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Sputum processing by mechanical dissociation: a rapid alternative to traditional sputum assessment approaches.

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Abstract

Sputum cytology is currently the gold standard to evaluate cellular inflammation in the airways and phenotyping patients with airways diseases. Sputum eosinophil proportions have been used to guide treatment for moderate to severe asthma. Furthermore, raised sputum neutrophils are associated with poor disease control and impaired lung function in both asthma and COPD and small airways disease in cystic fibrosis. However, induced-sputum analysis is subjective and resource heavy, requiring dedicated specialist processing and assessment; this limits its utility in most clinical settings. Indirect blood eosinophil measures have been adopted in clinical care. However, there are currently no good peripheral blood biomarkers of airway neutrophils. A resource-light sputum processing approach could thus help integrate induced sputum more readily into routine clinical care.

New mechanical disruption (MD) methods can rapidly obtain viable single cell suspensions from sputum samples. The aim of this study was to compare MD sputum processing to traditional methods for cell viability, granulocyte proportions and sputum cytokine analysis. Sputum plugs were split and processed using traditional methods and the MD method, and samples were then compared. The MD method produced a homogeneous cell suspension in 62 seconds; 70 minutes faster than the standard method used. No significant difference was seen between the cell viability ($p=0.09$), or the concentration of eosinophils ($p=0.83$), neutrophils ($p=0.99$) or interleukin-8 ($p=0.86$) using MD. This cost-effective method of sputum processing could provide a more pragmatic, sustainable means of directly monitoring the airway milieu. Therefore, we recommend this method be taken forward for further investigation.

249/250 words

Keywords: Eosinophil, Neutrophil, Asthma, Severe asthma, Chronic obstructive pulmonary disease, Sputum, Inflammometry

Introduction

Sputum inflammatory profiles have been used clinically in evaluating the heterogeneity of airways diseases such as asthma, chronic obstructive pulmonary disease (COPD) and idiopathic cough[1-4] as well as having potential for cystic fibrosis (CF) disease progression.[5] Sputum eosinophil proportional measures are recommended to guide treatment for moderate to severe asthma.[6-8] For example, steroid treatment can be tapered down when eosinophils are low[6] or increased to reduce exacerbation frequency.[2,8,9]

Raised sputum neutrophils are associated with lung function decline in both asthma[10-12] and COPD.[1,13] Binary measures used to describe eosinophilia and neutrophilia based on sputum cell profiles identify where disease control may be modifiable. Sputum differential cell counts are not currently adopted as standard clinical practice for CF, yet CF patients with severe small airways disease have higher neutrophil proportions than those with conserved small airways, hence differential cell counts may also guide understanding of CF disease progression.[5]

Sputum processing for differential cell counting is currently achieved by agitating sputum plugs with a mucolytic to release the cells.[14] This is a relatively rapid technique with incubation being as short as 15 minutes at room temperature. However, mucolytics act through the breakdown of disulphide bonds.[15] Therefore, further analysis of the fluid phase measures in supernatant is compromised. Bafadhel and colleagues showed that 14 proteins, which were sensitive to mucolytics, were measurable using a phosphate buffered saline (PBS) incubation step prior to mucus breakdown.[16] This increased analytical capacity of the concurrent method of sputum analysis and is favoured by many researchers, and as such this method is currently accepted as the standard.

The addition of a pre-mucolytic step in the concurrent sputum processing method increases sample processing time by around 45 minutes. Protracted sputum processing times and differential cell counts are resource heavy, demanding dedicated technicians to process and analyse samples.[17] These issues have prevented adoption in clinical practice and analysis is mostly confined to specialist centres with a history of respiratory research.[1,6,8] This restricted capacity to analyse sputum has highlighted the need for less intensive, technically straightforward means of phenotyping airway disease.[17] The identification of a less labour-intensive method of sputum processing would reduce the overall cost of analysis, expand the capacity to

directly monitor a broader panel of cells and mediators and offer the opportunity for more widespread adoption of sputum cell counts and targeted phenotypic-based treatment strategies.

