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Title page

Title:

A prospective study to evaluate a diagnostic algorithm for the use of fluid lymphocyte subset analysis in undiagnosed unilateral pleural effusions

Short title:

Lymphocyte subset analysis in undiagnosed unilateral pleural effusions

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Statement of contributions

All authors contributed to the writing of the manuscript. MTB and PFV undertook cytological and lymphocyte subset analysis respectively. GD, RB, NZE and AOC were responsible for data gathering. GD and RB were responsible for data analysis. ARLM and NAM were responsible for confirming each patient's final diagnosis. All authors have approved this submission.

Conflicts of interest:

None

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Abstract

Background

Haematological malignancy is an important cause of pleural effusion. Pleural effusions secondary to haematological malignancy are usually lymphocyte predominant. However, several other conditions such as carcinoma, tuberculosis and chronic heart failure also cause lymphocytic effusions. Lymphocyte subset analysis may be a useful test to identify haematological malignancy in patients with lymphocytic effusions. However, research into their utility in pleural effusion diagnostic algorithms has not yet been published.

Objectives

We aimed to determine the clinical utility of pleural fluid lymphocyte subset analysis, and whether it can be applied to a diagnostic algorithm to identify effusions secondary to haematological malignancy. The secondary aim was to evaluate the diagnostic value of pleural fluid differential cell count.

Methods

Consecutive consented patients presenting to our pleural service between 2008-2013 underwent thoracentesis and differential cell count analysis. We proposed an algorithm which selected patients with lymphocytic effusions (>50%) to have further fluid sent for lymphocyte subset analysis. Two independent consultants agreed the cause of the original effusion after a 12-month follow-up period.

Results

60 patients had samples sent for lymphocyte subset analysis. Lymphocyte subset analysis had an 80% sensitivity (8/10) and a 100% specificity for the diagnosis of haematological malignancy. The positive and negative predictive values were 100% and 96.1% respectively. 344 differential cell counts were analysed; 16% of pleural effusions with a malignant aetiology were neutrophilic or eosinophilic at presentation. A higher neutrophil and eosinophil count was associated with benign diagnoses whereas a higher lymphocyte count was associated with malignant diagnoses.

Conclusions

Lymphocyte subset analysis may identify haematological malignancy in a specific cohort of patients with undiagnosed pleural effusions. A pleural fluid differential cell count provides useful additional information to streamline patient pathway decisions.

List of abbreviations

AF	Atrial fibrillation
BAPE	Benign asbestos related pleural effusion
CABG	Coronary artery bypass graft
CLL	Chronic lymphocytic leukaemia
DLCBL	Diffuse large cell B-Lymphoma
HIV	Human immunodeficiency virus
HTN	Hypertension
IHD	Ischaemic heart disease,
LS	Lymphocyte subset

NHL	Non-Hodgkin’s lymphoma
T2DM	Type 2 Diabetes Mellitus
TB	Tuberculosis

Introduction

Haematological cancers are amongst the commonest causes of a malignant pleural effusion[1, 2]. Up to 16% of patients with Hodgkin and Non-Hodgkin Lymphoma will have a pleural effusion during their illness, occurring as either a presenting feature or later on in the disease course[3, 4]. The mechanisms of pleural effusion include pleural infiltration by the tumour, lymphatic obstruction, secondary heart failure, renal failure and hypoalbuminaemia [5]. Historically, the diagnosis of pleural involvement in haematological malignancy was based on simple cytological examination of pleural fluid, however reported diagnostic rates using this method alone can be highly variable [5]. Lymphocyte subset (LS) analysis, also referred to as flow cytometry, is amongst a number of more advanced cytological tests which can improve diagnostic yield [6]. LS has been suggested as a useful investigation in pleural effusions to identify those patients with haematological malignancy, although data remains limited and there may be significant costs associated with such tests [6]. Bangerter *et al* looked at both ascitic and pleural fluid and found the combined use of standard cytology and LS analysis achieved a sensitivity and specificity of 100% [7]. Despite this, there is currently no established guidance for clinicians as to where pleural fluid LS analysis may fit into a standard diagnostic algorithm.

