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## Genotype and phenotype in susceptibility to coal workers' pneumoconiosis. The use of cytokines in perspective

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*Genotype and phenotype in susceptibility to coal workers' pneumoconiosis. The use of cytokines in perspective. P.J.A. Borm, R.P.F. Schins. ©ERS Journals Ltd 2001.*

**ABSTRACT:** The readily available technique of screening for gene polymorphisms could be used to explain inter-individual variability in a classic occupational interstitial lung disease, such as coal workers' pneumoconiosis (CWP).

The objective of this paper is to describe candidate genes selected from the wide pool of cytokines and growth factors, and to discuss the applications and pitfalls when using them as biomarkers for susceptibility to CWP. The selection of candidate genes is mainly based on observed phenotypic changes in bronchoalveolar lavage (BAL) fluid or BAL cells of patients with CWP, or on animal experiments that use quartz as the fibrogenic agent.

This paper also reviews the studies that have been performed to validate tumour necrosis factor genotype and phenotype with respect to CWP.

Finally, it is proposed that a multiple marker approach to susceptibility to CWP should be used. This involves the measurement of two cytokines (tumour necrosis factor and transforming growth factor- $\beta$ ) to improve denomination of high- and low-risk groups.

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Despite the closure of coal mines in most of Western Europe, coal workers' pneumoconiosis (CWP) is still a frequently encountered interstitial lung disease (ILD) that affects only a minor percentage of exposed subjects. CWP is usually divided into two stages: simple pneumoconiosis (SP), in which fibrosis remains limited, and progressive massive fibrosis (PMF), characterized by perifocal extensive fibrotic responses and opacities >1 cm. Historically, there have been many different approaches to explaining the marked differences in susceptibility described in populations of miners exposed to equal dust types and concentrations. Recent approaches have evaluated polymorphisms in genes of the major histocompatibility complex (MHC) system [1, 2]. Since the original findings of HEPPLESTON and STILES [3], that silica-exposed macrophages produce factors that stimulate the production of collagen by fibroblasts, the field of growth factors and cytokines has developed tremendously and it has now emerged that cytokines play a role in particle-induced fibrosis [4–6]. These issues are also reviewed in the report by NEMERY *et al.* [7] in this Supplement.

When exposed to coal dust, lung tissue can initiate three types of reaction: 1) accumulation and activation of inflammatory cells in the lower respiratory tract; 2) fibroblast proliferation; and 3) enhanced synthesis and/or breakdown of extracellular matrix components. Chemokines, cytokines and growth

factors play a crucial role in the onset, progression and termination of these reactions. A body of research has concentrated on tumour necrosis factor (TNF)- $\alpha$ , a pro-inflammatory cytokine important during the early-onset inflammation induced by particles, lipopolysaccharide (LPS) and allergens. Crucial animal experiments were reported by PIGUET and coworkers [8, 9], who demonstrated that silica-induced lung fibrosis could be ameliorated using a specific anti-TNF antibody, and that the infusion of soluble TNF-receptors, which complexes free TNF, could prevent and reduce existing fibrosis. BRODY and coworkers [10–12] showed that TNF-receptor knock-out mice were protected against the fibrogenic effects of silica [10] and asbestos [11], and that inbred mice that failed to develop fibrosis in an asbestos model had reduced TNF and transforming growth factor (TGF)- $\beta$  expression in the lung [12]. Human studies have demonstrated increased levels of TNF and TNF-receptors in bronchoalveolar lavage (BAL), serum, and tissue specimens of subjects with various interstitial lung diseases. More specifically, in patients with CWP or PMF, changes of TNF release from alveolar macrophages, as well as interleukin (IL)-6, TGF- $\beta$ , monocyte chemoattractant protein (MCP-1) and platelet derived growth factor (PDGF) were noted [13]. In addition, both TNF and IL-6 messenger ribonucleic acid (mRNA) have been shown to be higher in lung

tissue biopsies from the lungs of coal miners, especially in geographical areas where coal dust was present [14].