The use of a mechanical dissociator uses mechanical disruption (MD) gentle enough to generate a live singe cell suspension from tissue in a fraction of the time needed for traditional cell dissociation. Dissociated cells using this method are viable for cell culture, flow cytometry and migration assays, but this technology is yet to be applied to sputum samples.[18] We aimed to run a pilot study to assess the feasibility of utilising a MD method using the GentleMACS cell dissociator for characterising sputum as an efficient alternative to the current sputum processing method. In order to investigate the utility of the MD method, we compared the composition of partially homogenised sputum samples segmented for side by side comparison with the Bafadhel processing method.[16] Sputum quality, cellular composition and interleukin (IL)-8 concentration were measured for each processing method. Demonstration of a streamlined methodology for analysing inflammatory profiles in the sputum could lead to greater adoption of sputum characterisation for use in guiding treatment in respiratory diseases.

Materials and methods

Sputum collection method

Spontaneous sputum samples were collected from 13 anonymised individuals to undertake a small pilot study including healthy controls (n =6), CF patients (n=4) and severe asthma patients (n=3). Healthy control and severe asthma samples were collected under ethics number 09/H0502/37, CF samples were collected under ethics number 08/H0502/126, both approved by Southampton and South West Hampshire Research Ethics Committee.

Sample processing methods

Sputum plugs were isolated from whole samples using fine tipped forceps and mixed until the sample appeared visibly homogenous. Homogenised plugs were then split for pairwise analysis of 2 processing methods; the widely accepted 2-step processing method described by Bafadhel and colleagues, referred to within this manuscript as the standard method, and the MD method.[16] Each technique provided a cell fraction and PBS supernatant. These methods are described in detail in Figure 1 and below.

for the standard method, samples were incubated for 30 minutes in 8 volumes of PBS, 4 volumes of supernatant were then removed for mediator analysis and replaced with 4 volumes of 0.2% DTT for a further

30-minute mucolytic incubation.[16] For the MD method, 8 volumes of PBS were added to the sample, this was then dissociated using the GentleMACS for 2 x 31 seconds on program B. These samples were subsequently stored on ice until samples prepared using the standard method were ready for side by side comparison of viability and counts.

In keeping with the current accepted practice, the cell fraction was resuspended and counted to assess cell viability and cell density using the trypan blue exclusion method on a Neubauer haemocytometer (Marienfeld Superior 640010). Cytospins were then generated from the remaining cell suspension and stained with haematoxylin and eosin. Cytospins were coded and counted by two operators who were blinded to the sample purification methods, a total of 400 respiratory cells were counted for each sample in each method by each operator. Pairwise differences in cell viability, cell yield, differential cell counts and IL-8 measured in PBS supernatant were analysed to identify where these measures differed according to the processing methods used. Patients were considered 'eosinophilic' where eosinophil proportions were greater than or equal to 2% sputum eosinophils[6,11,16] and 'neutrophilic' where neutrophil proportions were greater than or equal to 61% sputum neutrophils.[10,12,16]

Statistical analysis

Descriptive statistics were used to explore the normality of the data using IBM SPSS version 26, half of the measures fit a Gaussian distribution. Data were analysed using non-parametric analyses and presented as median values to accommodate non-parametric data distribution. Results of each measure were listed pairwise, pairs were analysed with Wilcoxon Signed-rank test using Graphpad prism version 8.0. Non-inferiority was assumed where no significant difference was seen between the standard and MD processing methods with $P \geq 0.05$.

Results

We sought to run a pilot study to compare the streamlined MD sputum processing method with the standard processing technique. The MD method used a single processing tube as compared to the standard method which required 4 tubes and the addition of a mucolytic reagent. The elimination of incubation, centrifugation, additional calculations and pipetting made the process 70 minutes faster per sample. There were 2 fewer tubes and pipette tips used with the MD method.