Traditionally, cytology reports comment upon the presence or absence of visible malignant cells. When the predominant cell type in pleural fluid is also reported, patients with haematological malignancy are typically found to have lymphocytic effusions. An accurate understanding of the cellular constituents of pleural fluid can help to improve differential diagnosis and allows targeted investigations in patients with undiagnosed pleural effusions. For example,

lymphocyte-predominant effusions are usually felt to warrant more invasive investigation, such as pleural biopsy, as the differential diagnosis includes malignancy and tuberculosis (TB) [8-10]. In contrast, neutrophilic effusions are more likely to represent an acute process such as infection [11].

The primary purpose of this study was to determine the effectiveness of a standardised algorithm, which focused on the role and utility of pleural LS analysis for those patients presenting with undiagnosed pleural effusions. As a secondary aim, we looked to investigate whether a differential cell count with a percentage breakdown of cellular constituents would provide any valuable additional clinical information.

Materials and Methods

The analysis utilised prospectively-collected data from patients presenting consecutively to a well-established pleural service between 2008 and 2013. Those with an undiagnosed unilateral pleural effusion were reviewed as part of a broader, actively maintained pleural database and associated study. The project received ethical approval from the South West regional ethics committee (08/H0102/11) and was registered with the UK Clinical Trials Register (UKCRN ID 8960). All patients provided informed written consent to take part in the study and have their details and samples stored.

As part of their initial work-up, patients had pleural fluid sent for routine analysis, including cytology. The study protocol also called for full pleural fluid differential cell count to be reported (Fig 1). All samples were examined by experienced cytopathologists, mainly M.B. In those with previous history of

haematological malignancy at presentation or clinical picture highly suggestive of haematological malignancy (such as radiological lymphadenopathy or “B” symptoms), LS analysis of the pleural fluid was also requested at presentation.

Full details of cytology reporting and LS analysis can be found in the supporting information. Effusions with >50% neutrophils or lymphocytes were categorised as neutrophilic and lymphocytic respectively, and those with >10% eosinophils were categorised as eosinophilic. Pleural effusions could therefore be defined simultaneously as both eosinophilic and lymphocytic or neutrophilic.

Figure 1. Proposed algorithm for selecting patients who require lymphocyte subset analysis.

Following initial investigations, those patients with a lymphocytic effusion on cytology, but with no firm tissue diagnosis of malignancy or clear alternative diagnosis (e.g. a transudative collection in a patient with known heart failure), had a second pleural fluid sample taken (Fig 1). This second sample was sent to a specific, experienced immunologist for LS analysis, as well as for repeat cytological examination. The final diagnosis for all pleural effusions was confirmed by two independent respiratory physicians after a minimum of 12 months of follow-up (or after death). These physicians were not blinded to the LS results. All diagnoses were classified into pre-defined groups to facilitate further analysis. The full diagnostic criteria can be found in the online supporting information. In those cases where there were felt to be multiple contributing factors to a pleural effusion, the likely causes were ordered to signify the greatest contributing factor first. For the purposes of this analysis, only the primary cause was used.

Statistical analysis was carried out using Microsoft Excel (2011), SPSS Statistics (9.5.0.0) and Social Science Statistics (www.socscistatistics.com).

Results

Patient demographics

A total of 509 patients were recruited during the study period. The most common final diagnoses were metastatic malignancy (n=188, 36.9%), infection (n=93, 18.3%), malignant mesothelioma (n=74, 14.5%) and cardiac failure (n=47, 9.2%). 408 effusions were exudates, 61 transudates and 40 had insufficient information to enable classification. Haematological malignancy was responsible for 14 cases of pleural effusion (2.8%) overall and 10/408 (2.5%) exudate effusions. Other diagnoses made up the remaining 93 cases (Table 1).

The clinical utility of lymphocyte subset analysis

Pleural fluid differential cell count identified 145 patients with a lymphocytic effusion. These patients were eligible to begin the diagnostic algorithm as described above, with the outcomes demonstrated in Figure 2. During initial investigations, non-haematological malignancy was confirmed via cytology or biopsy in 56 patients, and a further 33 had a clear alternative diagnosis. One patient had a haematological malignancy confirmed at this stage by biopsy at bronchoscopy. There were therefore 55 patients who were eligible for LS analysis on repeat pleural fluid samples. Three samples were not sent for analysis or were lost in transit.

