

# Haplotypes of Tumor Necrosis Factor Gene and Tracheal Aspirate Fluid Levels of Tumor Necrosis Factor- $\alpha$ in Preterm Infants

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**ABSTRACT:** Individual variability in the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) has been attributed to genetic factors. We examined whether alleles of TNF gene (lymphotoxin- $\alpha$ +250, TNF- $\alpha$ -308, and TNF- $\alpha$ -238) affect tracheal aspirate fluid (TAF) levels of TNF- $\alpha$  among preterm infants at risk of bronchopulmonary dysplasia. TAF samples were collected within 48 h of birth and 7, 14, 21, and 28 d later. Haplotypes [designated using the nucleotide bases in the chromosome order (lymphotoxin- $\alpha$ +250, TNF- $\alpha$ -308, TNF- $\alpha$ -238)] of TNF were correlated with levels of TNF- $\alpha$ . Diploypes of TNF (genotypes of haplotypes) classified as high, intermediate, or low based on their relation to TAF TNF- $\alpha$  levels were also correlated with TNF- $\alpha$  levels. The most frequent (and reference haplotype) was AGG. The GGG haplotype was associated with the lowest TAF TNF- $\alpha$  levels on day 7 among African American infants ( $p < 0.008$ ). Sequential changes in levels of TNF- $\alpha$  correlated with infants' diploype status [high (HH), intermediate (HL), low (LL)]. Fetal chorioamnionitis but not bronchopulmonary dysplasia was associated with infants' diploypes ( $p < 0.005$ ). Haplotypes of the TNF gene influence TAF levels of TNF- $\alpha$ . Diploypes of TNF are associated with fetal chorioamnionitis. (*Pediatr Res* 64: 165–170, 2008)

Prolonged elevations of several indices of inflammation have been documented in the tracheal aspirate fluid (TAF) of infants who develop bronchopulmonary dysplasia (BPD) (1). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is one of the proinflammatory cytokines that is increased in the TAF of infants at risk of BPD (2). This cytokine plays a vital role in the regulation of inflammation because it affects the release of other proinflammatory cytokines such as IL-1, IL-6, and several other mediators of inflammation including itself (3). Excessive production of TNF- $\alpha$  may overwhelm counter-regulatory processes that are involved in containing the inflammatory response, and may lead to prolonged inflammation and underlying tissue damage (4,5). Individual variability in the production of TNF was documented from family studies (6) and has been mostly attributed to inherited genetic factors (7). Production of TNF- $\alpha$  is regulated at the transcriptional and posttranscriptional levels (8). Promoter variants of the TNF gene are known to affect its transcription (9). Two single nucleotide polymorphisms in this region, more specifically, guanosine (G) to adenosine (A) transition variants at desig-

nated TNF- $\alpha$ -308 and TNF- $\beta$ +250 [also known as lymphotoxin- $\alpha$  (LT- $\alpha$ +250)] reflecting variants at -308 and +250, respectively, were associated with increased levels of production of TNF- $\alpha$  (10–12). Another G to A transition at position -238 was associated with a decreased transcription of the TNF gene *in vitro* (13). In this report, we investigated whether haplotypes of TNF gene variants affect levels of TNF- $\alpha$  in TAF specimen of preterm infants at risk of developing BPD. We also examined the relationship between TNF genotypes of haplotypes and risk of BPD and fetal chorioamnionitis.

## PATIENTS AND METHODS

Infants with a BW  $\leq$ 1250 g were recruited within the first 48 h of life from the Neonatal Intensive Care Nursery at Hutzel Women Hospital in Detroit, MI. After obtaining an informed consent from the infant's parent(s) or guardian, an EDTA preserved blood specimen (0.5 mL) was collected for the determination of TNF genotypes. DNA was extracted from peripheral blood mononuclear cells using the Genomic DNA purification Kit (Promega, Madison, WI). Genotypic analysis of TNF- $\alpha$  polymorphic sites [LT- $\alpha$ +250 (rs909253), TNF- $\alpha$ -308 (rs1800629), TNF- $\alpha$ -238 (rs361525)] was performed by using a polymerase chain reaction-restriction fragment length polymorphism technique, as previously described (12,14,15). The diagram in Figure 1 illustrates the method used in generating infants' haplotypes.