To assess the utility of the streamlined MD procedure in analysing inflammatory profiles in sputum compared with the standard method, we first assessed the comparative viability of purified cells. The cell viabilities between processing methods were not statistically different although slightly higher in samples purified using the standard method, with a median of 48.5% and 60.7% viability for MD and standard method, respectively (p=0.094) (Figure 2A). There was a positive correlation between the cell viability of paired samples processed using the standard and MD method. However, this was only a small positive association (rho=0.39) and not statistically significant (p=0.128) (Figure 2C).

We next assessed the cell yield of samples purified by MD as compared with the standard method. Samples purified by MD provided significantly higher cell yields as compared with those purified using standard method, with a median recovery of $8.37x10^6$ vs $6.04x10^6$ cells, respectively (p=0.027) (Figure 2B). Despite a higher yield from MD compared to standard there was a very strong, significant positive correlation in cell yield between the two processing methods (rho=0.97) (p=<0.0001) (figure 2D).

We subsequently assessed the comparative level of squamous cell contamination between samples processed using the two purification techniques. The level of squamous cell contamination showed a strong positive correlation between paired samples (rho=0.93, p<0.0001, Figure 3B). However, levels of squamous cell contamination were statistically lower in MD than standard method, with a median of 10.5% vs 30.5% of the total cell numbers, respectively (p=0.023) (Figure 3A).

Neutrophil and eosinophil proportions are important clinical markers in sputum and thus we compared MD and standard method to see if cells purified from sputum using these techniques were comparable to give consistent clinical phenotyping. Purification by MD and standard method gave similar proportions of neutrophils, with a median of 72.5% and 67.5 % of total cell count, respectively (p=0.987) (Figure 4A). There was a fair positive correlation between neutrophil proportions in these paired samples (rho=0.57) (p=0.04) (Figure 4C). Furthermore, MD and standard processing methods gave similar proportions of eosinophils with a median of 10.3% and 3.5% of total cell counts, respectively (p=0.825) (Figure 4B). Eosinophil proportions between these processing methods had a moderately strong, significant relationship (rho=0.65) (p=0.02) (Figure 4D).

The sputum classification of ‘eosinophilic’ remained stable in 93% (12/13 patients) and ‘neutrophilic’ in 76% (10/13) of the samples, using the clinical classifications of eosinophilia and neutrophilia being ≥ 2% and ≥61%

respectively. Proportions of the remaining cells in the sputum samples were also compared between each processing method (Table 1) and no significant difference was identified for each cell type measured.

Detection of inflammatory cytokines can also give important information about the inflammatory profile of a patient's airways and has been shown to be increased in samples purified using PBS as opposed to those using DTT.[16] We therefore compared IL-8 concentrations in the PBS supernatant samples separated by the standard method as compared with MD. IL-8 concentrations were not disimilar between the different processing methods, with medians of 13,144 pg/ml and 10,185 pg/ml for standard and MD, respectively (p=0.860) (Figure 5A). A strong, significant correlation was shown between the two processing methods (rho=0.86) (p=0.0006) (Figure 5B).

Discussion

Phenotyping sputum is important to guide treatment, however current methods are time-consuming and thus are not widely used in specialist care. Therefore, we aimed to compare the utility of a streamlined method with a widely used standard process to assess its value for broader adoption in guiding treatment of respiratory diseases. Our study shows that the MD method adequately liberated cells from mucus in order to perform differential cell counts with reasonably consistent granulocyte phenotypes. Furthermore, it gave similar yields and IL-8 concentrations to the longer non-streamlined standard sputum purification method.

Cell viability has been used as a measure of the value of sputum collection methods and thus may be considered an important measure of sputum quality.[19] However, no studies were found which provided conclusive stepwise differences in cell viability in relation to differential cell counts. Sputum viability counts tend to rely on visual inspection using trypan blue exclusion method on a Neubauer haemocytometer and are therefore subjective. Many studies involving sputum do not report cell viability, some report acceptable cell viability as greater than 70%[20] others have more inclusive quality control of greater than 50% viability.[21-23] In the absence of a conclusive measure of processing technique effectiveness we measured both cell viability and cell yield to compare processing methods. In this pilot study we processed spontaneous sputum which is known to be less viable than induced sputum[19], therefore values obtained are comparative and may not be referenceable to the values of induced sputum. Median cell viability was lower using the MD method but measures were not consistently lower and this was not statistically significant. Variability within the sample

may have contributed to part of the differences in viability. However, a larger future study would prove useful in more conclusively understanding potential differences in cell viability between these two methods.

