TUMOUR NECROSIS FACTOR GENE COMPLEX POLYMORPHISMS 
AND HAPLOTYPES IN CHRONIC OBSTRUCTIVE PULMONARY 
DISEASE

Running Title: Tumour necrosis factor polymorphisms in COPD
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ABSTRACT

We aimed to examine the role of tumour necrosis factor gene complex polymorphisms in subjects with chronic obstructive pulmonary disease (COPD). We hypothesized that individuals possessing polymorphic variants associated with higher TNF secretion would be more susceptible to and/or have more severe disease. Patients with COPD and population controls underwent detailed clinical phenotyping. Genotyping for the tumour necrosis factor-308 and the lymphotoxin alpha NcoI (LTα) polymorphisms was carried out by ‘blinded’ laboratory staff. 361 individuals (220 cases and 141 controls) were recruited. We showed an association between the LTαNcoI polymorphism and forced vital capacity (FVC) in a population of older adults with and without COPD. The LTαNcoI*2 allele was associated with poorer lung function, under a codominant model, with a fall in FVC (expressed as a percentage of its predicted value) of 3.7% for each copy of the LTαNcoI*2 allele possessed (for FVC, regression coefficient (95% CI) = -3.73 (-7.01 to -0.44), p=0.026; for FEV1 regression coefficient = -3.56 (-7.80 to 0.70), p = 0.101. However, there was no difference in genotype distribution between the case and control populations. This study adds weight to the suggestion that the TNF gene complex is involved in physiological alterations (FVC) that may affect the development and severity of COPD. The absence of a significant association between the TNF gene-complex polymorphisms in this study does not rule out a modest effect of these polymorphisms on the risk of COPD, as much larger studies are needed to detect modest gene effects on binary disease endpoints.

Keywords COPD, tumour necrosis factor, lymphotoxin alpha, polymorphisms, pulmonary function, haplotypes
Introduction
COPD is a common condition with significant morbidity and mortality\textsuperscript{1,2}. Smoking is a key environmental risk factor, but there is a marked difference in individual susceptibility to developing COPD\textsuperscript{3}. Evidence is accumulating that this susceptibility is at least in part genetically determined. Numerous family and twin studies have shown significant correlations in pulmonary function, particularly FEV\textsubscript{1}, between first-degree relatives of COPD patients, and in general population samples\textsuperscript{4-8}. There are a large number of possible candidate genes for COPD pathogenesis, but to date there is little evidence available from linkage studies that can reliably guide the selection of these candidate genes\textsuperscript{9,10}. The selection of candidate genes for association studies in COPD has therefore focused on a number of polymorphisms in apparent central roles in disease pathogenesis. One of these is the tumour necrosis factor gene complex on chromosome 6p.

Tumour necrosis factor is a pro-inflammatory cytokine and a number of polymorphisms have been described\textsuperscript{11-12}. One key candidate gene has emerged, a polymorphism of the tumour necrosis factor -308 polymorphism (TNF-308). A closely linked polymorphism of the lymphotoxin alpha promotor has also been widely studied. The tumour necrosis factor gene and the lymphotoxin alpha gene are in strong linkage disequilibrium within the human major histocompatibility complex III on chromosome 6p. Tumour necrosis factor alpha (TNF) is a soluble homotrimer of 17kD subunits each synthesized from 26kD trans-membrane pro-peptides\textsuperscript{13}. Initially described for its cytotoxic and anti-tumour activities, it is also known to have potent immunomodulating and pro-inflammatory effects. Its roles include adhesion of neutrophils to endothelial cells, enhanced IL-2 response, and induction of GM-CSF. It also appears to have effects on cell apoptosis\textsuperscript{14}. TNF is involved in early phase of bronchoconstriction via smooth muscle contraction and late phase via up-regulation of adhesion molecules and influx and activation of inflammatory cells. In addition, increased levels are found in induced sputum from patients with COPD\textsuperscript{15}.

The TNF -308 polymorphism is a biallelic restriction fragment length polymorphism (RFLP) originally described by Wilson AG (1992)\textsuperscript{16}. The common variant is termed TNF*1 and the rare variant TNF*2. The polymorphism results in an amino acid change in the promotor region of the TNF\textsubscript{α} gene at position -308 relative to the
transcription start site. The less common TNF308*2 allele has been associated with higher baseline and induced expression of TNF-α. Thus this variant might be expected to be associated with increased severity of disease or possibly increased susceptibility to COPD.