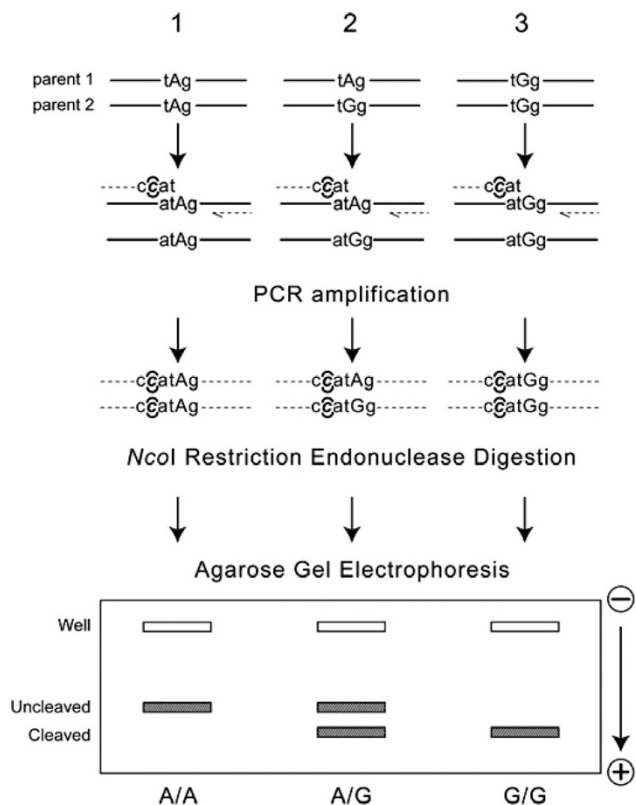
TAF samples were collected from ventilated infants at entry into the study and 7, 14, 21, and 28 d later, so long as the infant remained on mechanical ventilation. Whenever the infant needed suctioning, 1.0–1.5 mL of 0.9 NS was instilled in two aliquots into the infant's endotracheal tube, three to five breaths were delivered with a handbag and the tracheal lavage fluid was aspirated into a sterile aspirating tube (Luki, American Cyanamid Co, CT). The suction catheter was rinsed with a single pass into a 0.9 NS solution. The TAF was centrifuged and the supernatant was separated and stored at  $-80^{\circ}\text{C}$  for subsequent analysis. Levels of TNF- $\alpha$  in TAF were measured using a high sensitivity ELISA method that quantifies total TNF (free and bound to soluble receptors) (Quantakine HS human TNF- $\alpha$  system, R&D Systems, Minneapolis, MN). The sensitivity of the assay for the determination of TNF was 0.062 pg/mL with an interassay and intraassay coefficients of variation of  $<10\%$ . To correct for the diluting effect of the saline solution used in the tracheal suctioning, the total protein concentration in TAF was determined using bicinchoninic acid method (BCA protein assay, Pierce, Rockford, IL) and was used for standardization. Values of TNF- $\alpha$  are reported as picogram per milligram of protein. All protein determinations were based on interpolation within the standard curve.

Gestational age was determined from the maternal last menstrual period and/or first or second trimester antenatal ultrasound when available. For pregnant women with limited or no prenatal care, the infants' gestational age

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**Abbreviations:** BPD, bronchopulmonary dysplasia; HH, high secreting TNF diploype; HL, intermediate secreting TNF diploype; LL, low secreting TNF diploype; LTA, lymphotoxin alpha; TAF, tracheal aspirate fluid



**Figure 1.** Scheme showing genotyping of two different homozygotes and a heterozygote for a single SNP. DNA from three individuals (1, homozygote A/A; 2, heterozygote A/G; and 3, homozygote G/G) is shown indicating the SNP state on the two parental copies of the DNA (nucleotide in capital letter indicates the SNP base). Parents are identified as 1 and 2 because it is not possible to identify the paternal and maternal allele. PCR amplification was performed with a mismatched and matched primer. The mismatched c is encircled and italicized; its presence together with the G state in the SNP yields a product cleavable by the restriction enzyme *NcoI*. Products were separated by agarose gel electrophoresis and the SNP state identified by the sizes of the products.

