5 year survival data, due to the heterogeneity of Ki-67 cut-offs utilised and data presentation via Kaplan Meier curves, it was not possible to present this in a meaningful way. This was due to the 5 year survival estimates not being reported in all studies and could only be detected through Kaplan Meier plots.

Study characteristics and quality evaluation

Seven papers, published between 2013 and 2018 including 1268 patients (693 TC, 281 AC, 94 LCNEC and 190 SCLC) fulfilled the inclusion criteria for meta-analysis.(18–24) All included studies were retrospective and observational in nature, with no prospective studies identified. The cohort sizes varied between 82 and 399 subjects. Only one study (Rindi *et a*l) was inclusive of the full range of pulmonary NENs with most studies only including the well-differentiated NETs (typical and atypical carcinoids). Four of the studies included Italian cohorts, with France, Brazil, Finland and the UK each contributing a single study. The majority of studies used the MIB-1 antibody (4 of 7), although not all studies provided this information.

The majority (76.8%) of the patients had well-differentiated tumours (either in the form TC or AC) with only a minority (23.1%) having poorly differentiated NECs. 51% of the participants were female. The age range of participants varied between 15 and 83 years with one study failing to provide this information. Three studies did not report data for tumour stage. Across the remaining four studies, the majority of participants were noted to have early stage disease (54.3% of patients had stage I disease, 15.5% stage II, 14.2% stage III, 13.8% stage IV, 4.2% stage X). Median length of follow-up ranged between 9.6 and 70 months. The population characteristics of studies included in the meta-analysis are summarised in Table 1.

Quality evaluation revealed that the studies included in the meta-analysis were of an overall good quality. The median Newcastle-Ottawa Scale score was seven, with three papers scoring seven and eight each and one scoring six [Table 2]. Five and three studies made hazard ratio and confidence interval data available for OS and RFS respectively.

Meta-analysis of overall survival

In the meta-analysis of overall survival, five studies were included (Cusumano *et al* published a death HR, whilst Vesterinen *et al* offered a HR for disease specific mortality – both were deemed to be surrogate markers of OS).(17,18) Hazard ratios derived from univariate analyses were considered for meta-analysis over their multivariate counterparts in an effort to limit the heterogeneity resulting from

how hazard ratios are derived. The heterogeneity was high: I^2 = 69%. This necessitated use of a random effects model. The pooled HR for Ki-67 was 2.02 (95% CI 1.16 – 3.52) with a p-value of 0.01 [Figure 2].

Meta-analysis of recurrence free survival

In the meta-analysis of recurrence free survival (RFS), three studies were available (in one recurrence HR was available whilst a second study provided a time to progression HR – both were considered to be surrogate markers of RFS). Once again, the heterogeneity was high (I^2 = 89%) and therefore a random effects model was appropriate. The pooled HR was 1.42 (95% CI 1.01-2.00; p 0.04) [Figure 3].

Risk of bias

Despite the intention to assess the risk of bias using funnel-plot visual inspections, Begg's and Egger's test, this was not feasible due to the low number of studies included in the meta-analysis.

DISCUSSION

Prognostic biomarkers and tools play an important role in oncological management and decision making processes. In pulmonary NENs the dearth of prognostic biomarkers is notable and therefore oncologists often request Ki-67 indices in order to assist in therapeutic decisions despite the fact this has not been formally adopted. The primary aim of this study was to evaluate whether existing Ki-67 LI is associated with prognosis in pulmonary NENs as has been demonstrated in numerous other tumour types (e.g. GEP-NENs, urothelial carcinomas, breast cancer, lymphoma and lung cancer).

Ki-67 is most frequently evaluated immunohistochemically on paraffin sections using the MIB-1 antibody. Scoring is generally formulated by the percentage of tumour cells stained positively to the antigen (also known as the labelling index). Several methods are available to evaluate the Ki-67 labelling index (LI), including digital image analysis, eyeball estimation and manual counting. In digestive NENs, the method currently considered 'gold standard' is to evaluate the area with the most dense Ki-67 staining (i.e. histological 'hotspots') and to subsequently manually count a minimum of 500 cells, with best practice being to count 2000 cells or 2mm^2.(25,26) Manual counting is subject to limitations - not only can it become tedious, but it is time-consuming as counting 2000 cells can take approximately 40 minutes to complete. Utilising camera captured printed images reduces issues with inter-observer variability, although the issue of intra-tumoural heterogeneity remains as selecting which tumour area will be subjected to counting can be difficult to establish with consistency.(27) Therefore, some pathologists resort to eyeball estimations, resulting in poor reproducibility and inter-observer

variability relating to the pathologists experience.(28) Digital image analysis has been heralded as a means of deriving uniformity, but it is not currently widely employed as a result of a number of obstacles including technical issues (e.g. overcounting unwanted cells and underestimating negative cells) as well as its current lack of worldwide availability.

This meta-analysis provides tentative evidence demonstrating that high Ki-67 indices are associated with a 40% greater risk of recurrence amongst patients diagnosed with pulmonary NENs. This risk appears to be further exaggerated when considering overall survival where patients with a high Ki-67 have double the risk of death in comparison with patients with a lower Ki-67 LI. The strength of the association between Ki-67 LI and prognosis was only evaluated in studies that calculated hazard ratios using univariate analyses. As a result, no attempt has been made to account for confounding factors (such as stage, grade, and mitotic index).