After follow-up review, the main causes for the lymphocytic effusions were metastatic malignancy (n=58/145, 40.0%), cardiac failure (n=16/145, 11.0%), benign asbestos related pleural effusion (n=13/145, 9.0%), inflammatory pleuritis (n=12/145, 8.3%), malignant mesothelioma (n=12/145, 8.3%), infection (n=10/145, 6.9%) and haematological malignancy (n=10/145, 6.9%).

8/199 patients with non-lymphocyte predominant effusions had lymphocyte subsets analysed because of previous history of haematological malignancy or MGUS (myoclonal gammopathy of unknown significance) or clinical features strongly suggestive of haematological malignancy. 1 of these patients was subsequently diagnosed with an effusion secondary to diffuse large B cell lymphoma. In this case the differential cell count showed predominantly mesothelial cells and macrophages rather than a lymphocytosis. The remaining 7 had alternative diagnoses; 3 non-haematological malignancy, 3 malignant mesothelioma and 1 congestive heart failure.

Figure 2. Diagnostic algorithm outcomes for 509 patients presenting with a unilateral pleural effusion

MGUS = Monoclonal gammopathy of unknown significance, TB = Tuberculosis

Haematological malignancy was ultimately diagnosed in 10/145 (6.9%) patients with a lymphocytic effusion, comprising eight non-Hodgkin's lymphoma (of which three were diffuse large B cell lymphoma and one was Burkitt's lymphoma) and two chronic lymphocytic leukaemia. 1/10 patients were diagnosed by biopsy without LS analysis (as mentioned previously).

In total, ten patients (10/60, 16.7%) with LS analysis performed had an effusion secondary to haematological malignancy. 4 patients did not undergo lymphocyte subset analysis but had a subsequent diagnosis of haematological malignancy. In these cases the patients had symptoms, signs and radiological features to suggest haematological malignancy at presentation. The patients therefore had alternative investigations to confirm their diagnosis such as lymph node and/or bronchoscopy biopsy. Full patient details can be found in Table 2.

LS analysis was diagnostic for haematological malignancy in 8/10 patients. Diagnostic confirmation was subsequently obtained in 9/10 patients whereby other tissue was biopsied or other fluid (ascitic/blood) sent for subset analysis.

Lymphocyte subset analysis had a sensitivity of 80% and a specificity of 100% for the diagnosis of haematological malignancy (95% confidence intervals 44-96% and 91-100% respectively). The positive (PPV) and negative (NPV) predictive values were 100% (95% confidence interval 60-100%) and 96.1% (95% confidence interval 86-99%) respectively. In the two patients with non-diagnostic lymphocytic effusions diagnosis was made by bone marrow and lymph node biopsy.

A differential cell count confers additional useful clinical information

A differential cell count was available for 344/509 patients as 165 patients had an unsatisfactory sample. Of these, as described above, there were 145 patients with >50% lymphocytes, 54 with >10% eosinophils and 48 with >50% neutrophils. 23 patients had both lymphocytic and eosinophilic effusions. Therefore 120 effusions had a mixed cellular picture which did not fit into the aforementioned categories.

These included combinations of macrophages, neutrophils, lymphocytes, eosinophils, malignant and mesothelial cells.

The main diagnoses of patients with lymphocytic effusions have been set out above. The main diagnoses in the 54 patients with an eosinophilic effusion were; metastatic malignancy (n=20/54, 37.0%), malignant mesothelioma (n=8/54, 14.8%), pleural infection (n=7/54, 13.0%) and benign asbestos related pleural effusion (n=6/54, 11.1%). The main diagnoses in 48 patients with a neutrophilic effusion were; pleural infection (n=36/48, 75.0%), metastatic malignancy (n=6/48, 12.5%) and malignant mesothelioma (n=5/48, 10.4%).

The frequencies of benign and malignant effusions by cell type are set out in Table 3. Chi-squared comparison produces a p-value of <0.05.

Table 3. The frequency of benign and malignant effusions of 344 patients with a unilateral pleural effusion and a percentage differential cell count

187 patients with a malignant pleural effusion (including metastatic malignancy, mesothelioma and haematological malignancy) had a differential cell count available (Fig 3).

Figure 3. Differential cell count of 187 patients with a pleural effusion secondary to malignancy.