was determined by averaging estimates obtained from a late antenatal ultrasound and postnatal assessment using the Ballard examination (16). The diagnosis of fetal chorioamnionitis was based on histologic examination of the placenta (17). The pathologists were unaware of infants' TNF- $\alpha$  levels or genotypes. Symptomatic infants with respiratory distress syndrome were treated with surfactant (Survanta, Ross Laboratories, Columbus, OH). The diagnosis of patent ductus arteriosus was confirmed with a two-dimensional Doppler flow echocardiogram. Late onset sepsis was diagnosed in the presence of a positive blood or cerebrospinal fluid culture obtained after 5 d of age. BPD was defined as dependency on supplemental oxygen at 36 wk postmenstrual age with radiographic evidence of parenchymal lung disease. The study was reviewed and approved by the Institutional Review Board at Wayne State University in Detroit, MI.

Statistical analysis was performed using SPSS 13.5 for Windows (SPSS Inc., Chicago, IL) and with R, a language and environment for statistical computing (version 2.4.0) (18). Haplotype frequencies and probabilities were estimated using the HaploStats package in R which implements an approach that uses the expectation-maximization algorithm in conjunction with a general linear model approach and can take outcome variables and covariates into account (19,20). An individual has a pair of haplotypes, one from each parent, that together are called diplotypes. The diplotypes for individuals homozygous at all, or all but one, of the markers can be assigned unambiguously, *i.e.*, one and only one diplotype is possible for the individual [ $p(\text{diplotype, genotype data}) = 1$ ]. For individuals heterozygous at two or more markers, more than one diplotype is possible. For such individuals, the probability of more than one diplotype is estimated such that the sum of diplotype probabilities is 1. The TNF- $\alpha$  protein levels were made multivariate normal by a Box-Cox power transformation and were subsequently used as outcome variable in the haplotype estimation. Haplotypes were estimated for

all genotyped DNA specimens and stratified by race. Estimation was first performed in the absence of normalized TNF- $\alpha$  levels and subsequently with TNF- $\alpha$  levels as outcome for all days where TAF specimens were available. To stabilize the estimates, HaploStats uses the most common haplotype as the baseline or reference haplotype. The effect of the haplotype on the outcome variable (TNF- $\alpha$  protein levels) with respect to the reference haplotype (the most common haplotype) is indicated by the regression coefficient ( $\beta$ ). The linear model coefficients were examined to determine which haplotypes were associated with high (H) and which with low (L) TNF- $\alpha$  protein levels. Simplified diplotypes of H and L TNF haplotypes were scored on an ordinal scale as 0 (LL), 1 (HL), or 2 (HH) for infants using the diplotype probabilities for the TNF haplotypes.

Fisher's exact two-sided test was used to compare categorical variables such as BPD and fetal chorioamnionitis. Associations detected by Fisher's exact test were verified using the linear-by-linear association model to ensure that they fit the model of increased risk with an increase in the copy number of high-TNF haplotype (LL, HL, and HH).

**RESULTS**

Genotype data were determined on 154 infants. Five infants of Hispanic origin were excluded from the data analysis because of their limited number. Among the remaining infants ( $n = 149$ ), TAF samples were successfully obtained from 96 infants on day 1, 59 on day 7, 50 on day 14, 55 on day 21, and 59 on day 28. Characteristics of infants and their hospital course are shown in Table 1. Tumor necrosis factor haplotype frequencies among all infants, African American and white infants are shown in Table 2. We refer to all haplotypes using the nucleotide bases in the chromosome order, *e.g.*, A-G-A [A (LT- $\alpha$ +250)-G (TNF- $\alpha$ -308)-A (TNF- $\alpha$ -238)]. The most

**Table 1.** Infants characteristics and hospital course ( $n = 149$ )

Birth weight (g)*	891 $\pm$ 217
Gestational age (wks)*	27 $\pm$ 3
White/African American†	34 (23%)/115 (77%)
Male†	70 (47%)
Fetal chorioamnionitis†	40 (26%)
PROM $\geq$ 18 hrs†	36 (24%)
Antenatal steroids†	116 (78%)
Cesarean birth†	100 (67%)
Apgar Score $\leq$ 5 at 5 min†	23 (15%)
Ventilatory index at entry into study (VI)‡	3.0 (1.7–6.2) [108]
PDA†	62 (42%)
Grade 3 or 4 IVH†	22 (15%)
NEC stage $\geq$ 2†	21 (14%)
Late onset sepsis†	57 (38%)
BPD†	50 (34%)
Survived to hospital discharge†	112 (75%)

\* Values are mean  $\pm$  SD.