One of the major pitfalls of including Ki-67 in the classification of pulmonary NENs has in establishing the most appropriate thresholds or cut-offs that should be utilised when grading tumours. In the main, Ki-67 has not been used as a linear biomarker within the whole pulmonary NEN cohort, instead focusing on its utility within each categorical histological subtype. Whilst categorising NENs by grade is helpful in establishing management plans, it is likely that proliferative markers (such as Ki-67 and mitotic index) are continuous rather than categorical variables. Therefore, there may not be a single or absolute optimal cut off value to categorise tumours into distinct entities and a pragmatic approach is likely to be needed. In order to facilitate clinical clarity, it would be preferable to use the same thresholds as are utilised in GEP-NETs and any future studies should attempt to clarify this further. However, it is unclear whether attempting to implement a similar grading system in pulmonary NENs as GEP NENs does a disservice to the fundamental biological diversity between the two different tumour sites.(29) Examples of this diversity include the variability of genetic alterations seen as well as the differing rates of associated syndromes and hormone expression . (30-35)

Unfortunately only two studies involving SCLC and high grade neuroendocrine carcinomas of the lung were available. It is important to clarify that Ki-67 is not likely to be useful in subtyping these tumours prognostically. A number of biomarkers have been identified which may have greater utility in these patients. Nevertheless further research into Ki-67 is required in these tumour groups with such little evidence, especially in light of the fact that in GEP-NENs there is good evidence to suggest that Ki-67 is contributory with a cut-off of 55%.(36)

As with all studies, this meta-analysis is also subject to inherent limitations. None of the studies included in the meta-analyses were prospective in design; retrospective analyses are prone to error through issues with selection bias and reporting. Secondly, studies with a variety of endpoints (e.g. RFS analyses included studies where the endpoint was DFS and time to progression analyses etc.), diverse

cohort sizes, differences in the dilution of the primary antibody as well as variable Ki-67 cut-offs have all been amalgamated. Whilst some degree of heterogeneity is always to be expected, it diminishes the validity of the combined data-set and subsequent results. This is reflected in the I^2 statistics noted across both meta-analyses.

This study also preferentially utilised univariate analyses. Whilst multivariate analyses can be significantly distorted by differing in their approach to modelling or prognostic factors, univariate analyses fail to account for confounding variables. Furthermore, given the small number of studies identified as suitable for inclusion in this meta-analysis, it is clear that future international multi-centre efforts are needed to develop studies which are prospective with large cohorts to clarify whether Ki-67 labelling index is truly a prognostic biomarker in the setting of bronchopulmonary neuroendocrine neoplasms.

CONCLUSIONS

Although it is difficult to draw definitive conclusions, this meta-analysis of over 1250 patients with pulmonary NENs indicates that a high Ki-67 LI is associated with an adverse prognosis. Whilst these findings are subject to a number of limitations, they provide a valuable insight into a rare tumour and should be considered when producing new guidelines regarding the use of Ki-67 in pulmonary NENs.

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Patient and public involvement: Patients and the public were not involved in the development of this systematic review or meta-analysis.

Data Availability Statement: All data relevant to the study are included in the article or uploaded as supplementary information.

Ethics Statement: Given that this is a systematic review of existing literature, ethical approval is not required.

Figure Legends:

Figure 1: Study Selection

Figure 2: Forest plot of studies evaluating the association between Ki-67 expression and overall survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval

Figure 3: Forest plot of studies evaluating the association between Ki-67 expression and recurrence free survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval.

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Author (Year)	Trial Design (study centres)	Number of Subjects	Histological subtypes	Age Range	Gender M:F	Stage	Antibody	Methodology for calculating Ki-67	Median length of follow-up (months)	Ki-67 cut-off thresholds (%)	Outcome measure	Hazard Ratio (95% CI) from univariate analyses
Cusumano et al (2017)	Retrospective multicentre study (France, Italy)	195	TC (159); AC (36)	52.94 (TC); 60.16 (AC) [mean values]	89:106	I = 163; II = 16; III = 16; IV = 0	NR	NR	75 (mean)	NR	OS (Death HR) DFS	1.07 (0.97-1.17) 0.97 (1.01–1.2)
Rego et al (2017)	Retrospective, multicentre study (Brazil)	82	SCLC (82)	35-81 (mean 59)	48:34	I = 0; II = 0; III = 6; IV = 76	MIB-1 (1:1000)	Hotspot method; otherwise not specified	10.3	55	OS	1.15 (0.70-1.89)
Marchio et al (2017)	Retrospective multicentre study (Italy)	239	TC (171); AC (68)	NR	100:139	NR	NR	Manual counting of >1000 cells	NR	4	OS TTP	4.31 (1.624-11.45) 3.994 (1.58-10)
Filosso et al (2013)	Retrospective, single centre study (Italy)	126 [NB 110 included in Ki-67 analysis]	TC (83); AC (43). [In Ki-67 analysis TC (79); AC (31)]	15-82 (mean 60)	52:74	I = 90; II = 18; III = 16; IV = 2; X = 1	anti–Ki-67 antibody (DAKO) not further specified	NR	60	6	OS	2.08 (1.02-4.27)
Rindi et al (2014)	Retrospective, multicentre study (Italy)	399	TC (113); AC (84); LCNEC (94); SCLC (108)	63.26 (median)	245:154	I = 183; II = 90; III = 76; IV = 17; X = 33	MIB-1 antibody	Computer assisted manual count method 500-2000 cells	70.72	<4 vs 4-20	OS	1.26 (0.84-1.89)
Clay et al (2017)	Retrospective, single centre study (UK)	94 [NB survival analysis performed on 84]	TC (75); AC (19) [NB survival analysis performed 67 TC, 17 AC patients]	21-83 (median 60.5)	39:55	NR	MIB-1 antibody (1:50)	Manual count method 500-2000 cells in hot spot	35	NR	RFS	1.47 (1.25-1.74)
Vesterinen et al (2018)	Retrospective, single centre study (Finland)	133 (129 included in Ki-67 analysis)	TC (100); AC (33)		47:86	NR	MIB-1 antibody (1:100)	Manual and automated counting of 2000 cells	9.6	2.5	Disease specific mortality	10.51 (2.12-52.13)

Table 1: Main Characteristics of all studies included in the meta-analysis.

M = male, F=female, NR = not recorded, OS = overall survival, RFS = recurrence free survival, DFS = disease free survival, TTP = time to progression.

