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The Beneficial Effects of Oral Cotrimoxazole upon Likely Biomarkers of Oxidative Stress in Advanced Fibrotic Lung Disease

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Abstract

Introduction: In cases of idiopathic pulmonary fibrosis, we have observed an elevation in mean red cell volume, serum gamma glutamyl transferase and peripheral monocyte counts, initially in a pilot study but also in new incident cases. These changes could not be explained by drug therapy, vitamin deficiency or other diseases. **Method:** We compared the peripheral blood abnormalities in 149 patients with lung fibrosis to 448 age and sex matched controls. We also examined the effect of cotrimoxazole treatment for 12 weeks on these abnormalities. From the pilot study of cotrimoxazole in lung fibrosis patients, the relationship of the peripheral blood monocyte count and serum cytokine transforming growth factor beta-1 was examined. Epstein Barr viral status was examined in a selection of patients in case it explained our observations. **Results:** The findings confirm the elevation in mean red cell volume, gamma glutamyl transferase and peripheral monocyte counts in patients compared with matched controls. Oral cotrimoxazole ameliorated these 3 blood abnormalities. Serological evidence of Epstein Barr viral infection was present in tested patients but active viral replication was absent. The monocyte count had a linear relationship with the serum transforming growth factor beta-1 levels, which increased by 600 pg/ml for every of 0.1×10^9/l increase in the monocyte count. **Conclusion**: These observations may reflect oxidative stress which was reduced by cotrimoxazole. A related sulphonamide "dapsone" is known to reduce oxidative stress through direct effects on neutrophil and mo-nocyte function; similar effects may explain these findings and require a formal study.

Keywords

Oxidative Stress, Cotrimoxazole, Idiopathic Pulmonary Fibrosis, Monocytes, Gamma Glutamyl Transferase, Transforming Growth Factor Beta 1

1. Introduction

In 2008, we published our pilot study examining the effects of oral cotrimoxazole upon lung function, exercise capacity and quality of life scores in patients with advanced fibrotic lung disease who were treated with oral cotrimoxazole 960 mg BD and folic acid 5 mg 3 times a week [1].

During the selection of these patients, their blood tests' pre-randomisation showed three unexpected peripheral blood abnormalities with most patients displaying all three. The abnormalities were:

1) Raised red cell mean cell volume (MCV) on the peripheral blood count.

2) Raised serum gamma glutamyl transferase levels (GGT).

3) A raised peripheral blood monocyte count (Mo).

These abnormalities could not be explained by past or present medication or vitamin (B12/folate) deficiency. The elevation in GGT was isolated without explanation from alcohol intake, gallstones and past hepatitis (viral, auto-immune or drugs). All abdominal ultrasounds were returned as normal. The monocytosis was persistent, without any viral illness or active Epstein Barr Virus; nor other explanation and the blood film appeared normal.

Scrutiny of all newly presenting patients with idiopathic pulmonary fibrosis (IPF), revealed the same patterns at the initial diagnosis.

Literature searches have revealed data on lung oxidative stress and the metabolism of glutathione which could suggest a potential link for all three abnormalities as biomarkers of lung oxidative stress [2] [3] [4].

Glutathione (GSH) is the most abundant antioxidant in human lung and an important protector of the biomembrane [5] [6]. Levels in lung fluid are 140 times the plasma level where it functions to protect the lung against oxidative stress [4]. Clinical observations show that lung epithelial lining fluids from subjects with usual interstitial pneumonitis/idiopathic pulmonary fibrosis (UIP/IPF) were depleted of reduced glutathione, suggesting a redox imbalance (oxidant/anti-oxidant balance) in this disease [3]. This was shown to be improved by oral N-acetyl cysteine (NAC) which can generate glutathione, and led to the NAC clinical trials [7] [8]. Early published studies of this drug showed the vital capacity and transfer factors for carbon monoxide to diminish less in the NAC group compared with placebo at 12 months.

GGT is a plasma membrane ecto-enzyme that plays a key role in glutathione metabolism [9] [10] [11]. GGT catalyses the breakdown of glutathione to glutamate and cysteinyl glycine. These are then transported back into the cell for re-synthesis of glutathione. This is critical to prevent oxidative injury [12] [13]. While GGT has been considered an anti-oxidant, recent studies suggest that

GGT is up-regulated in response to oxidative stress and GGT cleavage of glutathione induces **R**eactive **O**xygen **S**eries (ROS) production [14] [15] [16]. In mice, the genetic absence of GGT (GGT-/-) protects the lung against bleomycin induced lung fibrosis through maintained glutathione levels [17].

In our present study, we compare our peripheral blood findings in patients with UIP/IPF or fibrotic nonspecific interstitial pneumonitis (NSIP) or mixed patterns with age and sex matched controls. We also examined the effect of cotrimoxazole treatment upon these parameters and the relationship between the peripheral blood monocyte count and the serum TGF beta-1 levels in the pilot study patients. A sample of patients had their Epstein Barr viral (EBV) status checked for active viral copy numbers in case EBV reactivation was related to the blood changes.