### Susceptibility studies: genotype

The pivotal role of cytokines in the pathogenesis of CWP, combined with the current ability to screen genes for polymorphisms, should incite a search for changes at loci in genes causing phenotypic changes in stability of the mRNA or translation products [15].

Table 1 summarizes a number of genes and candidate genes that could be associated with the pathogenesis of CWP, and outlines further screening candidates. This selection is mainly based on the phenotypic changes observed in the BAL or BAL-cells of patients with CWP, or on animal experiments that use quartz as the fibrogenic agent. From the listed factors, only TNF, glucose-6-phosphate dehydrogenase (G6PDH) and glutathione-S-transferase (GST)-polymorphs have been evaluated in relation to ILD. Polymorphisms are defined as structural modifications that occur at a specific gene location in >5% of individuals, and are preferentially related to altered levels of the transcription/translation product. GST and G6PDH have polymorphisms with an almost absent phenotypic activity, and have been associated with asbestosis and sarcoidosis [28]. In the TNF gene, polymorphisms at the -238, -308, -376 and +489 positions of the gene have been reported. It has previously been shown that the A-308 transition polymorph was seen more frequently in miners with SP [16]; another study showed that only the +489 polymorphism was different in patients with chronic

obstructive pulmonary disease (COPD) [17]. Interestingly, an increase in the A-308 polymorph was found in a case-control study comparing subjects with berylliosis to controls [31]. Polymorphisms have been observed in some factors that are active in the recruitment of inflammatory cells, such as the macrophage inflammatory proteins (MIPs), PDGF, TGF- $\beta$  and MCP-1.

MCP-1 is produced by several inflammatory cells, including epithelium, fibroblasts, monocytes and macrophages, and is a major chemoattractant for monocytes. Increased concentrations of MCP-1 were found in the BAL fluid of CWP patients, as well as in the supernatants of the *in vitro* culture of their alveolar macrophages. Immunohistochemical analysis indicated that in addition to the macrophages, fibroblasts and type II cells might be involved in the enhanced production of MCP-1 in CWP patients [20]. However, recently discovered polymorphisms (table 1) have not been linked to CWP or PMF.

IL-6 is produced by most nucleated cells, including monocytes, (alveolar) macrophages, endothelial cells, fibroblasts and B- and T-cells [32]. The gene encoding IL-6 is located on chromosome 7, and IL-6 molecules have different sizes ranging from 17–85 kD. A C/G polymorphism was found at the -174 site and the C-allele was associated with lower plasma levels of IL-6 [18]. Human macrophages were found to release IL-6 in response to coal dust, but interestingly, not in response to silica or titanium dioxide [33].

Three mammalian types of TGF- $\beta$  proteins have been described, *i.e.* TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, which share ~70% homology [34]. Genes encoding TGF- $\beta$  proteins are located on chromosome 19 and major sources of TGF- $\beta$  include blood platelets,

Table 1.—Candidate genes and mediators in susceptibility to coal workers' pneumoconiosis (CWP) and their link to phenotype or other end-points

Family	Polymorphism(s)	Relation to phenotype <sup>#</sup>	End-points	Reference
<b>Cytokines</b>				
TNF- $\alpha$	-238 (A/G), -308 (A/G) in promoter +489	A-238 ↓ ND	SP/PMF +(A-308) COPD	[16] [17]
IL-6	-174	C-174 ↓	Serum IL-6, arthritis	[18]
IL-10	-1082, -819, -519, all (A/G) in promoter	G-1082 ↑	No link with MS	[19]
MCP-1	-2518 (G/A), -2076 (A/T)	G-2518 ↑	ND	[20, 21]
RANTES	-403, -28	G-28 ↑	HIV-1 progress	[22, 23]
<b>Growth factors</b>				
TGF- $\beta$	+915 (signal sequence), codon 25 A/A -509 (promoter)	A-915 ↓ Homozygous ↑	Allograft fibrosis Asbestosis	[24, 25]
PDGF-A	IVS4 and IVS3 minisatelites	ND	SP (phenotype)	[26]
<b>Various</b>				
CYP450	CYP2A6, 1A2, 2D6, 2E1	Yes	Lung cancer	[27]
G6PDH	Deficiency in 15% of population	(0/+)	Sarcoidosis	[28]
CC10/16	-38 (G/A)	38AA ↓	Asthma	[29]
GST	GST-M1 (-/+), GST- $\theta$ (-/+), GST- $\pi$ (-/+)	(0/+)	Asbestosis (GST-M1)	[30]