Author (Year)	Representativeness of cohort (1 point)	Adequate definition of cases (1 point)	Assessment of exposure (1 point)	Outcome of interest not present at start of study (1 point)	Comparibility on the basis of the design or analysis (2 points)	Assessment of Outcome (death or recurrence) (1 point)	Adequacy of median follow-up for outcome (>2 years) (1 point)	Adequacy of follow-up of cases (<20% or reported) (1 point)	Total Quality Score
Cusumano et al (2017)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Rego et al (2017)	☆	☆	☆	-	☆	☆	-	☆	6
Marchio et al (2017)	☆	☆	☆	-	☆☆	☆	-	☆	7
Filosso et al (2013)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Rindi et al (2014)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Clay et al (2017)	☆	☆	☆	-	☆	☆	☆	☆	7
Vesterinen et al (2018)	☆	☆	☆	-	☆☆	☆	-	☆	7

Table 2: Quality assessment of included studies utilising the (modified) Newcastle-Ottawa Scale.

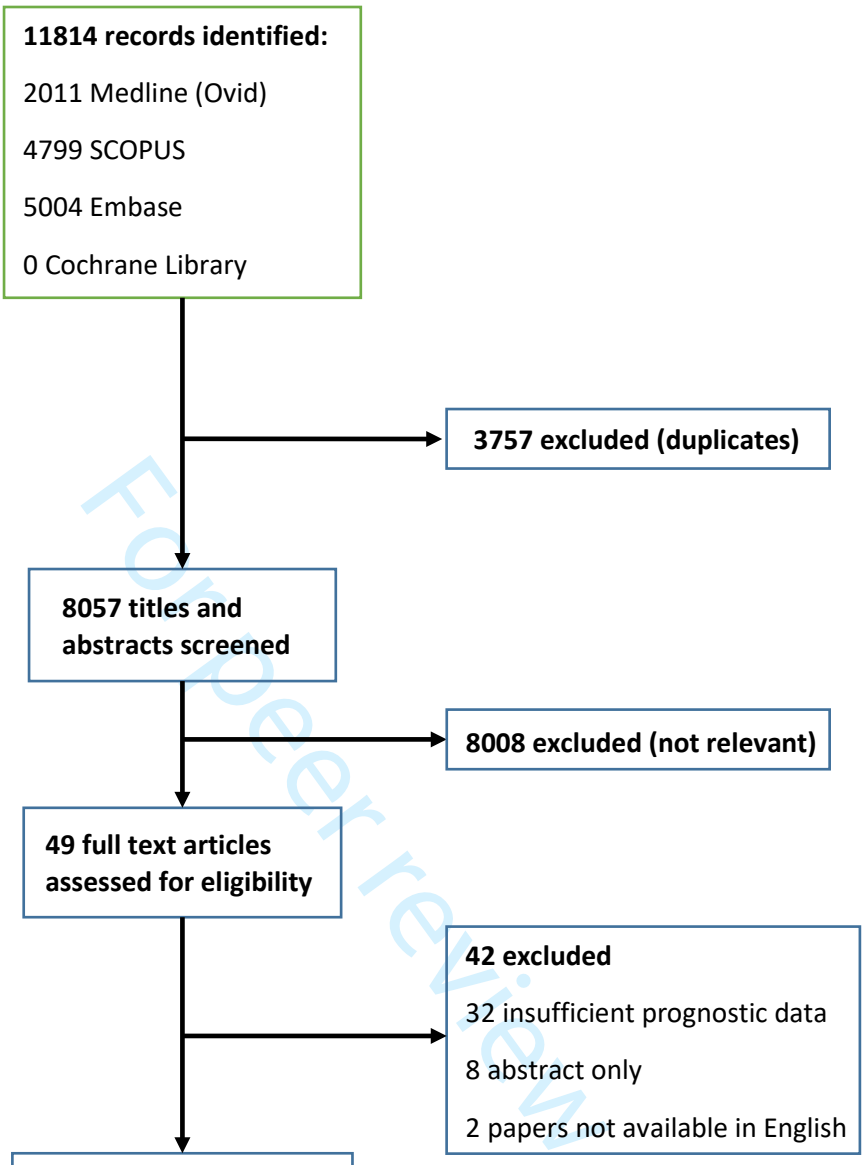

Figure 1: Study Selection

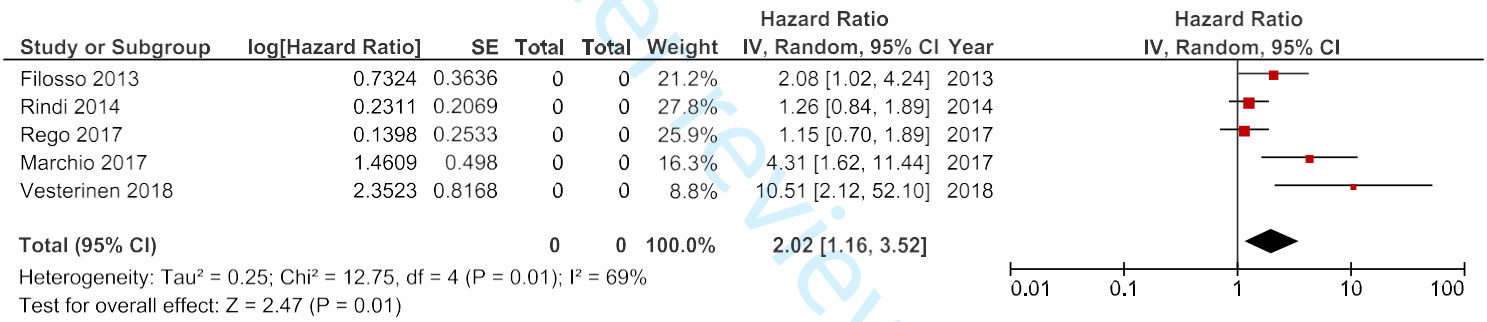

Figure 2: Forest plot of studies evaluating the association between Ki-67 expression and overall survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval

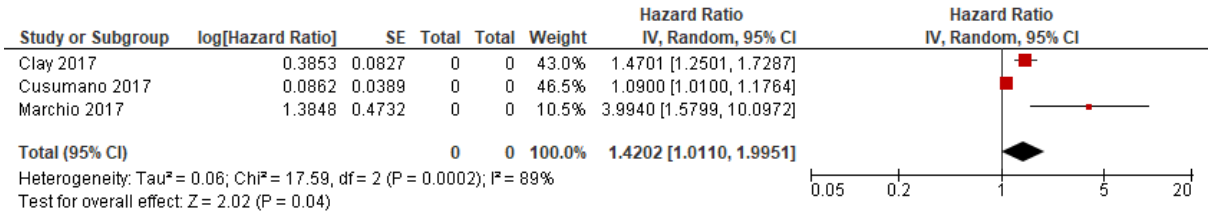

Figure 3: Forest plot of studies evaluating the association between Ki-67 expression and recurrence free survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval.