2. Methods

Study objectives:

1) To assess the 3 peripheral blood abnormalities in 149 new cases of IPF (UIP or fibrotic NSIP or mixed UIP/NSIP) and compare with 448 age and sex matched healthy controls measured in the same laboratory.

2) To examine the effects of cotrimoxazole treatment on the 3 parameters for all 73 treated patients.

3) To examine changes in the 3 blood parameters (red cell MCV, GGT and monocyte count) on time course graphs in response to cotrimoxazole treatment over 12 weeks from the pilot study data (2 weekly blood tests).

4) To examine the relationship between the peripheral blood monocyte count and the serum transforming growth factor beta 1 (TGF beta-1) levels in the pilot study data.

5) To examine EBV status including active viral copy numbers in a selection of patients.

The pilot study of cotrimoxazole was approved by the London-Surrey borders regional ethics committee 1999 [1] with expansion and reapproved by the same committee for a further study 2006, ISRCT80334919.

2.1. Blood Analysis

Each patient or control had their mean red cell volume and monocyte count measured by the Advia 120, BAYER Haematology systems Auto analyser. The GGT and liver function tests were measured in the Biochemistry department using an Advia 2400 Bayer chemistry systems. The GGT measurement uses synthetic gamma-glutamyl p nitroanilide and glycyl glycine as an accelerator for the gamma-glutamyl residue, from which p-nitroaniline is liberated. This liberated product has absorption near 400 nm and the rate of formation is measured photometrically at 410 nm. The advia 2400 method for serum GGT confirms also that it measures GGT involved in aspects of glutathione metabolism [18] in the biochemical data.

2.2. Serum Transforming Growth Factor Beta-1 (TGF Beta-1)

Serum TGF beta-1 was measured by Elisa kit supplied by R and D systems, Ab-

ingdon, Oxford, UK. This kit had specificity for TGF beta-1, TGF beta-1.2 in the serum with a detection level of 0.25 ng/ml. Samples were measured in duplicate. Coefficient of variation for assay was 4% - 7% for repeat measurements.

2.3. EBV Serology

The Trinity Biotech Captia™ Epstein-Barr Viral Capsid Antigen (EBV VCA (P-18)) IgG kit is an Enzyme-Linked Immunosorbent Assays (ELISA) for the qualitative determination of IgG antibodies in human serum to capsid and nuclear antigens which can indicate past infections with other Epstein-Barr as an aid in the diagnosis of infectious mononucleosis.

2.4. Epstein Barr Active Viral Copy Number Assessment (Table 1 and Table 2)

Quantitation/detection of EBV DNA was carried out at Micropathology Ltd. by nested real-time PCR as follows: DNA was extracted from 200 microliters specimen using a Qiagen MDx Bio-Robot running the QIA amp DNA Blood kit/protocol and was eluted in 200 microliters extraction buffer. 20 microliters of DNA extract from each specimen was subjected to 25 cycles of PCR using an outer primer set targeting the EBV nuclear antigen 1 (EBNA-1) gene. 1 microliter of amplicon from this PCR was used as template for a further 25 cycles of real-time PCR using an inner primer set, which was carried out using a Roche Light Cycler 480. PCR products were analysed by melt curve analysis. Where EBV DNA was detected this was quantified with respect to appropriate standards.

2.5. The Fibrosis Patients (Table 1 and Table 2)

A total of 149 new patients presenting clinical features of IPF (mean age 72, range 41 - 92 yrs, and 52% male) were prospectively identified from our respiratory clinics. Their diagnosis followed the guidelines at their time of selection (ATS/ERS consensus statement and British Thoracic Society 2008) [19] [20]. Patients under 50 yrs usually had a lung biopsy confirmation (5 cases).

Baseline blood results were documented (including B12 and folate, liver function, GGT and FBC) prior to any treatment along with review of their medical notes, clinical examination, medication, lung function, X rays and CT scans. No patients were taking NAC at the time of this data collection. Their HRCT scans were classified according to the method of Hansel and Wells by multidisciplinary (MDT) radiological review [21]. Current smokers were excluded from the analysis along with patients with significant emphysema on their HRCT scan.

2.6. The Controls (Table 1)

448 age and sex matched controls were selected prospectively during the same time period. The control group were selected from a large group of patients attending for their pre-operative assessment for day surgery. Patients under the day surgery unit must be judged healthy without any significant cardio-respiratory diseases

Table 1. Demographics of cases and controls and inclusion criteria.

