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Original Article

# Sputum neutrophils in cystic fibrosis patients display a reduced respiratory burst

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**Abstract**

*Background:* Few data exist on the functional activity of airway neutrophils in the milieu of the cystic fibrosis (CF) lung. We assessed reactive oxygen species (ROS) production by sputum neutrophils and the relationship to neutrophil viability. Identical assessments were made on peripheral blood neutrophils from CF patients.
*Methods:* ROS production in sputum neutrophils was assessed in 31 CF patients at varying phases of clinical disease using flow cytometry. Twenty patients provided blood samples (including 16 who also provided a matched sputum sample). Neutrophil viability was determined using dual annexin V (apoptosis) and propidium iodide (necrosis) staining. Comparative peripheral blood data were obtained from 7 healthy controls.
*Results:* ROS production was reduced in sputum compared to blood neutrophils and they demonstrated a higher level of necrosis. Subpopulations of neutrophils with different ROS production capacity were apparent in peripheral blood. Lung function was positively associated with both the proportion of blood neutrophils demonstrating increased ROS production and the proportion of apoptotic sputum neutrophils.
*Conclusions:* CF airway neutrophils display functional exhaustion. Healthier lungs in CF appear to be associated with subpopulations of blood neutrophils with increased oxidative burst capacity and evidence for increased neutrophil apoptosis within the airway.


*Keywords:* Cystic fibrosis; Neutrophils; Oxidative stress; Respiratory burst; ROS production

## 1. Introduction

Airway inflammation in cystic fibrosis (CF) is characterised by profound neutrophil infiltration, which is ineffective and fails to eradicate pathogens such as *Pseudomonas aeruginosa* (*P. aeruginosa*). There has been speculation as to whether the immune response is normal in CF, but the inability to clear bacterial infection strongly suggests functional abnormalities. In healthy, non-CF individuals the mechanism for removal of apoptotic neutrophils by macrophages following microbial destruction is very tightly regulated [1,2]. There is evidence of abnormal clearance of apoptotic airway neutrophils in CF with resultant progression to secondary necrosis contributing to ongoing inflammation and tissue damage by the release of proteolytic enzymes [3–5]. Finally, the oxidative burst of CF neutrophils may be exaggerated [6], which increases the risk of oxidative damage to lung tissues even when CF patients are clinically stable [7].

There are few studies of airway neutrophil function in adult CF disease once chronic infection has become established and most data have come from examination of peripheral blood

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and/or bronchoalveolar lavage samples in studies from mostly children [8,9]. In the current study, we have evaluated reactive oxygen species (ROS) production by sputum neutrophils and assessed the relationship to neutrophil viability in adult CF patients with chronic infection and bronchiectasis. In addition, we examined the oxidative burst capacity of peripheral blood neutrophils from CF patients compared to healthy controls. The primary outcome of interest was the relationship between the "health" of sputum and blood neutrophils and their capacity to generate ROS as part of the oxidative burst.

## 2. Methods

### 2.1. Subjects

We recruited 31 CF patients attending the adult CF Unit at the Royal Hobart Hospital in Tasmania and The Prince Charles Hospital (TPCH) in Brisbane. Patients were recruited on admission at the time of a pulmonary exacerbation, or from the outpatient clinic when clinically stable. Spontaneously expectorated sputum was collected and processed immediately for neutrophil functional assays. In patients recruited at the time of an exacerbation, sputum was obtained at varying time intervals during their hospital stay. Samples collected during the first week of intravenous (IV) antibiotic treatment (usually within 48 h of admission) were recorded as 'acute' and samples collected during the second week of hospitalisation, when patients were clinically improving, were designated 'post-acute'. Stable CF patients with no history of a recent exacerbation (within one month) were recruited through outpatient clinics. Blood samples were also obtained from 20 CF patients (including 16 patients who provided a matched sputum sample, and four patients who provided blood only). A further 11 patients provided a sputum sample only. Seven healthy non-smoking and non-CF individuals with no history of lung or systemic disease provided blood for comparison (controls). Each patient provided a maximum of one acute, post-acute or stable sample, respectively. Routine microbiological testing of sputum identified all major pathogens with 24 of the 31 patients chronically infected with *P. aeruginosa* (see Supplementary data for full culture and antibiotic sensitivity profiles for each patient). The Tasmanian Health and Medical Human Research Ethics Committee and TPCH Human Research Ethics Committee (Queensland) approved the study and written informed consent was obtained from all patients.

### 2.2. Sputum processing

Sputum processing was commenced within 30 min of collection. Saliva free plugs were selected, mixed with an equal volume of 10% Sputolysin® (dithiothreitol, Calbiochem, San Diego, CA, USA). A 1 ml aliquot of the homogenised sample was used for flow cytometric (FCM) analysis of neutrophil apoptosis and oxidative burst. A total cell count was performed on the remainder of the sample using a Neubauer haemocytometer. Cytospin slides were stained with Haem Kwik (Clinipure, Wetherill Park, NSW, Australia) for the calculation of differential cell counts.

The assay sample was washed twice with PBS and passed through a 100 μm pore nylon mesh filter. A cell count was performed and viability determined with Trypan blue. The sample was then resuspended in PBS to give a concentration of approximately $1 \times 10^6$ cells per 100 μl assay volume. This final cell suspension was used for both oxidative burst and apoptosis assays. The use of FCM precluded the use of further neutrophil isolation procedures as this method allowed us to gate out contaminating red blood cells, mononuclear cells and debris on the basis of size, granularity and cell surface markers. Furthermore, examination of the cytospin preparations confirmed that neutrophils were the predominant cellular component of the sputum samples. Because of the highly viscoelastic nature of CF expectorated sputum and concerns about the effect of chemical and mechanical agents on the functionality and physical properties of neutrophils [10–13], we conducted preliminary studies to compare the activation status of neutrophils in CF sputum homogenised by either Sputolysin® or mechanical disruption. The latter entailed syringing the sample through a wide bore needle as advocated by Hector and colleagues [11]. Consistent with the findings of these authors, there was no significant difference in ROS production between the different processing methods. Similarly our experiments indicated that there was no difference in the rates of apoptosis depending on homogenisation method used.

### 2.3. Isolation of peripheral blood neutrophils

Venous blood was collected and placed in lithium heparin coated tubes. Neutrophils were isolated from whole blood utilising a one-step double Histopaque® (Sigma-Aldrich, Sydney, Australia) gradient technique allowing their separation from the erythrocyte and mononuclear cells. Three milliliter of Histopaque®-1077 was layered over an equal volume of Histopaque®-1119 followed by 6 ml of whole blood as per product insert. Neutrophils banding at the interface of the two Histopaque® layers were washed in PBS and a cell count performed. Viability of the neutrophils was assessed using Trypan blue and the cell concentration adjusted to approximately $1 \times 10^6$ cells per 100 μl assay volume. This final cell suspension was used for both apoptosis and oxidative burst assays. Mononuclear cells were aspirated from the Histopaque®/plasma interface layer for cryopreservation and later studies. The need for concurrent retrieval and storage of lymphocytes precluded the use of whole blood for neutrophil analysis by FCM [14]. Red cell lysis in whole blood using an ammonium chloride solution was trialled and found to markedly increase the level of neutrophil apoptosis (data not shown). Extreme care was undertaken to prevent artificial activation of neutrophils, including minimising centrifugation and washing steps, gentle pipetting and avoiding temperature fluctuations. Whilst erythrocyte contamination was nominal using the double Histopaque® gradient, remaining red blood cells and cell debris were largely excluded from the FCM analysis by adjusting the forward light scatter threshold.