A benign diagnosis was found in 12/16 effusions with >30% eosinophils compared to 15/25 of effusions with values between 10 and 20%. Highly neutrophilic effusions were more likely to be benign. However 23% (7/30) of effusions with >80% neutrophils were associated with malignancy. A higher lymphocyte count was associated with malignancy as effusions with >80% lymphocytes had a 63.4%

(26/41) chance of being malignant. One patient with an eosinophilic effusion had a traumatic haemothorax.

Of those patients with pleural infection and a lymphocytic effusion 6/10 patients had experienced symptoms for two or more weeks. Additionally, 3/10 had a concurrent diagnosis of cardiac failure, one patient had a concurrent diagnosis of malignancy and one patient was infected with mycobacterium avium. Of the 16 patients with >90% lymphocytes 75% (n=12) had effusions that were caused by malignancy.

Discussion

Targeted lymphocyte subset analysis

This is the first study to prospectively analyse the use of LS exclusively in unilateral pleural effusions. In our study population, haematological malignancy was responsible for 14 pleural effusions. This relatively small incidence, coupled with the time and labour intensive nature of LS analysis, makes it impractical to be applied to all undiagnosed effusions. There are currently limited data exploring the clinical utility of routine lymphocyte subset analysis, with the most recent national guidelines unable to propose how LS analysis should be incorporated into the investigation algorithm for undiagnosed pleural effusions [6]. Those studies which have previously looked at the utility of LS analysis have not focussed specifically on pleural effusions [7, 12].

Our targeted algorithm was designed to restrict LS analysis to those patients with a previous history of haematological malignancy, or with undiagnosed lymphocytic effusions. This ensured that the test remained practical in day-to-day

clinical use and was only applied to a group who were felt to be most likely to benefit from repeat sampling. We were able to demonstrate high sensitivity and specificity for the diagnosis of haematological malignancy, at 80% and 100% respectively. This suggests that there may be a place for the addition of LS in the routine diagnostic pathway for new pleural effusions. By following this approach, LS analysis was only required in 60/509 (11.8%) patients presenting to our service during the study period, suggesting it may be applied in a relatively selective manner.

In our centre differential cell count results are not usually available for 48 hours post-thoracentesis by which time sample degradation rules out adequate LS analysis. However, if this result could be obtained more rapidly it may allow for targeted LS analysis on the first thoracentesis sample avoiding the need for a second procedure. We would suggest that individual centres could alter the algorithm according to their local service provision.

In patients who had lymphocyte subset analysis performed without a lymphocyte predominant effusion 1/8 had haematological malignancy confirmed as the cause of their effusion. Further research is required to determine whether lymphocyte subset analysis is indicated within this group.

Differential cell count aids diagnosis

Standard practice in differential cell count reporting is to provide a cellular description or predominant cell type. Our work has indicated that a specific percentage differential cell count can provide useful clinical information, helping to narrow the differential diagnosis at presentation and potentially alter management pathways.

It has previously been reported that highly neutrophilic or eosinophilic effusions are pathognomic of benign processes [11, 13]. However, our data suggest an effusion with >80% neutrophils has a one in four chance of having an underlying malignant aetiology. Whilst we found that highly eosinophilic effusions were less likely to be malignant, in one case we found eosinophil counts of up to 65% in a malignant effusion. Overall we found that 16% of malignant pleural effusions in this series were neutrophilic or eosinophilic. It must therefore be stressed that highly neutrophilic or eosinophilic effusions are not always associated with a benign aetiology. One patient in this series with a eosinophilic effusion had a traumatic haemothorax, although the presence of eosinophils in pleural fluid has previously been shown to be associated with air or blood in the pleural space[14].

Lymphocytic effusions often raise diagnostic concern, especially regarding the presence of malignancy. Indeed, malignancy and TB have been reported to be the cause of around two thirds of lymphocytic effusions in areas of moderate TB prevalence [15]. In our patient population, the prevalence of TB was much lower, with just 10/509 patients diagnosed, 6 of whom had lymphocytic effusions. Studies have previously suggested several causes of an effusion with >80% lymphocytes including lymphoma, rheumatoid, post-coronary artery bypass graft (CABG), malignancy and TB [9]. Lymphocytes are also known to predominate in longstanding effusions of over 2 weeks duration.