Lymphotoxin alpha (LTα) is a 25-kb soluble protein, synthesized primarily by stimulated T cells. Lymphotoxin alpha has a similar range of immunoregulatory effects as TNFα, although a broader range of bioactivities has been described for TNFα. The Ncol polymorphism lies in the first intron of the lymphotoxin alpha gene and is also biallelic. Messer G (1991) showed that the polymorphism was associated with a nucleotide substitution at position 26. It is debated if the Ncol polymorphism is of any functional significance in terms of alteration in both lymphotoxin and TNFα production or activity. Messer G (1991) have shown that in vitro LTα Ncol*1 was associated with increased level of lymphotoxin response, but no association was found with respect to TNFα production. Conversely the LTα Ncol*2 allele has been associated with different TNFα secretory phenotypes in other studies. Both Molvig J (1990) and Pociot F (1993) have shown LTα Ncol*2 allele to be associated with higher TNFα levels. Despite these inconsistent findings the Ncol restriction site polymorphism continues to be a useful marker for disease predisposition in association and linkage studies.

Wilson and colleagues describe an extended haplotype for the TNF-308/LTNcol/HLA al/B8/DR3. To date there have been few studies examining the role of haplotypes in disease causation and modification and on pulmonary function. Several studies have examined the role of both of these polymorphisms, particularly the -308 polymorphism, in subjects with asthma, atopy and latterly, COPD. Our primary hypotheses were that these polymorphisms and common haplotypes determined susceptibility to COPD and/or severity of COPD, by quantitative effects on the pulmonary function. Specifically we hypothesised that the polymorphisms have modifying effects on FEV1 and FVC. Our secondary hypothesis was that the polymorphisms determine the severity of emphysema, as measured by the gas transfer coefficient, in subjects with COPD.
Recently published linkage data for pulmonary function in COPD would support this model\textsuperscript{9,10}. However, recently published linkage data for pulmonary function in a general population sample also showed linkage of FEV1 and thus genetic effects could not only be apparent in those with established disease, but also plausibly be within the whole population\textsuperscript{23}. Both models were examined in the primary analysis. Our secondary outcome variable was that the polymorphisms determine the severity of emphysema, as measured by the gas transfer coefficient, in subjects with COPD.

**Methods**

**Study population**
Subjects with COPD were recruited from outpatient departments of Respiratory Medicine and General Medicine. They all had definite airflow obstruction FEV1/FVC ratio $<70\%$ and FEV1$< 80\%$ of its predicted value, with a senior hospital physician diagnosis of COPD. All cases had a smoking history of at least 10 pack-years and symptom onset after 45 years of age. Individuals with significant bronchodilator reversibility were not excluded provided their spirometry did not return to normal following bronchodilators. Cases were excluded if they had a prior doctor diagnosis of asthma, known or suspected bronchiectasis or any other medical condition that might adversely affect pulmonary function. Controls were recruited from general practitioner patient lists and were age, sex and geographically matched with cases. The controls had no history of respiratory disease and normal spirometry. They were excluded if they had any condition that might adversely affect pulmonary function. It was not possible to match exactly for smoking history and this was therefore adjusted for in the final analysis, using regression models. All subjects were Caucasian. The study was approved by two local research ethics committees and participants gave written informed consent.

**Clinical phenotyping**
All subjects completed spirometry to American Thoracic Society specifications\textsuperscript{24}. Additionally the majority of cases also performed full pulmonary function testing including a gas transfer measurement by single breath carbon monoxide diffusion.
The transfer factor (TLCO) and coefficient, (KCO) expressed as a percentage of their predicted values were used as quantitative markers of emphysema.

**Genotyping**
DNA was extracted from whole blood using a DNA extraction system (Gentra, PURGENE DNA purification kit). Genotyping was performed for the lymphotoxin alpha polymorphism and the tumour necrosis factor polymorphism adapted from previously published protocols\(^{12,16}\). Negative and positive controls of known genotype were included with each set of digests. A random selection of samples (8 %) were sequenced to confirm genotyping. No discrepancies were detected by sequencing.