EXAMPLE OF FULL SEARCH STRATEGY

The systematic review will employ the following search terms for Medline OVID, and EMBASE:

Ki-67 antigen/ OR (Ki67 or Ki-67 or mib-1 or mib1).mp.

AND

neuroendocrine tumors/ or carcinoid tumor/ or carcinoma, neuroendocrine/ OR small cell lung carcinoma/ OR ((tumo?r* or neoplas* or carcinoma or cancer* or malignan*) adj3 (neuroendocrine or carcinoid or small cell)).mp.

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5-6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5-6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5-6

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5-6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5-6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6-7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	6-7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	7
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	7-8
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	7-8
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	6

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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THE UTILITY OF KI-67 AS A PROGNOSTIC BIOMARKER IN PULMONARY NEUROENDOCRINE NEOPLASMS: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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Secondary Subject Heading:	Oncology
Keywords:	Respiratory tract tumours < THORACIC MEDICINE, Pathology < NATURAL SCIENCE DISCIPLINES, Endocrine tumours < DIABETES & ENDOCRINOLOGY

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THE UTILITY OF KI-67 AS A PROGNOSTIC BIOMARKER IN PULMONARY NEUROENDOCRINE NEOPLASMS: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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Word Count: 2915

ABSTRACT

Objectives: Ki-67, a marker of cellular proliferation, is associated with prognosis across a wide range of tumours including gastroenteropancreatic neuroendocrine neoplasms, lymphoma, urothelial tumours and breast carcinomas. Its omission from the classification system of pulmonary neuroendocrine neoplasms is controversial. This systematic review sought to assess whether Ki-67 is a prognostic biomarker in lung neuroendocrine neoplasms (NENs) and if feasible, proceed to a meta-analysis.

Research Design and Methods: Medline (Ovid), Embase, Scopus and the Cochrane library were searched for studies published prior to 28 February 2019 and investigating the role of Ki-67 in lung NENs. Eligible studies were those that included more than 20 patients and provided details of survival outcomes, namely hazard ratios with confidence intervals according to Ki-67 percentage. Studies not available as a full text or without an English manuscript were excluded. This study was prospectively registered with PROSPERO, number CRD42018093389.

Results: Of 11814 records identified, 7 studies met the inclusion criteria. These retrospective studies provided data for 1268 patients (693 TC, 281 AC, 94 LCNEC and 190 SCLC) and a meta-analysis was carried out to estimate a pooled effect. Random effects analyses demonstrated an association between a high Ki-67 index and poorer overall survival (HR of 2.02, 95% CI 1.16 – 3.52) and recurrence free survival (HR 1.42; 95% CI 1.01-2.00).

Conclusion: This meta-analysis provides evidence that high Ki-67 labelling indices are associated with poor clinical outcomes for patients diagnosed with pulmonary NENs. This study is subject to inherent limitations, but it does provide valuable insights regarding the use of the biomarker Ki-67, in a rare tumour.

Prospero registration: CRD42018093389

Strengths and Limitations of this study

This systematic review and meta-analysis provides a comprehensive synopsis of the literature published up to February 2019.

The protocol adheres to PRISMA guidelines, and was published in the *BMJ Open* ensuring transparency.

Heterogeneity in methodologies, diverse cohort sizes and types and variety of endpoints considered may limit comparison across studies.

INTRODUCTION

Bronchopulmonary neuroendocrine neoplasms (NENs) encompass a group of malignancies which exhibit considerable diversity and behave in an extremely heterogenous manner. Pulmonary NENs are classified through a combination of morphological neuroendocrine characteristics together with additional histological parameters by the 2015 World Health Organisation (WHO) classification.(1) This classification separates pulmonary NENs into four distinct groups ranging from typical and atypical carcinoids to large cell neuroendocrine carcinomas (LCNECs) and small cell lung carcinomas (SCLCs). Typical carcinoids (TC) are well differentiated, slow growing, indolent tumours which rarely metastasize. By way of contrast, SCLCs are aggressive, poorly differentiated tumours which have frequently metastasized at the point of presentation. Clinical outcomes are also markedly different; the 10-year survival for TCs is reported to be 82-87%, whilst the prognosis for untreated metastatic small cell lung cancer is 6-12 weeks.(2–4)

Originally identified in the 1980s by Gerdes *et al*, the DNA binding nuclear protein, Ki-67, is expressed during all phases of the cell cycle barring the rest phase (G_0).(5) *MKI67,* the gene which encodes the Ki-67 protein is located on chromosome 10q26.(6) Whilst a number of studies initially implicated Ki-67 in ribosomal RNA synthesis, more recent evidence suggests its main role is as a biological surfactant to disperse mitotic chromosomes.(7) In the setting of malignancy, Ki-67 has become established as a robust biomarker of cellular proliferation given its characteristic property of being rapidly degraded during anaphase and telophase with a short half life of 1 to 1.5 hours. Across multiple tumour sites, numerous studies have determined an association between the Ki-67 LI and patient survival.(8–12) Furthermore, evidence in other solid tumours suggests that Ki-67 is also a useful predictive biomarker, predicting response to treatment such as chemotherapy; in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) Ki-67 LI is not only integral to grading and classification but subsequently also assists oncologists to determine how best to sequence treatments for patients.