Sputum differential cell counts remain the gold standard analysis for moderate to severe asthma.[6-8] Yet, there are few centres able to provide sputum cell analysis.[6,8] Arron and colleagues identified the need for technically straightforward means of phenotyping asthma because current sputum processing techniques are time and resource heavy.[17] Yet, Brightling and colleagues argue that the healthcare burden of exacerbations, key drivers of respiratory disease progression, exceed the cost of sputum analysis and recommend sputum analysis be available in all specialist centres.[1,4,24-26]

As our understanding of the diseased lung evolves, a cost effective, technically straightforward and direct window to the lung would provide a broader, more cost-effective analytical platform than seeking surrogate indirect measures for each emerging treatable trait of each respiratory disease.[1,5,10-12] Any additional mediator measures available from sputum supernatant would provide additional potential to understand emerging knowledge of the airway milieu in disease.

Unlike differential cell counts in blood which are often automated, sputum differential cell counts are performed by visual inspection and manual counting of over 400 cells per expectoration. This measure therefore remains subjective and requires specialist training. Fluid phase markers measured in PBS sputum supernatant can distinguish between granulocytic phenotypes in both asthma and COPD[16,27] and appear stable.[28] Although differential cell counts are a useful measure of granulocyte presence they give no indication of granulocyte activation. Fluid phase markers of granulocyte activity correlate more strongly with asthma control and lung function than granulocyte proportion.[29] As expected, IL-8 remained detectable and consistent when measured in PBS supernatant from the standard and MD processing methods. If the other markers reported by Bafadhel[16] are also intact in the MD sample processing method this could eliminate the need for any subjective, specialist analysis of sputum in the absence of useful automated differential cell counts.

The MD method is 70 minutes faster and a more technically straightforward process for obtaining sputum cells and fluid phase markers in PBS for analysis. Studies requiring a differential cell count and cytokines may be able to use the MD method to increase processing capacity, facilitating clinical lung research. The MD method requires an initial investment of a mechanical cell dissociator. However, the overall reduction in resource demands using the MD processing methods could make sputum analysis available to a wider patient

population which is likely to improve patient care. Future development and validation of fully automated techniques will, however, be essential for full introduction of sputum analysis into everyday care.

The limitations of this pilot study include the small number of spontaneous sputum samples at a single study site. This, therefore, made it difficult to demonstrate reproducibility and conclusively rule out potential differences in viability and cell type proportions between the two methods which may be distinguishable in a future larger multi-centre study. Spontaneous sputum samples were used for this pilot study, yet cell viability is lower in spontaneous sputum[19], further analysis would benefit from the use of induced sputum. Differential cell counts did, however, have a fair to strong positive correlation between MD and standard processing methods. Samples were not split for dual analysis using the same technique, nor were patients requested to produce repeated samples, samples were not spiked to demonstrate uniformity when sputum samples were split. Therefore, any differences may be attributable to differences in the plugs. Sputum plug consistency may also vary across disease pathologies through differences in cell concentration or properties of the mucus. Larger studies of sputum from a breadth of diseases encompassing these additional considerations will be useful to investigate the power of these analyses in a range of different samples in the future.

In summary the MD method is a potential alternative technique for sputum processing compared to the current slow and resource-intensive method. In this pilot study, this new method was quicker and more streamlined and showed no inferiority to the current processing methods with regards to cell viability, cell yield, granulocytic phenotype or protein stability.

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Data availability statement

Any additional data or information related to the study is available upon reasonable request to the corresponding author.