Demographics	IPF cases	Pre-operative day-case controls
No of cases	149	448
% male	52	58
Mean age and range	72 (41 - 92)	73 (40 - 80)
Caucasian	81%	75%
Smoking status	90% ex-smoker	Non-smoker or <10 pack years
Chest X ray	Abnormal	normal
Normal ECG	Variable	100%
Past cardiorespiratory disease	Yes	None
Diabetes	33%	None
Alcohol intake units/week	0 - 6	0 - 1
Age match	Number of cases-149	Number of cases-448
35 - 40 yrs	2	40
45 - 54 yrs	10	70
55 - 64 yrs	20	106
65 - 75 yrs	40	110
75 yrs+	77	122
Criteria for IPF cases (major and minor criteria) as per ATS/ERS criteria	Insidious onset Bibasilar inspiratory crackles Progressive dyspnoea Duration > 3 months CT consistent with UIP/NSIP or mixed ± lung biopsy Restrictive pulmonary function of reduced VC, FVC, TLCO, KCO Exclusion of other causes of interstitial lung disease	

that could preclude day-case anaesthesia and early discharge post-operatively. These assessments included blood tests, ECG and chest x rays, history and examination and medication check by the supervising specialist nursing sister. Non-smoking patients or ex-smokers (<10 pack years) without significant alcohol intake and a normal chest X ray were selected if of suitable age and sex for our control group. Their pre-operative blood tests were measured in the same laboratory which offered the best possible match for the IPF patients.

2.7. The Effects of Cotrimoxazole Treatment on the 3 Blood Parameters at 12 Weeks (Table 3)

All 73 patients who received cotrimoxazole (46 male, 27 female) had their pre-treatment blood tests compared after 12 weeks (cotrimoxazole 960 mg BD and folic acid 5 mg 3 times a week).

The pilot study: cotrimoxazole time course studies the change in MCV, GGT and Mo has been plotted from the pilot study data (where it was collected double-

Table 2. Peripheral blood markers (means ±SD) for MCV (mean cell volume), GGT (gamma glutamyl transferase) and Mo (monocyte count) in controls and fibrosis patients.

Parameters (normal range)	MCV (84-98 fL)	P Value Vs C	GGT (7-40U/L)	P Value Vs C	Mo (<0.6 x10^9/L)	P Value Vs C
Controls (C) n = 448 mean ± SD	88 ± 4.4		23±13		0.44 ± 0.2	
Fibrosis (all cases) n = 149 mean ± SD	93.4 ± 6.7	<0.0001	53.0 ± 38	<0.009	0.64 ± 0.2	<0.0001
UIP n = 104 mean ± SD	93 ± 6.8	<0.0001	54.6 ± 29	<0.0001	0.65 ± 0.2	<0.0001
NSIP n = 31 mean ± SD	93 ± 7.7	<0.0001	38.2 ± 26.6	<0.0001	0.60 ± 0.2	<0.0001
Mixed UIP/NSIP n = 14 mean ± SD	94.4 ± 5.3	<0.0001	44.8 ± 29	<0.0001	0.68 ± 0.2	<0.0001

blind) every 2 weeks in the active n = 10 and placebo n = 10 groups.

Monocyte/TGF beta-1 regression analysis

TGF beta-1 levels (measured only in the pilot study) and the peripheral monocyte counts were examined by linear regression. The peripheral monocyte count (pre-randomisation n = 20) were compared with the TFG beta-1 serum levels from the same blood sample to examine the relationship between monocyte count and TGF beta-1 levels.

2.8. Statistics

2.8.1. Comparison of Controls and Fibrosis Patients

The fibrosis patients (n = 149) had their 3 blood parameters compared with the age and sex matched controls blood results (n = 448) by unpaired t tests. All the variables were tested for normality using the Shapiro-Wilk W test for normal data. Given the multiple testing procedures, the Bonferroni correction has been applied making the reference level for statistical significance 0.016 for these comparisons. This included subgroup analysis for UIP and fibrotic NSIP patients.

2.8.2. Assessment of Cotrimoxazole Treatment at 12 Weeks

Blood results for the 73 patients treated with cotrimoxazole were compared at 12 weeks using a paired t test. All the variables demonstrated normality using the Shapiro-Wilk W test for normal data, permitting a paired t-test for the differences in pre and 12 week means if normality applied. Identical p values were obtained by Wilcoxon-Mann-Whitney test for the difference in the distributions of the pre-and 12 week measurements. Given the multiple testing procedures, the Bonferroni correction was applied and the reference level for statistical significance has been dropped to 0.05/3 = 0.016.

Table 3. The effects of cotrimoxazole in lung fibrosis on the peripheral blood changes showing means ± SD before treatment and at 12 weeks in ILD MCV (mean cell volume), GGT (gamma glutamyl transferase), Mo (monocyte).