Pulmonary NENs are classified on the basis of morphological characteristics including mitotic activity and the presence or absence of necrosis (2015 WHO classification). As outlined above, they are stratified into the well differentiated NETs (TC and atypical carcinoids [ACs]) and the poorly differentiated NECs (LCNECs and SCLCs). Despite each of these subtypes being endowed with behavioural heterogeneity, these tumours are not further sub-categorised according to tumour grade.(13) This places pulmonary NENs at odds with GEP-NENs where the Ki-67 index together with the mitotic rate are important considerations when determining the grade of disease and also significantly influences how therapies are sequenced. The updated 2019 WHO classification of digestive NENs has progressed further, by formally recognising the heterogeneity of grade 3 NENs - a well-differentiated grade 3 NET group has been included for the first time differentiated them from their poorlu differentiated counterparts.(14)

Whilst a number of studies have been conducted to examine the prognostic utility of Ki-67 in pulmonary NENs, its omission from the pulmonary NEN classification system remains controversial. No consensus has been established for the routine use of Ki-67 in pulmonary NENs. Nevertheless, oncologists continue to request this in the belief that this marker is predictive and/or prognostic.(15) Therefore, the primary aim of this systematic review and meta-analysis is to determine whether existing evidence supports or refutes the use of Ki-67 as a prognostic biomarker in pulmonary NENs.

METHODS

This study was prospectively registered with the International Prospective Register of Systematic Reviews (PROSPERO) website (registration number CRD42018093389) following the production of a protocol in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A copy of the PRISMA protocol is also available via the *BMJ Open*.(16)

Search strategy and selection criteria

A systematic review was conducted evaluating the prognostic relevance of the Ki-67 LI in patients with bronchopulmonary NENs. MEDLINE Ovid, Embase, the Cochrane Library and Scopus were searched to look for relevant studies published from the inception of each database to 28 February 2019. The following search terms were employed: “Ki-67”, “mib-1”, “neuroendocrine tumor”, “carcinoid” and “small cell lung carcinoma”. References of articles included in the analysis were also screened to ensure a complete dataset was available for review. An example of the full search strategy is available in Supplementary File 1.

To be eligible, studies had to provide details of prognostic outcomes (hazard ratios with confidence intervals or 5-year overall survival) in more than 20 subjects with pulmonary NENs according to Ki-67 LI. Studies which did not provide sufficient prognostic details for the pulmonary NEN cohort, studies not published in English, or not available as a full manuscript were excluded. Articles which contained only predictive outcomes were also excluded.

Two independent reviewers (SN and CH) screened the title and abstracts against the pre-defined eligibility criteria independently of each other. Where discrepancies arose, a third reviewer (GP) served as arbitrator and a collective decision was then reached. Data from the studies was extracted (SN) and reviewed (GP).

Data analysis

For each study included in the meta-analysis, the following study characteristics were extracted wherever possible: first author, year of publication, country where the study was carried out, study design, number of patients, histological subtypes, mean age, disease stage, gender distribution, length of follow-up and methodology for calculating Ki-67. Hazard ratios (HR) with 95% confidence intervals (CIs) were sought as the primary outcome measure from each study in terms of overall survival (OS), disease free survival (DFS) and recurrence free survival (RFS). Secondary outcomes for each study were five year survival rates. Disease free survival denotes the length of time between primary treatment and first relapse, whereas recurrence free survival refers to the time between primary treatment and local or regional relapse.

The Newcastle-Ottawa Scale (as recommended by the Cochrane Non-Randomised Studies Methods Working Group) was utilised to appraise the quality of studies eligible for meta-analysis.(17) This involved appraising the selection, comparability and outcome of each study with scores ranging from 0 to 9. Scores of 0-3 indicate a low quality study, 4-5 and 6-9 are considered medium and high quality respectively. Only medium and high quality studies were considered for inclusion in the meta-analysis.

Statistical analysis

The statistical analyses were performed using the RevMan 5.3 software (Cochrane Collaboration, Copenhagen, Denmark). The generic inverse variance model was employed to pool and weight hazard ratios. In order to assess the heterogeneity of results between studies, Higgins I^2 statistic was used. Where there was evidence of high levels of heterogeneity (i.e. $I^2 > 50\%$), a random effects model was utilised. It was intended to assess the risk of bias using funnel-plot visual inspections together with Begg's and Egger's test.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

The database searches identified 11814 publications. Following the exclusion of duplicates, 8057 studies remained. 8008 articles were excluded following initial screening of titles and abstracts. The

remaining 49 articles were retrieved for full text review. 42 further articles were excluded, with the main reason for exclusion being insufficient prognostic data to facilitate a meta-analysis. A flowchart of the study selection process is shown in Figure 1. Although the planned protocol had intended to also capture 5 year survival data, due to the heterogeneity of Ki-67 cut-offs utilised and data presentation via Kaplan Meier curves, it was not possible to present this in a meaningful way. This was due to the 5 year survival estimates not being reported in all studies and could only be detected through Kaplan Meier plots.

Study characteristics and quality evaluation

Seven papers, published between 2013 and 2018 including 1268 patients (693 TC, 281 AC, 94 LCNEC and 190 SCLC) fulfilled the inclusion criteria for meta-analysis.(18–24) All included studies were retrospective and observational in nature, with no prospective studies identified. The cohort sizes varied between 82 and 399 subjects. Only one study (Rindi *et a*l) was inclusive of the full range of pulmonary NENs with most studies only including the well-differentiated NETs (typical and atypical carcinoids). Four of the studies included Italian cohorts, with France, Brazil, Finland and the UK each contributing a single study. The majority of studies used the MIB-1 antibody (4 of 7), although not all studies provided this information.

The majority (76.8%) of the patients had well-differentiated tumours (either in the form TC or AC) with only a minority (23.1%) having poorly differentiated NECs. 51% of the participants were female. The age range of participants varied between 15 and 83 years with one study failing to provide this information. Three studies did not report data for tumour stage. Across the remaining four studies, the majority of participants were noted to have early stage disease (54.3% of patients had stage I disease, 15.5% stage II, 14.2% stage III, 13.8% stage IV, 4.2% stage X). Median length of follow-up ranged between 9.6 and 70 months. The population characteristics of studies included in the meta-analysis are summarised in Table 1.

Quality evaluation revealed that the studies included in the meta-analysis were of an overall good quality. The median Newcastle-Ottawa Scale score was seven, with three papers scoring seven and eight each and one scoring six [Table 2]. Five and three studies made hazard ratio and confidence interval data available for OS and RFS respectively.