**Statistical Methodology**
Contingency table analysis using the chi-square test was used to determine if the polymorphisms had any effects on disease susceptibility. The quantitative primary outcome variables were FEV1 and FVC, both expressed as a percentage of their predicted values based on European Community Coal and Steel tables. The secondary outcome variable was KCO expressed as a percentage of the predicted value. The quantitative variables were examined using multiple regression analysis, with age, sex and smoking history examined as covariates in all regression models. Analysis was carried out for these quantitative variables in the whole population. In the absence of clear prior evidence informing the genetic model, genotype was analysed as a three level factored variable (AA=1, AB=2, BB=3, where A is the common allele and B the rare allele), corresponding to an additive codominant model where the heterozygote lies midway between the two homozygotes. This corresponds to a comparison of the effect of the B versus A allele ( an “allele dosage” effect) in the linear regression model. Associations nominally significant at the P<0.05 level under were also analysed under dominant (AB and BB versus AA baseline) and recessive (BB versus AB and AA baseline) models.

Haplotype analysis was undertaken using haplotype estimates derived from the Expectation-Maximization algorithm\(^ {25}\). Data was analysed using SPSS version 10.0 and STATA 7.0. Statistical significance defined as p < 0.05. Power calculations were based upon a sample size of 200 cases and 200 controls. This is sufficient to detect a
difference between proportions of, for example, 11 and 2% for the LTαNcol1,1 genotype ($\alpha = 0.05, \beta = 0.2$). These differences between cases and controls are similar in magnitude to those reported in younger asthmatic populations.

**Results**

220 cases and 141 controls were genotyped for the lymphotoxin alpha Ncol and tumour necrosis factor -308 polymorphisms. Other data from these subjects (with others) have been previously presented\textsuperscript{26}. Demographic data for the case and control populations is summarized in Table 1. The case and control populations were in Hardy-Weinberg Equilibrium. Allele frequencies for LTαNcol allele 1 were 0.33 and for TNF -308 allele 2 were 0.15. The two polymorphisms lay in strong linkage disequilibrium (chi square = 151, $p <0.001$). There was no difference in genotype distribution between the case and control populations for either of the polymorphism.

Our primary hypothesis was that the polymorphism might have an effect on pulmonary function. **Table 2** shows the relationship between mean FEV1 and mean FVC, both expressed as a percentage of their predicted values, by genotype for the study population. A significant trend in FVC ($P=0.026$) was seen across the LTA genotypes with an average fall of 3.7% in FVC for each additional copy of the LTαNcol *2 allele (Table 2). A similar trend was observed in cases alone (Table 3), which was of borderline significance ($P=0.051$). A non-significant ($P=0.101$) trend was also evident in FEV1 across the LTA genotypes was observed, with an average fall of 3.35% in FEV1 for each copy of the LTαNcol *2 allele in the study population as a whole (Tables 2). The effect of the TNF -308 allele 2 polymorphism on either FVC or FEV1 was less clear.

The secondary hypothesis sought to explore the relationship between the degree of emphysema and the two genotypes and haplotypes. Gas transfer measurements (KCO and TLCO) were available for 159 cases with COPD (82%). These were examined as a quantitative variable, as a percentage of their age and sex matched predicted values. There were no statistically significant differences in KCO measurements between the TNF-308 genotype or the lymphotoxin alpha genotype (Table 3). Analysis was also
performed using TLCO (as a percentage of its predicted value). No significant differences were observed.

The haplotype analysis showed no difference in haplotype distribution between the case and control populations (results not shown), although only certain haplotypes were common in the population. A quantitative analysis of common haplotypes to both markers of pulmonary function with adjustment for covariates showed no statistically significant effects of haplotype on pulmonary function.

**Conclusions**
This is the one of the largest studies to date examining the role of the LTαNcol polymorphism and the TNF -308 polymorphisms and common haplotypes in subjects with COPD and population controls. We have shown that the frequencies of the LTαNcol polymorphism and the TNF -308 polymorphism are the same order of magnitude as reported in other recent studies involving Western Caucasian populations. The first study to report a significant association between pulmonary function and TNF gene complex polymorphisms in a COPD population, showed a strong effect at the -308 locus, but was based on only 42 cases and 141 controls. Small genetic association studies have been especially prone to reports of positive associations that could not be replicated in larger studies. This underlines the importance of studies, such as ours, that aim to replicate such results in larger populations.