Parameters (normal range)	MCV 84 - 98 fL	p Value Pre-post treatment	GGT 7 – 40 U/L	p Value Pre-post treatment	Mo $<0.6 \times 10^9$/L	p Value Pre-post treatment
pre-treatment n = 73 (UIP/NSIP) mean ± SD	93.7 ± 6.5		49.6 ± 35		0.67 ± 0.2	
cotrimoxazole treated week 12 n = 73 (UIP/NSIP) mean ± SD	90.1 ± 5.0	<0.0001	30.6 ± 31	<0.0011	0.44 ± 0.2	<0.0001
UIP patients pre-treatment n = 53 mean ± SD	94.3 ± 6.1		51.04 ± 37		0.65 ± 0.2	
UIP patients Week 12 cotrimoxazole n = 53 mean ± SD	90.5 ± 4.8	<0.0001	31.1 ± 31	<0.0076	0.430.2	<0.0001
NSIP patients pre-treatment n = 20 mean ± SD	92.5 ± 7.4		46.1 ± 21		0.72 ± 0.3	
NSIP patients week 12 week co-trimoxazole n = 20 mean ± SD	88.1 ± 5.4	<0.0001	29.4 ± 31	<0.0001	0.45 ± 0.2	<0.0001

Pilot study graphs the 2 weekly group means for the10 active and 10 placebos were calculated and compared for each time point by Mann Whitney U test with significance at the 5% level. With comparison of baseline measurements (week 0 with week 12) were made by unpaired t test. Graphs were plotted by Graph Pad prism for MCV, GGT and Mo.

2.8.3. Assessment of Monocytes and TGF Beta-1 by Linear Regression

TGF beta-1 values in pg/ml and monocyte counts (10^9/L) were plotted by Graph Pad Prism computer software. The line of best fit and 95% prediction intervals showed by the dotted lines are shown along with calculation of the slope. The p value is displayed on the graph with significance at the 5% level. The correlation coefficient was calculated from the regression line.

3. Results Section

The findings are displayed in tables and figures as described below.

3.1. Peripheral Blood Parameters

Table 2 shows the laboratory units, normal ranges, means ± SD with p values for

the 3 blood measurements in the age/sex matched controls (n = 448) and fibrosis patients (n = 149). There were 3 matched controls for each fibrosis patient over the age of 65 yrs and 4 matched controls for those less than 65 yrs of age. The age ranges and percentage of males were well matched.

Mean volume cell was lower in the healthy control group 88fL (95% CI 87.9 - 88.9) compared with the fibrosis group 93.41fL (95% CI 92.8 - 95.4) $p < 0.0001$, including fibrotic subtypes (Table 2).

Gamma Glutamyl transferase (Table 2)

The mean GGT level for the healthy control group was 23U/L (95% CI 21.7 - 24.3), compared with the fibrosis group mean 53 U/L (95% CI 41.9 - 64.7) $p < 0.009$.

Peripheral monocytes (Table 2) Mean Mo counts in the healthy controls were 0.44×10^9/L (95% CI 0.42 - 0.46), with fibrosis group mean 0.64×10^9/L range (95% CI 0.59 - 0.68), $p < 0.0001$.

3.2. Table 3 Analysis of All Fibrosis Patients Treated with Cotrimoxazole Treatment (n = 73)

Mean MCV (Table 3) pre-treatment was 93.7 fL (95% CI 92.2 - 95.2), and fell to 90.1 fL (95% CI 88.9 - 91.3) after 12 weeks of treatment $p < 0.0001$. Findings were similar for the subgroups. Statistically significant differences between pre and post means of 3.30 (95% CI 2.43 - 4.77) were obtained.

GGT levels (Table 3) pre-treatment means were 49.6 U/L (95% CI 35.1 - 64.2) and fell by 40% to 30 U/L (95% CI 23.3 - 38.0) after 12 weeks of cotrimoxazole treatment, $p < 0.0076$.

The distributions of both pre-treatment GGT and post-treatment GGT values exhibit strong departures from normality ($p < 0.0001$) and remains so even after log transformation. Therefore, the Wilcoxon-Mann-Whitney test reveals strong evidence against the null hypothesis ($p < 0.0001$).

Monocytes (Mo) Table 3

Peripheral monocytes showed a pre-treatment mean count of 0.67×10^9 /L (95% CI 0.61 - 0.74) which fell by 35% to 0.44×10^9/L (95% CI 0.38 - 0.50) at 12 weeks $p < 0.0001$. This was similar for the subtypes analysis (UIP reduced by 34%, fibrotic NSIP 37%) $p < 0.0001$ respectively.

The distributions of both pre-treatment Mo and post-treatment Mo counts exhibit strong departures from normality ($p < 0.0001$) but after log transformation they were consistent with normal distributions ($p > 0.05$). The Wilcoxon-Mann-Whitney test applied to the original scale data ($p < 0.0001$) demonstrated a difference in the pre and post Mo distributions. This is confirmed by the paired t-test applied to the log transformed data ($p < 0.0001$) which quantify the difference between the means of the log transformed variables: mean difference (pre-post) = 0.50 (95% CI 0.37 - 0.62).