TNF- $\alpha$ : tumour necrosis factor- $\alpha$ ; IL: interleukin; MCP-1: monocyte chemotactic protein-1; TGF- $\beta$ : transforming growth factor- $\beta$ ; PDGF-A: platelet derived growth factor; CYP450: various cytochrome P450 isozymes; G6PDH: glucose-6-phosphate dehydrogenase; CC10/CC16: Clara cell protein; GST: glutathione-S-transferase; ND: not determined; SP: simple pneumoconiosis; PMF: progressive massive fibrosis; MS: multiple sclerosis; COPD: chronic obstructive pulmonary disease; HIV-1: human immunodeficiency virus type 1; A: adenosine; G: guanine; T: thymine; RANTES: regulated on activation, normal T-cell expressed and secreted. <sup>#</sup>: Phenotype defined as altered production of (active) protein or messenger ribonucleic acid (mRNA)-product; ↑: increase; ↓: decrease.

Table 2. -- Overview of studies on cell-derived tumour necrosis factor (TNF) release as a biomarker in coal miners and controls (exposed miners)

Cell type	TNF release	Primary findings	Confounders		Reference
			Smoking	Exposure	
PBM, 18 h	0.75±0.14 (baseline, controls) 3.0±0.4 (LPS, 3 ng·mL <sup>-1</sup> )	Priming of release; increase of coal-dust induced TNF at early (suspect) stages of CWP	No effect	No relation	[39]
AM, 72 h	2.5±1.0 (spontaneous, controls)	Spontaneous release only; PMF higher than simple CWP. Exposed controls higher than references	No effect	NE	[40]
PBM, 18 h	1.77±2.06 (baseline, controls) 9.53±7.33 (LPS, 3 ng·mL <sup>-1</sup> )	TNF not related to exposure. Positive relation between profusion and TNF release within PMF group	Minor effects	No relation	[41]
PBM, 18 h	0.20±0.10 (baseline, controls) 2.20±0.4 (LPS, 3 ng·mL <sup>-1</sup> )	Effect of age on TNF release in retired (age >60 yrs) miners Initial TNF release predictive of progression of CWP (RR=8.1 at levels greater than mean±2 SD)	Small effects	No relation	[36]
PBM, 18 h	0.10±0.13 (baseline, controls)	Increase of coal-induced TNF (and interleukin-8) in miners with small or large opacities; also serum levels increased	NE	NE	[42, 43]
Whole blood, 16 h	0.19±0.31 (CMD, controls) 3.39±1.92 (LPS, 3 ng·mL <sup>-1</sup> )	CMD-induced TNF release significantly induced in PMF versus matched controls Number of leukocytes increases in PMF	No effect	No effect	[44]

For comparison of studies, where possible, the unstimulated (baseline) release from cells was used for comparison. Data on TNF release are in ng·mL<sup>-1</sup> from control miners and usually obtained by incubation of 5×10<sup>5</sup> cells. PBM: peripheral blood monocytes; AM: alveolar macrophages (from bronchoalveolar lavage); CWP: coal workers' pneumoconiosis; CMD: coal mine dust; PMF: progressive massive fibrosis; LPS: lipopolysaccharide (endotoxin); RR: risk ratio; NE: not evaluated.