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Tables

Table 1 – The effect of processing method on sputum macrophages, lymphocytes and columnar epithelial cells

Proportion of each cell type	n	Median [IQR]		Spearman's rho		Wilcoxon signed rank test
		Standard method	MD Method	Correlation coefficient (rho)	Significance (p)	Significance (p)
% Macrophages	13	16.0 [22.5]	18.5 [23.85	0.776	**0.003**	**0.455**
% Columnar epithelial cells	13	0.00 [0.75]	0.15 [1.65]	0.505	0.085	**0.281**
% Lymphocytes	13	0.00 [1.0]	0.65 [0.75]	0.359	0.226	**0.180**

Median, [Inter quartile range]. Correlations between the standard and mechanical disruption (MD) methods analysed using Spearman's rho. Differences between methods used analysed using Wilcoxon signed rank.

Figure Legends

Figure 1 Summary of steps of each sputum processing used. Standard method as described by Bafadhel, and the mechanical disruption (MD) method trailed as a rapid alternative method.[16]

Figure 2– Effect of processing method on cell viability (A and C) and yield (B and D).

Graphs A and B analysed using Wilcoxon signed rank test, blue dots represent measures from the standard method of processing and red triangles represent measures from the mechanical disruption (MD) method. Lines show paired samples. * p=0.027. Graphs C and D analysed using Spearman's rho. Graph D axes are not equal y axis is 3-fold greater than x axis

Figure 3 – Effect of processing method on squamous cell contamination.

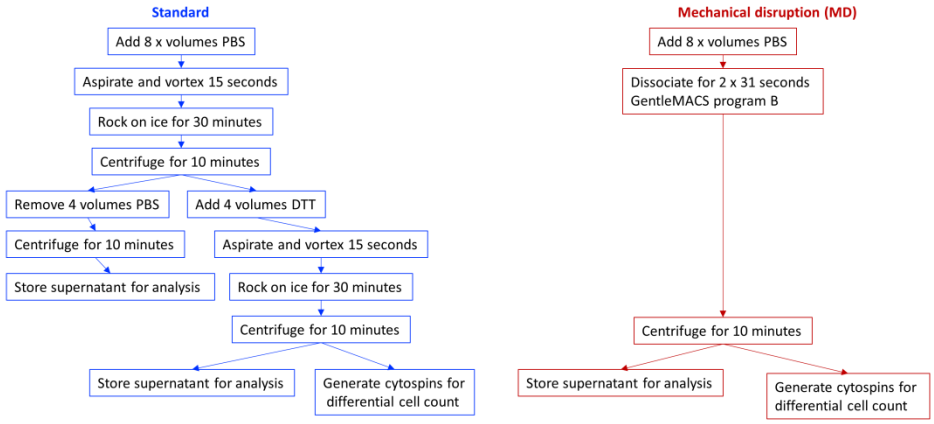
Graph A analysed using Wilcoxon signed rank test, blue dots represent measures from the standard method of processing and red triangles represent measures from the mechanical disruption (MD) method. Lines show paired samples. * p=0.023. Graph B correlation of the percentage of squamous contamination between the 2 methods analysed using Spearman's rho.

Figure 4– Effect of processing method on sputum neutrophil (A and C) and eosinophil (B and D) proportions.

Graphs A and B analysed using Wilcoxon signed rank test, blue dots represent measures from the standard method of processing and red triangles represent measures from the mechanical disruption (MD) method. Lines show paired samples. Graphs C and D analysed using Spearman's rho.

Figure 5– Effect of processing method on IL-8 detection.

Graph A IL-8 concentrations in PBS sputum supernatant using each processing technique analysed using Wilcoxon signed rank test, blue dots represent measures from the standard method of processing and red triangles represent measures from the mechanical disruption (MD) method. Lines show paired samples. Graph B IL-8 concentrations in sputum supernatant analysed using Spearman's rho.