Meta-analysis of overall survival

In the meta-analysis of overall survival, five studies were included (Cusumano *et al* published a death HR, whilst Vesterinen *et al* offered a HR for disease specific mortality – both were deemed to be surrogate markers of OS).(17,18) Hazard ratios derived from univariate analyses were considered for

meta-analysis over their multivariate counterparts in an effort to limit the heterogeneity resulting from how hazard ratios are derived. The heterogeneity was high: I^2 = 69%. This necessitated use of a random effects model. The pooled HR for Ki-67 was 2.02 (95% CI 1.16 – 3.52) with a p-value of 0.01 [Figure 2].

Meta-analysis of recurrence free survival

In the meta-analysis of recurrence free survival (RFS), three studies were available (in one recurrence HR was available whilst a second study provided a time to progression HR – both were considered to be surrogate markers of RFS). Once again, the heterogeneity was high (I^2 = 89%) and therefore a random effects model was appropriate. The pooled HR was 1.42 (95% CI 1.01-2.00; p 0.04) [Figure 3].

Risk of bias

Despite the intention to assess the risk of bias using funnel-plot visual inspections, Begg's and Egger's test, this was not feasible due to the low number of studies included in the meta-analysis.

DISCUSSION

Prognostic biomarkers and tools play an important role in oncological management and decision making processes. In pulmonary NENs the dearth of prognostic biomarkers is notable and therefore oncologists often request Ki-67 indices in order to assist in therapeutic decisions despite the fact this has not been formally adopted. The primary aim of this study was to evaluate whether existing Ki-67 LI is associated with prognosis in pulmonary NENs as has been demonstrated in numerous other tumour types (e.g. GEP-NENs, urothelial carcinomas, breast cancer, lymphoma and lung cancer).

Ki-67 is most frequently evaluated immunohistochemically on paraffin sections using the MIB-1 antibody. Scoring is generally formulated by the percentage of tumour cells stained positively to the antigen (also known as the labelling index). Several methods are available to evaluate the Ki-67 labelling index (LI), including digital image analysis, eyeball estimation and manual counting. In digestive NENs, the method currently considered 'gold standard' is to evaluate the area with the most dense Ki-67 staining (i.e. histological 'hotspots') and to subsequently manually count a minimum of 500 cells, with best practice being to count 2000 cells or 2mm^2.(25,26) Manual counting is subject to limitations - not only can it become tedious, but it is time-consuming as counting 2000 cells can take approximately 40 minutes to complete. Utilising camera captured printed images reduces issues with inter-observer variability, although the issue of intra-tumoural heterogeneity remains as selecting

which tumour area will be subjected to counting can be difficult to establish with consistency.(27) Therefore, some pathologists resort to eyeball estimations, resulting in poor reproducibility and inter-observer variability relating to the pathologists experience.(28) Digital image analysis has been heralded as a means of deriving uniformity, but it is not currently widely employed as a result of a number of obstacles including technical issues (e.g. overcounting unwanted cells and underestimating negative cells) as well as its current lack of worldwide availability.

This meta-analysis provides tentative evidence demonstrating that high Ki-67 indices are associated with a 40% greater risk of recurrence amongst patients diagnosed with pulmonary NENs. This risk appears to be further exaggerated when considering overall survival where patients with a high Ki-67 have double the risk of death in comparison with patients with a lower Ki-67 LI. The strength of the association between Ki-67 LI and prognosis was only evaluated in studies that calculated hazard ratios using univariate analyses. As a result, no attempt has been made to account for confounding factors (such as stage, grade, and mitotic index).

One of the major pitfalls of including Ki-67 in the classification of pulmonary NENs has in establishing the most appropriate thresholds or cut-offs that should be utilised when grading tumours. In the main, Ki-67 has not been used as a linear biomarker within the whole pulmonary NEN cohort, instead focusing on its utility within each categorical histological subtype. Whilst categorising NENs by grade is helpful in establishing management plans, it is likely that proliferative markers (such as Ki-67 and mitotic index) are continuous rather than categorical variables. Therefore, there may not be a single or absolute optimal cut off value to categorise tumours into distinct entities and a pragmatic approach is likely to be needed. In order to facilitate clinical clarity, it would be preferable to use the same thresholds as are utilised in GEP-NETs and any future studies should attempt to clarify this further. However, it is unclear whether attempting to implement a similar grading system in pulmonary NENs as GEP NENs does a disservice to the fundamental biological diversity between the two different tumour sites.(29) Examples of this diversity include the variability of genetic alterations seen as well as the differing rates of associated syndromes and hormone expression . (30-35)

Unfortunately only two studies involving SCLC and high grade neuroendocrine carcinomas of the lung were available. It is important to clarify that Ki-67 is not likely to be useful in subtyping these tumours prognostically. A number of biomarkers have been identified which may have greater utility in these patients. Nevertheless further research into Ki-67 is required in these tumour groups with such little evidence, especially in light of the fact that in GEP-NENs there is good evidence to suggest that Ki-67 is contributory with a cut-off of 55%.(36)

As with all studies, this meta-analysis is also subject to inherent limitations. None of the studies included in the meta-analyses were prospective in design; retrospective analyses are prone to error through issues with selection bias and reporting. Secondly, studies with a variety of endpoints (e.g. RFS analyses included studies where the endpoint was DFS and time to progression analyses etc.), diverse cohort sizes, differences in the dilution of the primary antibody as well as variable Ki-67 cut-offs have all been amalgamated. Whilst some degree of heterogeneity is always to be expected, it diminishes the validity of the combined data-set and subsequent results. This is reflected in the I^2 statistics noted across both meta-analyses.

This study also preferentially utilised univariate analyses. Whilst multivariate analyses can be significantly distorted by differing in their approach to modelling or prognostic factors, univariate analyses fail to account for confounding variables. Furthermore, given the small number of studies identified as suitable for inclusion in this meta-analysis, it is clear that future international multi-centre efforts are needed to develop studies which are prospective with large cohorts to clarify whether Ki-67 labelling index is truly a prognostic biomarker in the setting of bronchopulmonary neuroendocrine neoplasms.