alveolar macrophages, monocytes and neutrophils [35]. Since TGF-β is secreted in a latent form that cannot bind to its receptors, its activation *in vivo* (e.g. by proteolytic enzymes) is considered to be an important regulatory step. VANHÉE *et al.* [5] reported increased TGF-β concentrations in the BAL fluid of patients with simple CWP, as compared to the BAL fluid of patients with PMF and of nonexposed control supernatants. They also showed that macrophage supernatants from simple CWP patients, but not PMF patients, inhibited fibroblast growth *in vitro*, and that this "antifibrotic" effect could be abrogated by anti-TGF-β antibodies [13]. In addition, other studies have demonstrated that TGF-β has antifibrotic properties and that its pro/antifibrotic action may be concentration-dependent [36]. Clearly, further studies are needed to clarify these paradoxical findings. A report has recently been published in abstract form, which links phenotype and genotype in ILD [24], while others have shown a link with allograft response after lung transplantation [25] or with blood pressure [37].

Clara cell secretory protein (CC16) is a 16 kD anti-inflammatory protein primarily expressed by non-ciliated bronchiolar secretory cells, which was recently identified as a genotypic and phenotypic risk factor in asthma [29]. The 38A/G transition was associated with reduced plasma CC16 levels and an increased prevalence of asthma (odds ratio (OR)=4.75, p<0.04). The observation of Clara cell hyperplasia in rats after chronic exposure to various coal dusts and quartz [38] should stimulate further investigation of CC16 polymorphisms in CWP. Finally, it should be realized that studies to identify new risk factors are usually designed as case-control studies, and even when the number of subjects is high enough, the results may be made less reassuring by poor matching for exposure variables, such as smoking, occupational exposure and constitutional factors. These factors need to be taken into account in study design.

**Susceptibility to coal workers' pneumoconiosis: phenotype studies and their pitfalls**

Previous examples of TNF and CC16 [16, 29] have shown that finding an increased genotype does not necessarily affect phenotype expression, and that it is largely the phenotype that determines the risk. In the case of TNF, the -308G/A transition, which was more frequent among miners with CWP, was not associated with coal-induced TNF release from coal dust stimulated monocytes, a phenotype earlier established as a risk marker for progression of SP [36]. Similarly, patients with a homozygous 38A transition in the gene for Clara cell protein, had reduced plasma CC16 levels, and correction for plasma levels led to a different risk estimate (OR=1.83) for asthma than that purely based on genotype (OR=4.78). As environmental exposure is the primary determining factor in the incidence of CWP, the fact that both exposure and the presence of disease can affect the phenotypic marker must be taken into consideration.

### Validation of tumour necrosis factor as a biomarker in coal workers' pneumoconiosis

A number of studies have evaluated monocyte or macrophage TNF release in relation to exposure to coal dust, defined as years of underground employment, respirable exposure or other indices (table 2). From these studies, several conclusions can be drawn on the impact of exposure on the marker itself, without the interference of disease. First, indirect evidence suggests that during exposure to coal dust in coal miners without CWP, a systemic macrophage and monocyte preactivation occurs [39, 40]. Unlike spontaneous monocyte TNF release, the TNF release in response to coal dust was higher in active control miners compared to nondust-exposed controls. Furthermore, this difference was no longer apparent when the miners' exposure to coal dust ceased [36]. This priming has also been reported in peritoneal macrophages after intratracheal instillation of silica in rats [45], and therefore, these cells experienced no direct contact with particles. This could be explained by the release of a more stable cytokine into the bloodstream, causing upregulation of monocyte TNF-expression. Although priming should be considered as a secondary effect of exposure to coal dust, and not

necessarily related to disease, there is strong evidence that TNF release from monocytes or TNF in plasma are not associated with actual or cumulative exposure. Most studies (table 2) that have attempted to evaluate the relationship between TNF release and individual exposure have used underground-yrs as an exposure estimate and have found no significant relationships in multiple linear regression models [36, 40, 41, 44]. It should also be considered that exposure-yrs is not a particularly accurate exposure estimate, as illustrated by a more refined exposure estimate of the cases and controls previously matched on underground-yrs ( $20 \pm 5$  yrs). Whereas exposure-yrs led to a variation of 15–25 underground-yrs, a subsequent job-exposure matrix analysis in the same miners revealed a variation of exposure of 10–200  $\text{g}\cdot\text{h}\cdot\text{m}^{-3}$  [36]. In addition, this more acute estimate did not lead to a significant correlation between individual exposure estimate and monocyte TNF release.