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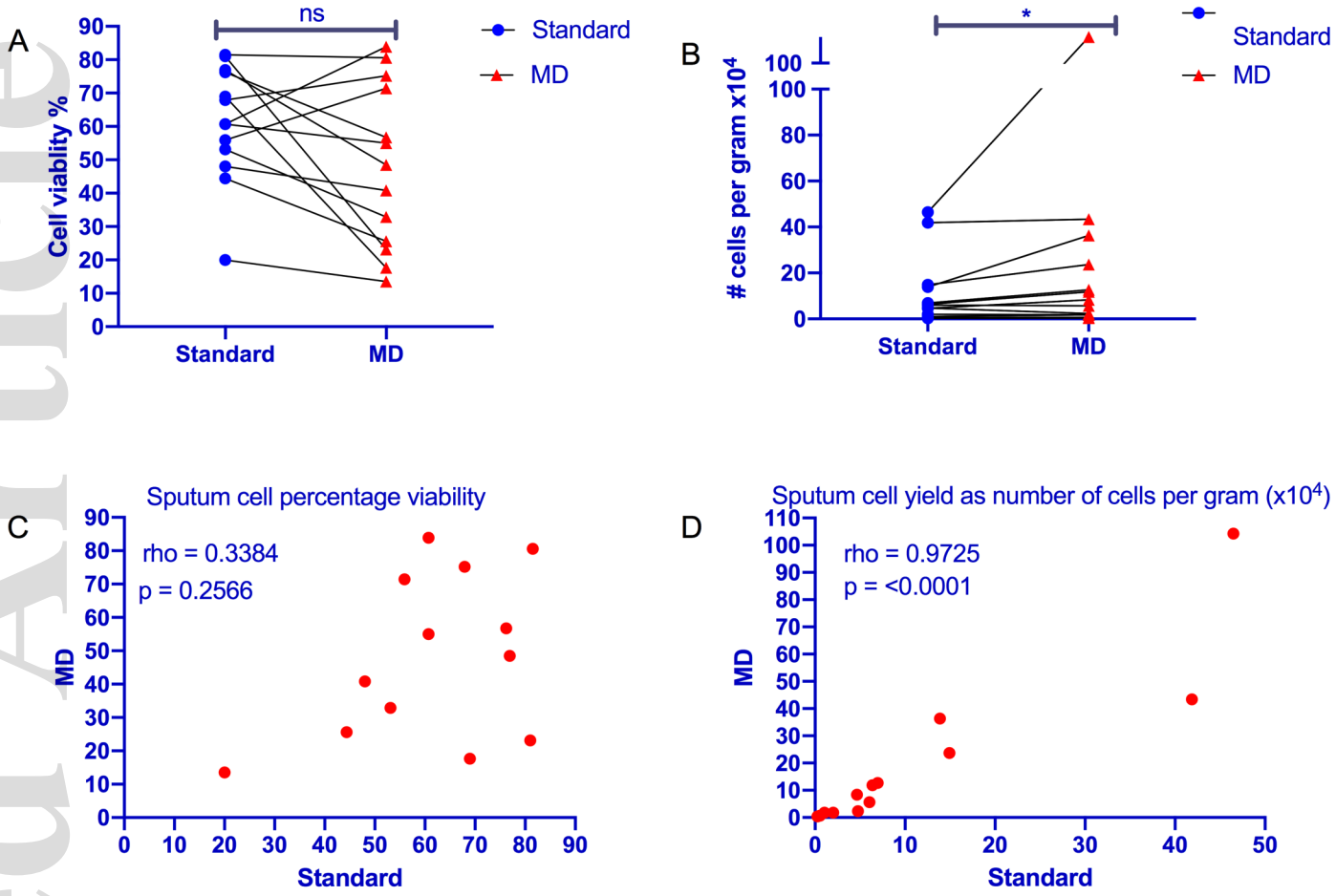