3.3. Pilot Study Time Course Data for Peripheral Blood Markers

Pilot study Time Course graphs: MCV (Figure 1) shows the group means ± SEM for week 0 - 12 for the 10 active and 10 placebo treated patients. The pla-

cebo group shows no consistent change, while the active group shows a decline which is significant after week 6. Comparison of week 0 with week 12 for the active group confirms a significant reduction $p < 0.0001$ which is not seen with the placebo group $p = 1.0$.

Time course Graphs: GGT (Figure 2) shows the group means ± SEM. The placebo group shows no consistent change $p = 0.97$. The active group decreases significantly over the 12 weeks ($p < 0.0145$).

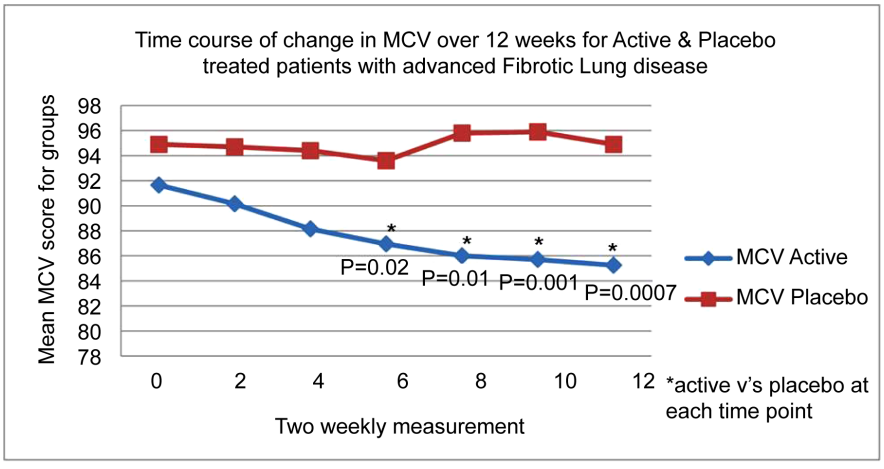
Time course graphs: Mo (Figure 3) shows the group means ± SEM. The placebo group shows no change (week 0 - 12) $p = 0.64$, while the active group declines from a mean of 0.9×10^9/l to 0.35×10^9/l ($p < 0.003$). At week 0 the Mo count is significantly above the placebo $p = 0.036$, while at 12 weeks it is significantly below $p = 0.035$.

3.4. Monocytes and the Relationship to Serum Transforming Growth Factor Beta-1 from Pilot Study Data: Figure 4

Linear regression analysis with the line of best fit and 95% prediction intervals are shown for all pre-randomised patients in the pilot study ($n = 20$). This gave a slope of 5101 ± 766 $p < 0.0001$ with a correlation coefficient of **r = +2.1**. TGF beta-1 levels increased by 600 pg/ml for every 0.1×10^9/L increase in peripheral blood Mo count.

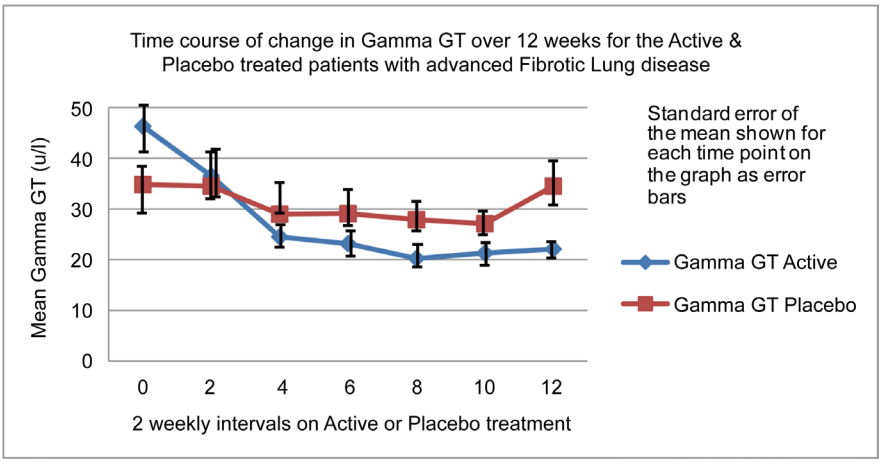
After 12 weeks the active group ($n = 10$) Mo counts were reduced (Figure 3), but the relationship and slope of monocytes to TGF beta-1 levels remained unchanged and significant ($p = 0.0165$, slope 4851 ± 1606).

For the placebo treated group at 12 weeks, the relationship between monocyte count and TGF beta-1 remained identical to pre-randomisation and significant (slope 5190 ± 1226 $p < 0.002$). Data are not shown.