We have also suggested potential benefit in knowing the percentage of lymphocytes in those with lymphocytic effusions. We found that with increasing lymphocyte percentages, the likelihood of malignancy rose. Patients with highly lymphocytic effusions, in the right clinical context, should therefore be routinely

considered for tissue biopsy via image guided techniques or thoracoscopy, as well as for lymphocyte subset analysis.

Limitations

There are a number of limitations to our study. The primary limitation is the small number of patients with pleural effusions caused by haematological malignancy, which LS analysis is intended to detect, although the proportions found in our cohort overall are similar to those in other large scale studies [16]. Additionally, there will always remain a group of patients in which LS analysis may be appropriate regardless of the proposed algorithm, and these must be addressed on a case-by-case basis. Examples of these might be patients with significant undiagnosed lymphadenopathy, classical symptoms and signs of haematological malignancy, or non-specifically suspicious cytology. In our study four patients were found to have haematological malignancy causing a pleural effusion and were not accommodated by the proposed algorithm.

It could also be argued that the 16.7% of patients who had the test performed and who went on to be confirmed as having a haematological malignancy, is too low a proportion to justify its routine clinical use. Whilst clearly not ideal, we feel the selection of patients based upon the finding of lymphocyte-predominant fluid is the only approach which can practicably be applied to the current investigation pathways for those presenting with undiagnosed effusions.

Although our study focussed on the role of lymphocytes, neutrophils and eosinophils in pleural effusions, there are several cell types which were not present in large enough numbers frequently enough to comment upon in this series. It has been suggested previously that effusions with >10% basophils have an increased

risk of leukaemia and are also associated with pneumothoraces and pneumonias [17, 18]. The presence of macrophages in effusions is generally thought to be non-diagnostic as they are difficult to distinguish from mesothelial cells due to overlapping morphological characteristics [17, 19]. As mentioned above, the presence of air or blood in the pleural space has been associated with eosinophilic effusions[14]. The results presented here represent the first thoracentesis procedure carried out by the study group, however a small number of patients may have undergone thoracentesis at a different centre prior to presentation to our pleural service, which may account for a minority of eosinophilic effusions.

A large number of patients (165/509) in our study had unsuitable pleural fluid samples and did not go on to have a differential cell count. In these patients samples were either predominantly blood or had too few cells to enable sufficient analysis. Within the 165 patients with no differential cell count the major diagnoses were; metastatic malignancy (n=53/165, 32.1%, malignant mesothelioma (n=34/165, 20.6%) and pleural infection (n=31/165, (18.8%). The high proportion of malignancy here should reiterate the need for further investigation of this patient cohort. Despite this, 344 patients had a differential cell count reported and analysed.

Whilst the study participants were prospectively recruited to the study the algorithm was retrospectively applied to the cohort. The participants were recruited as part of a part of a wider study described in the “Materials and Methods”.

9/10 patients with haematological malignancy who had lymphocyte subset analysis in this study also required additional biopsy or investigation to confirm the diagnosis (Table 2). We would suggest LS analysis diagnosed the cause of the effusion and the disease was subsequently confirmed on further investigation.

Further confirmation was required to enable haematologists to make appropriate decisions regarding the treatment of the haematological malignancy.

Conclusions

Lymphocyte subset analysis appears to have a high sensitivity and specificity for the identification of haematological malignancy as a cause of pleural effusion in those with lymphocyte predominant effusions. A differential cell count can help to guide further investigation and is helpful in the diagnosis of undiagnosed unilateral pleural effusions. Furthermore, a neutrophil or eosinophil predominant effusion is not always an indicator of a benign process. In addition to this, a higher percentage of pleural fluid lymphocytosis is associated with a malignant aetiology. Patients with a lymphocytic pleural effusion and no obvious alternative diagnosis after initial testing may benefit from repeat fluid sampling and lymphocyte subset analysis. In order to confirm the clinical utility of our proposed algorithm, further prospective analysis including a greater number of patients with effusions secondary to haematological malignancy will be required.

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434 **Supporting information 1. Cytology reporting and**

435 **lymphocyte subset analysis methodology**.

436 **Supporting information 2. Diagnostic criteria for**

437 **identifying the cause of pleural effusion.**

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