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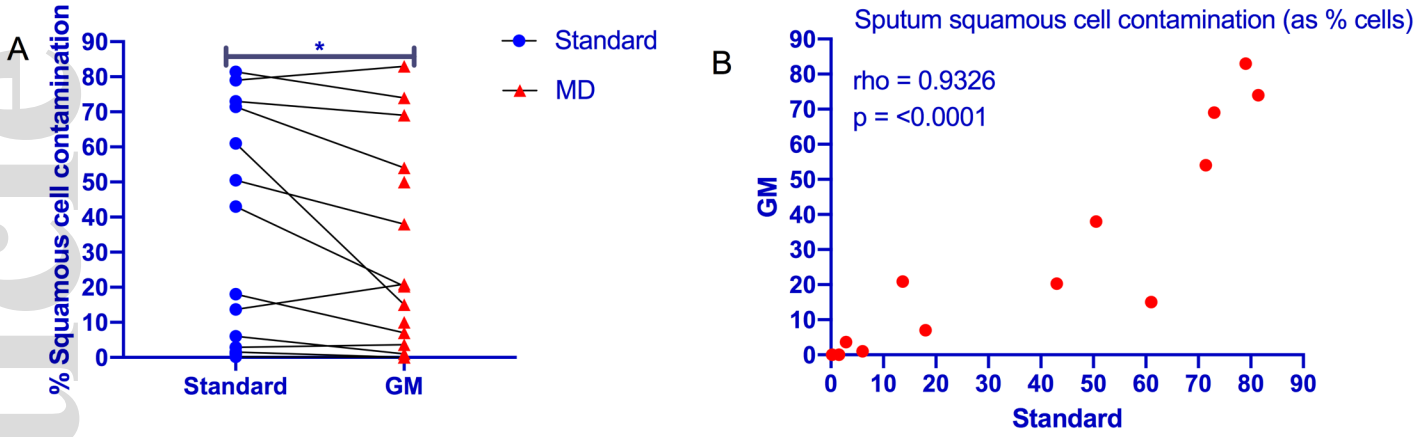

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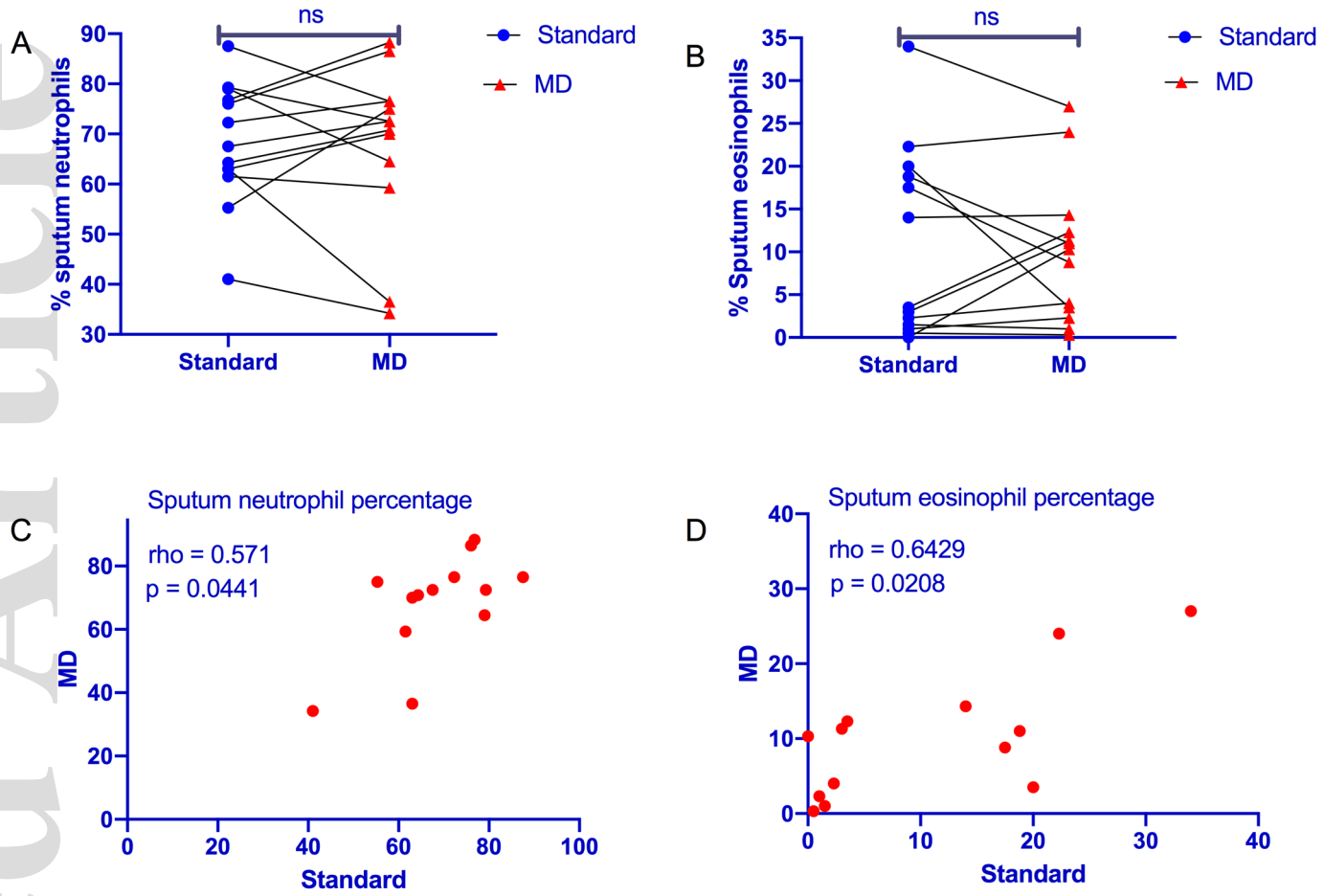