There are some indications that monocyte TNF release as a phenotypic biomarker is affected by the disease process. Unfortunately, these data originate from cross-sectional studies in miners at different stages of CWP/PMF [39–41], and few follow-up studies have been conducted that could discriminate between the individual adaptation of the marker or the effect of disease progression.

The only follow-up study that has been conducted so far shows that the miners ( $n=6$ ) that had disease progression during 5 yrs [36] already had high levels of dust-induced TNF release at the start and did not change notably during follow-up (fig. 1a). This suggests that dust-induced monocyte TNF release is a constitutional marker, which is not highly affected by the disease itself. Conversely, low-dose ( $3 \text{ ng}\cdot\text{mL}^{-1}$ ) LPS-induced release of TNF decreased in five individuals and only rose in an individual that also showed deviant behaviour in dust-induced TNF release (fig. 1b). As was seen in the total cohort, spontaneous TNF release was reduced significantly in this subgroup ( $p=0.046$ , Wilcoxon rank test), which is attributed to cessation of exposure.

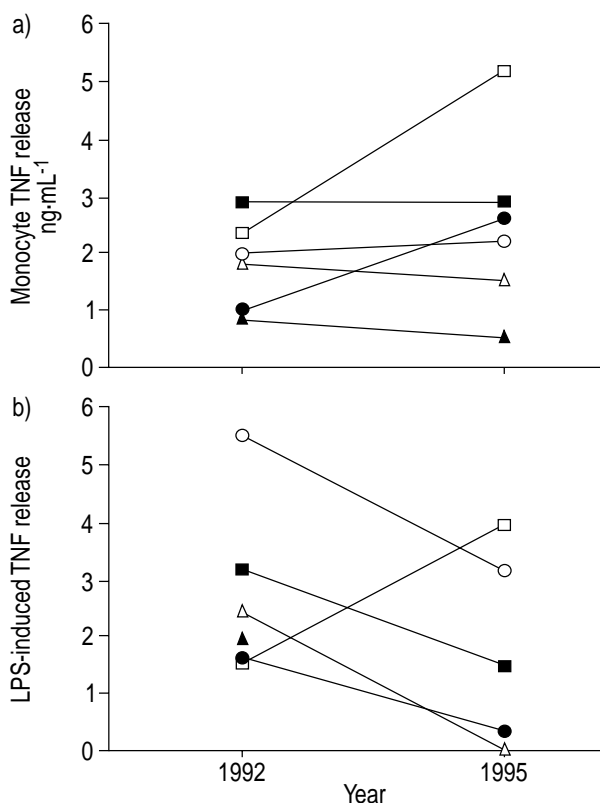


Fig. 1.—Change a) in dust-induced monocyte tumour necrosis factor (TNF) release and b) low lipopolysaccharide (LPS)-induced ( $3 \text{ ng}\cdot\text{mL}^{-1}$ ) monocyte TNF release in six coal miners who showed progression of simple coal workers' pneumoconiosis during a 5-yr follow-up period. Individual data previously presented in group averages in [36]. No significant difference was seen in a) while the decrease with ongoing disease was significant in b) ( $p<0.05$ , Wilcoxon test).

### Multiple marker approach: the future

One approach that minimizes interactions with exposure/disease and achieves better risk estimates, is the use of multiple markers in the same individual, whether using genotype, phenotype or both. Since peripheral blood monocytes are also important sources of TGF- $\beta$ , its release in the same monocyte supernatants previously used to determine TNF release [36] was evaluated. The primary aim of the study was to evaluate the interindividual differences in cytokine release among nondust-exposed controls *versus* subjects chronically exposed to coal dust, as well as in simple CWP. A further purpose of the study was to relate such patterns of cytokine release to mineral dust exposure, and to determine whether these cytokines, either independently or in combination with TNF, can be used as "multiple markers" in CWP. Monocytes were isolated and stimulated as previously described [36]. Adherent monocytes were stimulated with coal