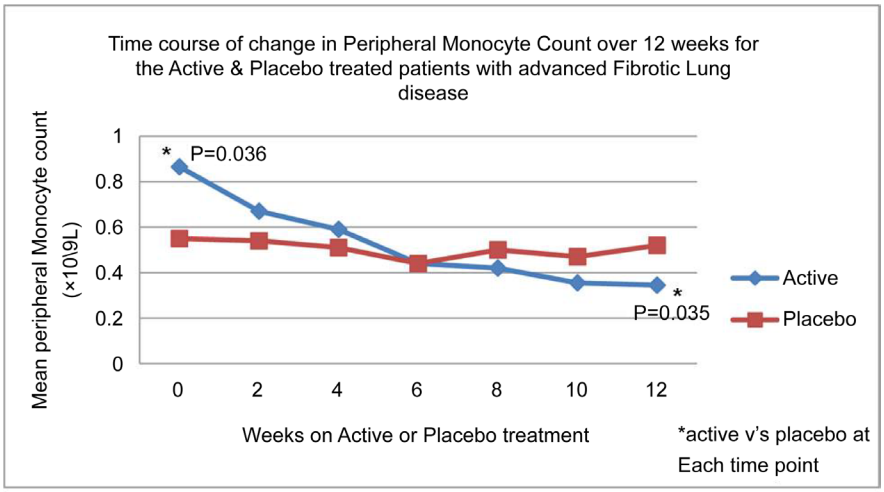
Active SEM	1.2	1.5	1.9	1.7	1.9	1.7	1.6
Placebo SEM	1.9	1.8	1.8	1.6	2.3	1.8	1.8

Figure 1. Group means ± SEM for mean cell volume (MCV) measured 2 weekly in the 10 active and 10 placebo treated patients from the original 12 weeks pilot study.



		Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
Active	95% CI	24.2-67.8	19.6-53.3	17.3-31.7	17.5-28.7	15.3-25.1	14.9-27.6	15.2-28.4
Placebo	95%CI	15.6-53.9	15.4-53.6	16.2-41.7	17.6-40.5	18.4-37.3	17.5-36.6	18.1-50.3

Figure 2. Group means ± SEM for gamma glutamyl transferase (GGT) measured 2 weekly in the pilot study (10 active and 10 placebos) over 12 weeks. SEM is show on the graph and the 95% confidence intervals in the table below.



Active SEM	0.13	0.07	0.07	0.06	0.05	0.04	0.03
Placebo SEM	0.05	0.06	0.09	0.08	0.05	0.06	0.07

Figure 3. Group means ± SEM for peripheral monocyte counts (Mo) measured 2 weekly in the pilot study (10 active, 10 placebos) over 12 weeks of cotrimoxazole treatment.

TGF beta-1 levels (active group) were 4394 pg/ml and fell to 3633 ng/ml at week 12 (p = ns). In the placebo group 3387 pg/ml at start and 3115 ng/ml at 12 weeks (p = ns) with no difference found between the active and placebo groups at 12 weeks.

EBV virology screens; All patients with IPF were positive EBV capsid antigen

and nuclear IgG. 30 patients were examined for active viral DNA copy numbers in their white cells but none showed evidence active replication.

Figure 5, a schematic diagram of the likely mechanisms of oxidative stress and its possible relationship to glutathione metabolism and the 3 observed blood changes.

4. Discussion

The underlying trigger for IPF and fibrotic NSIP is not yet recognised, but oxidative

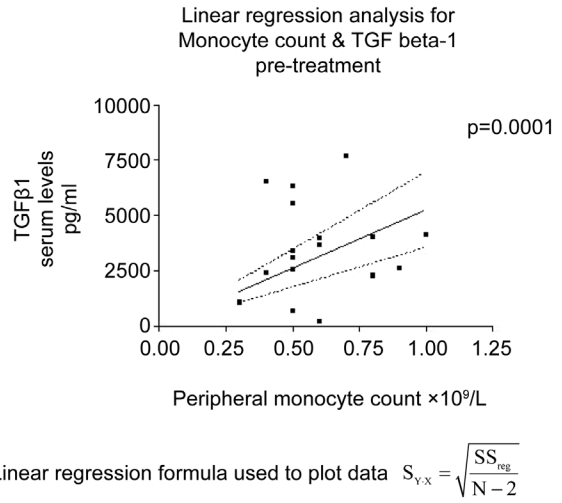


Figure 4. Linear regression analysis for the relationship between pre-treatment Monocyte counts and the corresponding serum TGF-beta1 measurements for the pilot patients (n = 20). The dotted lines show the 95% prediction intervals of the regression line. The formula for the calculation of the linear regression by prism is shown on the graph.