CONCLUSIONS

Although it is difficult to draw definitive conclusions, this meta-analysis of over 1250 patients with pulmonary NENs indicates that a high Ki-67 LI is associated with an adverse prognosis. Whilst these findings are subject to a number of limitations, they provide a valuable insight into a rare tumour and should be considered when producing new guidelines regarding the use of Ki-67 in pulmonary NENs.

Conflict of interest statement: The authors have declared no competing interests.

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Patient and public involvement: Patients and the public were not involved in the development of this systematic review or meta-analysis.

Data Availability Statement: All data relevant to the study are included in the article or uploaded as supplementary information.

Ethics Statement: Given that this is a systematic review of existing literature, ethical approval is not required.

Contributors: SN contributed to the study question, protocol design, screening, data collection, data analysis, dissemination of results including preparation of the manuscript. CH contributed to the screening and data collection. LT, NP, BG and EJ contributed to protocol design. LP assisted with the statistical analysis. CHO, JC and GP were responsible for the study question; all authors have been involved in the preparation of the manuscript.

Figure Legends:

Figure 1: Study Selection

Figure 2: Forest plot of studies evaluating the association between Ki-67 expression and overall survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval

Figure 3: Forest plot of studies evaluating the association between Ki-67 expression and recurrence free survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval.

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Author (Year)	Trial Design (study centres)	Number of Subjects	Histological subtypes	Age Range	Gender M:F	Stage	Antibody	Methodology for calculating Ki-67	Median length of follow-up (months)	Ki-67 cut-off thresholds (%)	Outcome measure	Hazard Ratio (95% CI) from univariate analyses
Cusumano et al (2017)	Retrospective multicentre study (France, Italy)	195	TC (159); AC (36)	52.94 (TC); 60.16 (AC) [mean values]	89:106	I = 163; II = 16; III = 16; IV = 0	NR	NR	75 (mean)	NR	OS (Death HR) DFS	1.07 (0.97-1.17) 0.97 (1.01–1.2)
Rego et al (2017)	Retrospective, multicentre study (Brazil)	82	SCLC (82)	35-81 (mean 59)	48:34	I = 0; II = 0; III = 6; IV = 76	MIB-1 (1:1000)	Hotspot method; otherwise not specified	10.3	50	OS	1.15 (0.70-1.89)
Marchio et al (2017)	Retrospective multicentre study (Italy)	239	TC (171); AC (68)	NR	100:139	NR	NR	Manual counting of >1000 cells	NR	4	OS TTP	4.31 (1.624-11.45) 3.994 (1.58-10)
Filosso et al (2013)	Retrospective, single centre study (Italy)	126 [NB 110 included in Ki-67 analysis]	TC (83); AC (43). [In Ki-67 analysis TC (79); AC (31)]	15-82 (mean 60)	52:74	I = 90; II = 18; III = 16; IV = 2; X = 1	anti–Ki-67 antibody (DAKO) not further specified	NR	60	6	OS	2.08 (1.02-4.27)
Rindi et al (2014)	Retrospective, multicentre study (Italy)	399	TC (113); AC (84); LCNEC (94); SCLC (108)	63.26 (median)	245:154	I = 183; II = 90; III = 76; IV = 17; X = 33	MIB-1 antibody	Computer assisted manual count method 500-2000 cells	70.72	< vs 4-20	OS	1.26 (0.84-1.89)
Clay et al (2017)	Retrospective, single centre study (UK)	94 [NB survival analysis performed on 84]	TC (75); AC (19) [NB survival analysis performed 67 TC, 17 AC patients]	21-83 (median 60.5)	39:55	NR	MIB-1 antibody (1:50)	Manual count method 500-2000 cells in hot spot	35	NR	RFS	1.47 (1.25-1.74)
Vesterinen et al (2018)	Retrospective, single centre study (Finland)	133 (129 included in Ki-67 analysis)	TC (100); AC (33)		47:86	NR	MIB-1 antibody (1:100)	Manual and automated counting of 2000 cells	9.6	2	Disease specific mortality	10.51 (2.12-52.13)

Table 1: Main Characteristics of all studies included in the meta-analysis.

M = male, F=female, NR = not recorded, OS = overall survival, RFS = recurrence free survival, DFS = disease free survival, TTP = time to progression.

Author (Year)	Representativeness of cohort (1 point)	Adequate definition of cases (1 point)	Assessment of exposure (1 point)	Outcome of interest not present at start of study (1 point)	Comparibility on the basis of the design or analysis (2 points)	Assessment of Outcome (death or recurrence) (1 point)	Adequacy of median follow-up for outcome (>2 years) (1 point)	Adequacy of follow-up of cases (<20% or reported) (1 point)	Total Quality Score
Cusumano et al (2017)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Rego et al (2017)	☆	☆	☆	-	☆	☆	-	☆	6
Marchio et al (2017)	☆	☆	☆	-	☆☆	☆	-	☆	7
Filosso et al (2013)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Rindi et al (2014)	☆	☆	☆	-	☆☆	☆	☆	☆	8
Clay et al (2017)	☆	☆	☆	-	☆	☆	☆	☆	7
Vesterinen et al (2018)	☆	☆	☆	-	☆☆	☆	-	☆	7

Table 2: Quality assessment of included studies utilising the (modified) Newcastle-Ottawa Scale.