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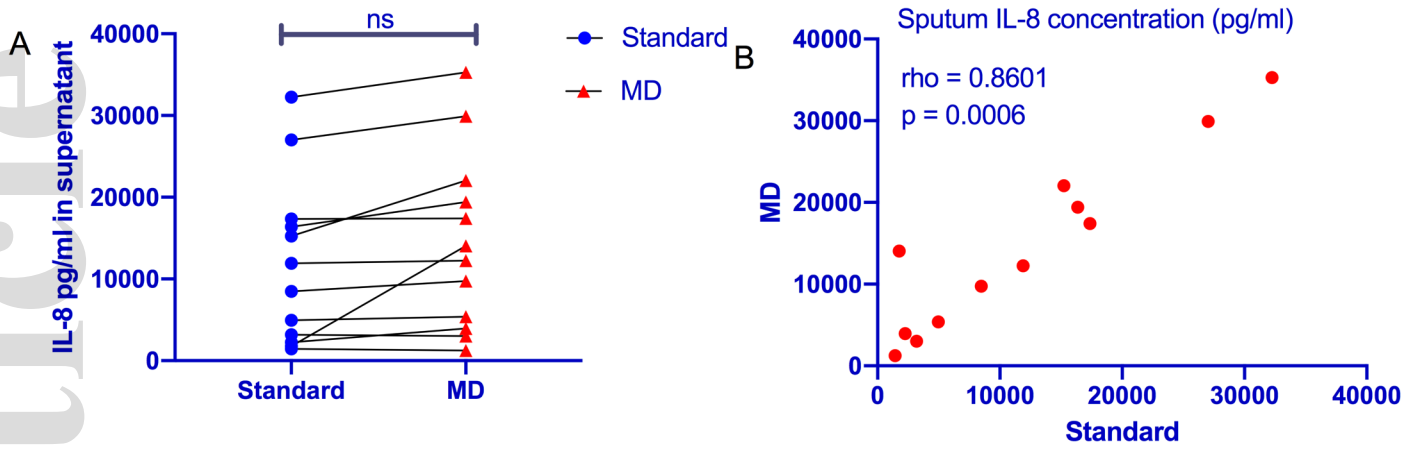

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