Table 3. – Demographic characteristics and cytokine release of the coal miners cohort in 1992 (n=133)

	Nonexposed referents	Control miners	Miners with CWP
Subjects n	29	76	28
Age yrs	50.3±1.0*	47.7±0.6	49.9±1.1
Smoking pack-yrs	115±20	111±12	150±24
Cumulative dust g·h·m <sup>-3</sup> †		91.1±6.4	130.9±9.3**
Years underground		22.2±0.4	25.0±0.9**
Years since first exposure		28.9±0.6	30.7±1.0
Months retired		46.3±2.1	53.9±2.1
Coal-dust induced cytokine release ng·mL <sup>-1</sup>			
TNF-α	1.10±0.15	1.92±0.16 <sup>#</sup>	1.99±0.26 <sup>#</sup>
TGF-β total	0.32±0.05	1.56±0.17 <sup>#</sup>	1.32±0.23 <sup>#</sup>
TGF-β active	0.08±0.01	0.12±0.02	0.08±0.01

Adapted from [25]. Values are mean±SEM. CWP: coal workers' pneumoconiosis; TNF-α: tumour necrosis factor-α; TGF-β: transforming growth factor-β. †: Cumulative dust exposure was calculated by summation of products of the yearly mean total dust concentrations for the colliery or job where the miner was appointed. Total dust was converted into respirable dust as described in [36]. \*: p<0.05; \*\*: p<0.01 from control miners; and <sup>#</sup>: p<0.05 from nonexposed referents (Mann-Whitney U-test).

dust (5 mg·mL<sup>-1</sup>) or silica particles (0.5 mg·mL<sup>-1</sup>). Each individual's baseline and 18-h stimulated cytokine release was measured without addition of dusts. The experimental conditions are based on the optimal conditions for the release of TNF [35, 39]. TGF-β1 (active and latent forms) was measured by an enzyme-linked immunosorbent assay (ELISA), as previously described [13].

As previously reported, TNF release following coal dust stimulation of monocytes was higher in miners compared to nonexposed subjects (table 3). In line with the observations of GOSSET *et al.* [33], silica-stimulated TNF release was lower than coal dust-stimulated release at the concentrations used. Spontaneous, as well as coal and silica dust-stimulated TGF release, was significantly higher in miners compared to nonexposed individuals. However, at the concentrations used, total TGF-β1 release from dust- or silica-stimulated monocytes was significantly lower than their baseline release, suggesting that some absorption might have taken place. Correlations between individual TNF and TGF-β1 levels were dependent on the group, which led to the development of a cross-table of specific combinations of these two cytokines in relation to disease, age and exposure.

Table 4. – Classification of nonexposed referents, control miners, and miners with coal workers' pneumoconiosis (CWP) versus individual release of tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β from dust-stimulated monocytes

TNF/TGF	Group			
	I Low/ low	II High/ low	III Low/ high	IV High/ high
Subjects n	14	12	14	7
Nonexposed referents	12	3		
Control miners	2	6	9	7
Miners with CWP		3	5	

Subjects were grouped in combinations of upper (high) and lower (low) tertiles of TNF and TGF release.

Individuals were divided into one of four groups, based on high and low release from tertile scores; subjects in the middle range were discarded.

This analysis showed that none of the nonexposed individuals had a high release of transforming growth factor, while only three out of 12 had a high release of tumour necrosis factor (table 4). In contrast, none of the miners with coal workers' pneumoconiosis showed either low or high release of both tumour necrosis factor and transforming growth factor-β simultaneously. Based on the above results, it is suggested that, in addition to tumour necrosis factor release, the production of transforming growth factor-β by monocytes is associated with a lower susceptibility to coal workers' pneumoconiosis, and that combined measurement of transforming growth factor-β and tumour necrosis factor is a powerful marker of coal workers' pneumoconiosis.

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