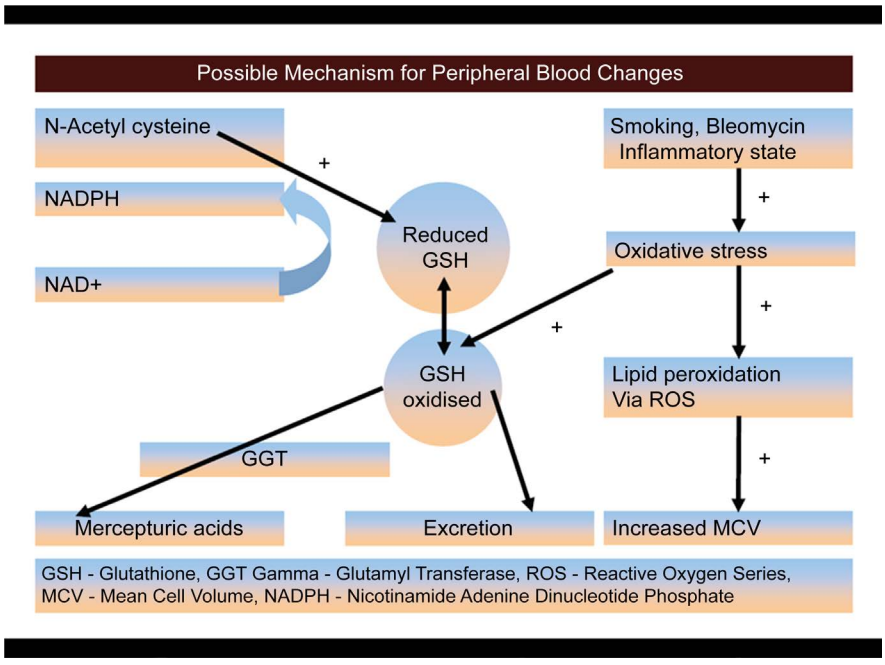


Figure 5. A schematic diagram of the possible mechanisms involved in the peripheral blood changes and their relationship to oxidative stress.

stress and the generation of oxygen free radicals is thought to play a role in the pathogenesis and progression of the disease. This is well described in bleomycin models where superoxide and hydroxyl radicals are generated [22] [23].

Our findings show a pattern of peripheral blood abnormalities in patients with lung fibrosis, irrespective of their subtype which is not seen in the age and sex matched controls. An association between IPF and EBV infection was one of the original hypotheses as a causal agent for IPF. EBV is a herpesvirus prevalent in man with a number of disease associations which include B cell and Burkitts lymphoma, Hodgkin's disease and nasopharyngeal carcinoma [24]. Interestingly the observed blood changes in IPF are similar to those that may be seen in infective mononucleosis (glandular fever). Furthermore, IPF is associated with enlargement of mediastinal lymph nodes, the cause of which is not been explained. EBV has been shown to stimulate the fibrotic cytokine TGF-beta-1 and epidermal-mesenchymal transition which is associated with IPF [25]. From our selected fibrosis patients, all showed antibody evidence of past exposure to EBV but none were positive for active EBV copy numbers in their white blood cells to suggest current active viral replication. A previous study in IPF has shown rearranged EBV genomes (termed WZhet) in 61% lung tissue from IPF patients and 59% of IPF buffy coats while only 4% of healthy blood donors show such a re-arrangement, suggesting an association of WZhet with IPF patients [26].

The published literature on lung oxidative stress could also explain our findings in the absence of active EBV and link them to abnormalities in the glutathione pathways particularly affecting red cell size and GGT levels (**Figure 5**). Glutathione metabolism and its role in IPF have gained interest through the NAC studies. Glutathione catabolism is up-regulated in response to oxidants and cytokines [27] [28], and has an important role in protecting membranes from free radical damage [29]. If mitochondrial glutathione is depleted, permeabilization and death can occur [30]. GGT is a membrane bound enzyme linked to the breakdown of glutathione. The prevention of bleomycin induced lung fibrosis in GGT-/-deficient mice may be related to maintained glutathione levels which reduce apoptosis and fibrosis [17]. This has interesting links to our observations where cotrimoxazole treatment and clinical improvement occurred with a fall in serum GGT. Since glutathione homeostasis is disturbed in IPF, it may indicate that GGT is pro-inflammatory in man also.

Raised serum GGT > 50 U/L is also reported in relapsing cryptogenic organising pneumonias, without evidence of cholelithiasis or liver disease [31] [32], and here chronic lung injury and fibrosis can also occur.

The elevation of MCV may reflect membrane oxidative stress and lipid peroxidation in a similar fashion to liver disease [33] and be connected with the raised Ethane levels described in IPF [34] [35]. Red cells are more vulnerable to free radicals due to their iron content compared to white blood cells [36]. Measurements of Ethane are not routinely available, but are elevated in IPF and are suggested to be valuable as markers of lipid membrane peroxidation [34].