We have shown an association between the LTαNcol polymorphism and FVC in a population of older adults with and without COPD. The LTαNcol*2 allele was associated with poorer lung function. From a pathobiological perspective one would predict that the LTαNcol*2 allele would be associated with more severe disease. It is possible that being homozygous for the rare allele may accelerate inflammatory processes, in the presence of cigarette smoke. In vitro experiments have shown that TNF can cause an increase in apoptosis in alveolar epithelial cells in culture, further accelerated by exposure to ultraviolet light. It is possible therefore that TNF can induce cellular changes making alveolar cells more prone to apoptosis by any stressor, including tobacco smoke.
Importantly, the regression model explained only a moderate proportion of the variability of FVC and FEV1 suggesting that additional factors determining pulmonary function in this population and these are likely to be a combination of genetic and environmental influences. We do not report any significant difference in pulmonary function with the TNF-308 genotype, although there was a trend towards worse lung function with possession of the TNF-308*2. This variant has also been associated with higher TNF secretory levels. Given the frequency of TNF-308 2,2 homozygotes our failure to find a statistically significant difference may not be that surprising. The haplotype analysis showed no significant associations between common haplotypes and pulmonary function. However, for regions in which linkage disequilibrium is strong, a haplotype-based approach may be less powerful (owing to an increase in degrees of freedom) than tests based on a single marker.

In our large Caucasian population of patients with COPD and population controls, we have not found that these polymorphisms determine the susceptibility to COPD. Our findings are in contrast to the findings of a number of smaller association studies\textsuperscript{28,31,32}. These studies have shown the TNF-308 polymorphism to be associated with both disease susceptibility and disease severity. However, three recent studies have failed to show such associations\textsuperscript{22,27,29}. Our results suggest, if these polymorphisms do determine the susceptibility to COPD, then these effects are likely to be modest, such that they cannot readily be detected in a study based on 220 cases. Our study had greater power to detect quantitative traits underlying lung function as compared to binary disease endpoints, and this alone may explain our findings. However, the failure to detect associations for some quantitative traits can be subject to attrition by the effects of treatment, and this may be an explanation for the lack of a statistically significant association observed between FEV1 and LTαNcol.

We showed no significant difference in the degree of emphysema and TNF gene complex polymorphisms in our population. A recent study by Sakao (2002) showed a trend towards a more emphysematous disease picture in those individuals who were homozygous for the rare TNF-308 allele\textsuperscript{33}. Eighty-four patients with COPD were scored for the presence of emphysematous changes observed on high resolution
computerised tomography scan of the chest (HRCT). A visual scoring system to quantify the degree of emphysema was developed using the scoring method of Goddard and co-workers[^34]. A trend towards higher emphysema scores was shown with possession of allele 2, but conventional levels of statistical significance were not reached.

There are a number of explanations for the inconsistent findings between the various studies. It is of interest that two of the statistically significant studies, although small in size, were both carried out in populations of Asian extraction[^31,32]. There is some evidence to suggest that the TNF gene complex polymorphism result in functional changes in cytokine production. It is possible however that the association is indirect and that the polymorphism acts as a marker in linkage disequilibrium with a functional variant. Linkage disequilibrium varies between populations and where an indirect association is detected in one population it may be difficult to replicate in another. The potential lack of power has already been discussed, and remains a limitation for interpretation of results from many candidate gene association studies.

In summary, the TNF gene-complex polymorphism LTαNcol appears to play an important role in determining FVC, and may therefore also influence the development of COPD. However, much larger studies (probably in the region of 5,000 cases) are required to detect the modest effects of genes on binary disease endpoints. The failure to detect an association between TNF gene-complex polymorphisms in our study would not rule out a modest effect of these polymorphisms on the risk of COPD. In contrast, our study adds weight to the suggestion that the TNF gene complex is involved in physiological alterations (FVC) that may affect the development and severity of COPD. Although we tested only two genetic polymorphisms in this study, the results of any genetic association study should be interpreted with appropriate caution, since multiple testing may lead to “false-positive” associations[^30]. However, when considered in the context of the overall evidence available for the involvement of genes in this pathway in the development of COPD, there is sufficient evidence to suggest that the TNF gene complex polymorphisms appear to modify lung function in older adult smokers, (both cases and controls) in our study.
Conflict of interest- None