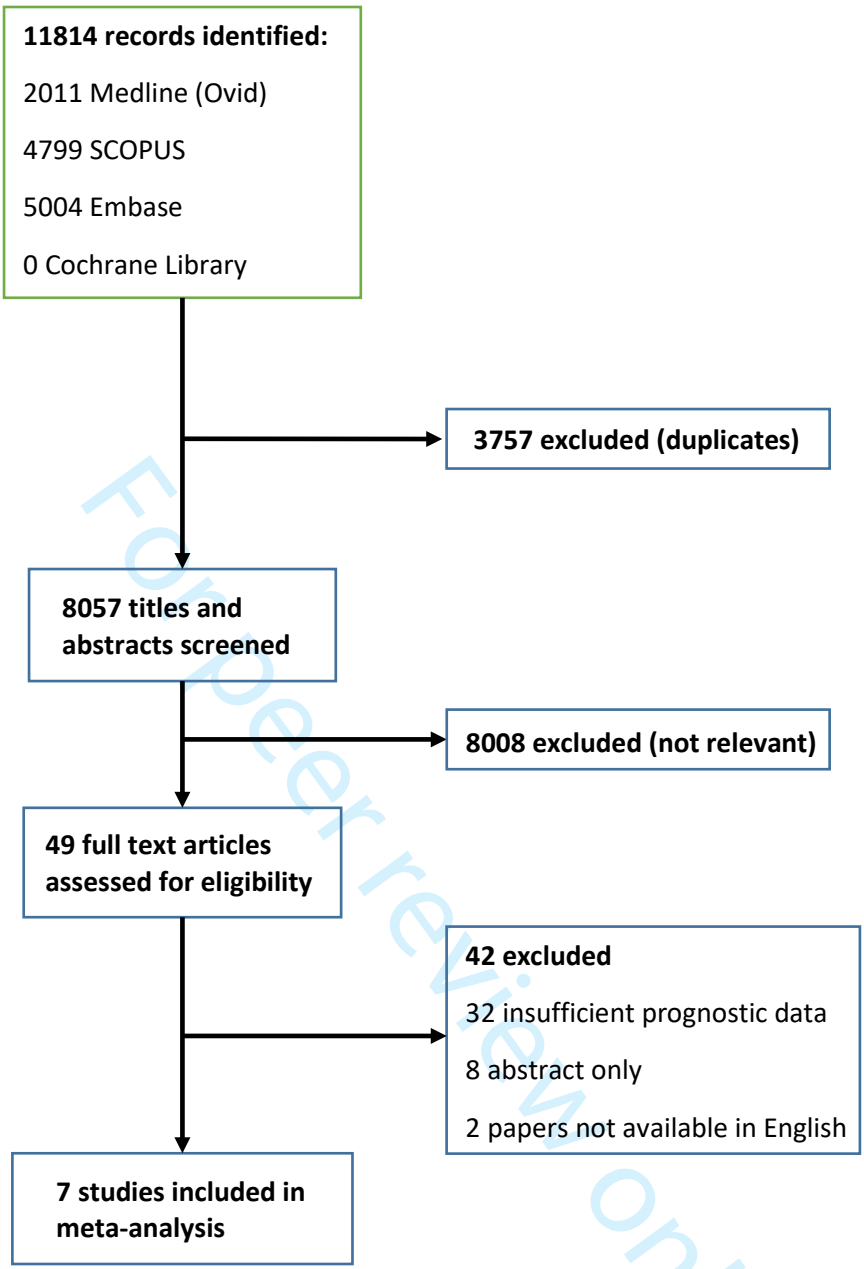

Figure 1: Study Selection

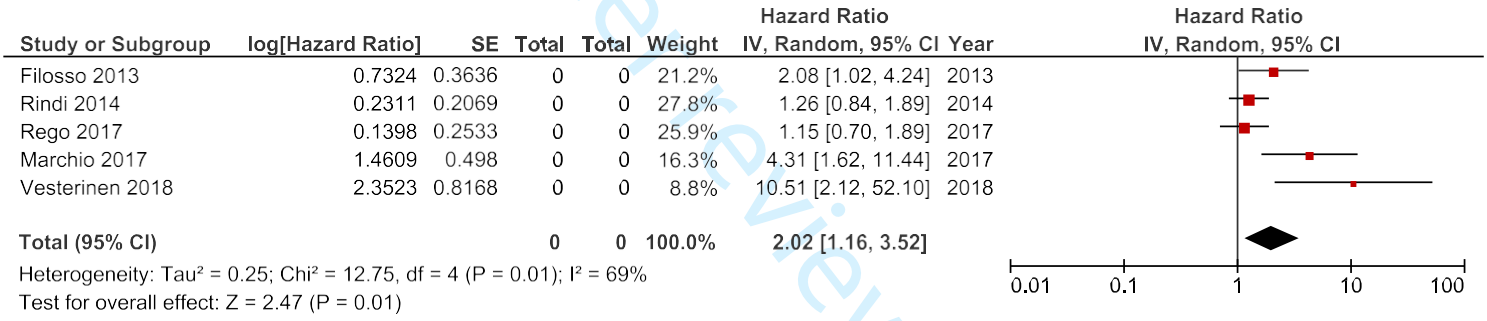

Figure 2: Forest plot of studies evaluating the association between Ki-67 expression and overall survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval

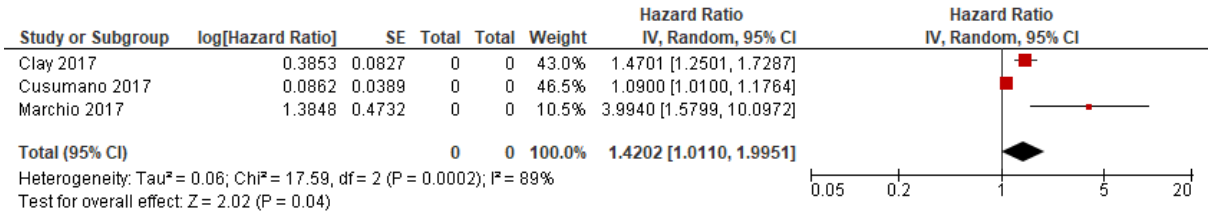

Figure 3: Forest plot of studies evaluating the association between Ki-67 expression and recurrence free survival in pulmonary neuroendocrine neoplasms.

CI = confidence interval.

EXAMPLE OF FULL SEARCH STRATEGY

The systematic review will employ the following search terms for Medline OVID, and EMBASE:

Ki-67 antigen/ OR (Ki67 or Ki-67 or mib-1 or mib1).mp.

AND

neuroendocrine tumors/ or carcinoid tumor/ or carcinoma, neuroendocrine/ OR small cell lung carcinoma/ OR ((tumo?r* or neoplas* or carcinoma or cancer* or malignan*) adj3 (neuroendocrine or carcinoid or small cell)).mp.

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5-6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5-6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5-6

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5-6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5-6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6-7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	6-7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	7
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	7-8
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	7-8
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	6

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