Monocytes are intimately involved with wound repair and fibrosis. M2 ma-

crophages, are the counterparts to TH2 cells and generally anti-inflammatory and produce growth factors including TGF beta-1 and platelet derived growth factors [37]. TGF beta-1 enhances fibroblast differentiation and the synthesis of collagens by myofibroblasts, with high levels reported in honeycomb lung [38] [39] [40]. Monocytes themselves can generate oxygen free radicals to regulate other inflammatory cells [39]. Oxidative stress disturbs normal cytokine regulation and promotes fibrogenesis [40] [41] [42]. In human and animal models of fibrotic organ injury, the site of tissue damage triggers the recruitment of monocytes often via granulocyte monocyte colony stimulating factor [43]. Blocking of these monocytes in animal studies arrests fibrotic processes [44] [45].

The reduced peripheral monocyte count seen with cotrimoxazole treatment could be due to anti-inflammatory effects on the lung cytokine milieu. This could reduce monocytes recruitment and monocyte derived ROS [46] [47].

The linear relationship between the peripheral monocyte count and the serum TGF beta-1 levels in the pilot study are interesting due to the potential significance of this cytokine. After cotrimoxazole treatment, both values fell but the relationship remained the same with an increase of 600 pg/ml of TGF-beta-1 for every increase in monocyte count of 0.1×10^9. One possible explanation is that TGF beta-1 is derived from the blood monocytes. This is an important fibrotic cytokine, linked to the production of ROS, with resistance to apoptosis and epithelial-mesenchymal differentiation [48] [49] [50].

Cotrimoxazole in IPF has gained further attention recently following the publication of the TIPAC study [51]. A double blind randomised placebo controlled study of cotrimoxazole or placebo added to standard care medication (prednisolone, azathioprine, NAC and mycophenolate mofetil); showed no significant improvement in forced vital capacity, transfer factors or 6minute walk test, but a 5-fold reduction in all cause mortality in those adherent to treatment. There were improved health related scores with reduced sudden exacerbations.

The study did not address the mechanism, but the possible antibacterial properties of cotrimoxazole were raised. Bacterial infection in newly presenting cases of IPF has been examined by bronchoscopy with quantitative culture to establish bacterial levels in the lower airways [52] [53]. Pathogens were isolated in 36% of IPF patients but not in healthy controls. The authors questioned whether unrecognised colonisation is a determinant in this disease [54].

Sulphonamides such as sulfamethoxazole and dapsone are structural analogues of para-aminobenzoic acid, and competitively inhibit the first step in the synthesis of folic acid (dihydro-folate) affecting growth and replication of microorganisms. Trimethoprim blocks the next step in folic acid metabolism affecting nucleic acid and protein synthesis [55]. Cotrimoxazole has good penetration to the bronchial secretions. It is active against Pneumocystis jiroveci, streptococcus pneumoniae and a wide range of aerobic gram negative microorganisms [55]. It is used long term in HIV disease and organ transplantation with a good safety record and no significant problems of bacterial resistance/overgrowth.

Dapsone shares cotrimoxazole's sulphonamide ring with similar actions on

folic acid synthesis and bacteria. Detailed studies have shown Dapsone to also have extensive actions on the immune system and oxidative stress. It prevents the formation of oxygen free radicals at sites of infection by inhibiting myeloperoxidase in mononuclear cells and neutrophils through direct effects on these cells [56]. Dapsone has been shown to block lipid peroxidation and maintain glutathione levels in animal models of neurotoxicity [57]. Detailed *in-vitro* studies show substantial anti-oxidative properties attributed largely to inhibition of monocyte and neutrophil ROS production [58], but comparable studies on cotrimoxazole have not been conducted.

5. Conclusion

These findings suggest that the blood abnormalities noted in lung fibrosis patients could fit with oxidative stress leading to monocyte recruitment, rather than active EBV disease. The effect of cotrimoxazole on mononuclear cells may be similar to that described for dapsone, with reductions in oxygen free radicle generation reducing lipid peroxidation and the fibrotic cytokine TGF beta-1. This reduction in oxidative stress may explain the reduced mortality from sudden exacerbations of IPF seen in patient on regular cotrimoxazole. Further studies of cotrimoxazole effects on mononuclear cells and oxidative stress along with exhaled ethane levels are indicated and may increase the understanding of IPF.

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Abbreviations

GSH	Glutathione
GST	Glutathione-S-transferase
MCV	Mean cell volume
GGT	Gamma glutamyl transferase
Mo	Monocytes in peripheral blood
HRCT	High resolution CT scan
UIP	Usual interstitial pneumonitis
NSIP	Non specific interstitial pneumonitis
TGF beta-1	Transforming growth factor beta-1
ROS	Reactive oxygen species
NAC	N acetyl cysteine
GMCSF	Granulocyte monocyte colony stimulating factor
EBV	Epstein barr virus
IPF	Idiopathic pulmonary fibrosis