Acknowledgments Professor WOCM Cookson, Dr M Moffatt, Professor P Burton.
<table>
<thead>
<tr>
<th></th>
<th>Cases (number = 220)</th>
<th>Controls (number = 141)</th>
<th>Statistical summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (SD)</td>
<td>67.4 (9.9)</td>
<td>68.5 (8.5)</td>
<td>ttest = -1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.263</td>
</tr>
<tr>
<td>Gender ( % male)</td>
<td>59.1%</td>
<td>58.9%</td>
<td>Chi-square = 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.966</td>
</tr>
<tr>
<td>Percentage ever:ex:current smokers.</td>
<td>0:70:30</td>
<td>30:52:18</td>
<td>Chi-square = 75.3,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>= &lt; 0.005</td>
</tr>
<tr>
<td>Median pack-year history (interquartile range)</td>
<td>30-40 pack years (20-30 pack-years-50-60 pack-years)</td>
<td>10-20 pack years (0 pack years-30-40 pack years)</td>
<td>Chi-square test for trend = 144, p &lt; 0.005</td>
</tr>
<tr>
<td>Mean FEV1(SD)</td>
<td>46.6 (19.3)</td>
<td>93.3 (16.3)</td>
<td>ttest t = -22.9, p &lt; 0.005</td>
</tr>
<tr>
<td>Mean FVC(SD)</td>
<td>76.5 (21.4)</td>
<td>97.7 (16.8)</td>
<td>ttest t = -9.9, p &lt; 0.005</td>
</tr>
</tbody>
</table>

**Table 1**

Characteristics of the case and control populations.
<table>
<thead>
<tr>
<th>Genotype (number of subjects)</th>
<th>Mean FEV1 (SD)</th>
<th>Mean FVC (SD)</th>
<th>Genotype (number of subjects)</th>
<th>Mean FEV1 (SD)</th>
<th>Mean FVC (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-308 1,1 (251)</td>
<td>64.0(30.2)</td>
<td>84.4(22.7)</td>
<td>LTα 2,2 (158)</td>
<td>62.0(30.3)</td>
<td>82.3(23.0)</td>
</tr>
<tr>
<td>TNF-308 1,2 (97)</td>
<td>66.7(28.5)</td>
<td>86.3(21.6)</td>
<td>LTα 1,2 (161)</td>
<td>66.7(30.1)</td>
<td>86.1(22.4)</td>
</tr>
<tr>
<td>TNF-308 2,2 (10)</td>
<td>62.6(27.6)</td>
<td>82.5(21.1)</td>
<td>LTα 1,1 (42)</td>
<td>68.7(24.7)</td>
<td>89.7(17.9)</td>
</tr>
<tr>
<td>Regression coefficient (95% CI); P</td>
<td>p = 0.610</td>
<td>p = 0.614</td>
<td>Linear regression model</td>
<td>β = -3.56 (-7.80 - 0.70)</td>
<td>β = -3.73 (-7.01 - 0.44)</td>
</tr>
</tbody>
</table>

† Additive codominant model equivalent to a ‘per-allele’ dosage effect.
§ Age, sex and smoking history included as covariates in all regression models.

Table 2
Relationship between genotypes and primary outcome variables for study population (cases and controls)
<table>
<thead>
<tr>
<th>Genotype (number of cases)</th>
<th>Mean FEV1 (SD)</th>
<th>Mean FVC (SD)</th>
<th>Mean KCO (SD)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTα 2,2 (71)</td>
<td>43.8 (19.2)</td>
<td>73.7 (21.8)</td>
<td>80.1 (28.7)</td>
</tr>
<tr>
<td>LTα 1,2 (73)</td>
<td>48.1 (19.8)</td>
<td>77.1 (20.9)</td>
<td>82.5 (26.7)</td>
</tr>
<tr>
<td>LTα 1,1 (15)</td>
<td>52.8 (15.7)</td>
<td>86.0 (19.7)</td>
<td>78.3 (27.3)</td>
</tr>
</tbody>
</table>

Regression coefficient, significance

|                          | β = -3.04     | β = -4.25, p = 0.051 | β = -0.51, p = 0.881 |

† data based on 159 subjects

Table 3
Relationship between LTα genotype and pulmonary function for subjects with COPD
References


19. Pociot F, Briant L, Jongeneel CV et al. Association of TNF and class II major histocompatability complex alleles with the secretion of TNFα and TNFβ by human mononuclear cells; A possible link to IDDM. Eu J Immunol 1993(a);23:224-231