† Values are count (percentage) of infants.

‡ VI = MAP  $\times$  FiO<sub>2</sub>; values are median (25–75th interquartile range); number of infants in brackets.

**Table 2.** Haplotype frequencies among infants

Haplotype (LT- $\alpha$ +250, TNF- $\alpha$ -308, TNF- $\alpha$ -238)	All infants ( $N = 149$ )	White ( $N = 34$ )	African American ( $N = 115$ )
A-A-A	0.004		0.005
A-A-G	0.004	0.017	
A-G-A	0.047	0.029	0.055
A-G-G	0.513	0.689	0.453
G-A-A			
G-A-G	0.115	0.086	0.130
G-G-A	0.014		0.018
G-G-G	0.302	0.179	0.339

**Table 3.** Estimated haplotype frequencies among infants with TAF TNF- $\alpha$  levels on day 7\*

Haplotype (LT- $\alpha$ +250, TNF- $\alpha$ -308, TNF- $\alpha$ -238)	All infants (N = 58)	White (N = 11)	$\beta$ coefficient	African American (N = 47)	$\beta$ coefficient	High/low
A-A-A	0.008			0.011	-1.66	L
A-A-G						
A-G-A	0.039	0.046	1.587	0.036	0.191	H
A-G-G	0.538	0.682	0	0.506	0	H
G-A-A						
G-A-G	0.102	0.046	-3.420	0.117	-0.512	L
G-G-A	0.021			0.028	-0.300	L
G-G-G	0.293	0.227	0.921	0.302	-0.726†	L

The effects of haplotypes on TAF TNF- $\alpha$  protein levels are indicated by the regression coefficients ( $\beta$ s).

\* HaploStats incorporates genotypic information from the infants without outcome data.

†  $p < 0.008$  for GGG vs AGG associated TAF TNF- $\alpha$  levels.

H, high secreting haplotype; L, low secreting haplotype.

common TNF haplotype for both African American and white infants was A-G-G and was used as the reference haplotype in all subsequent analyses. The effect of haplotypes on TNF- $\alpha$  protein levels is indicated by the regression coefficients ( $\beta$ s) of the linear model (Table 3). Association of TNF- $\alpha$  protein levels with haplotypes was tested for all days (1, 7, 14, 21, and 28). On day 7, the G-G-G haplotype was associated with the lowest TAF TNF- $\alpha$  levels relative to the reference A-G-G haplotype among African American infants (Table 3,  $p < 0.008$ ). The white subgroup of infants was too small to produce stable estimates. Examination of the regression coefficients among African American infants revealed that they were clustered into two groups, those above and below  $\beta = 0$  for the reference haplotype A-G-G. Haplotypes associated with high levels of TNF- $\alpha$  protein (indicated by H in Table 3) can be characterized as A-G-X, *i.e.*, only the LT- $\alpha$ +250 and TNF- $\alpha$ -308 seem to contribute to the elevation in the TNF- $\alpha$  protein levels. Haplotypes associated with low levels of TNF- $\alpha$  protein seem to include mostly those with G allele of LT- $\alpha$ +250, although this observation did not hold for the A-A-A haplotype, which had the lowest frequency of all haplotypes among African American infants, and the estimate was derived from two haplotypes only.

We assigned diplotypes, *i.e.*, genotypes of LL, LH, HH pairs of haplotypes to all African American infants ( $n = 115$ ), including those who did not have TNF- $\alpha$  protein levels measured on day 7, but had levels measured on one or more other days. Diplootype probabilities revealed that for all but five African American infants, the diplotypes were unambiguous (*e.g.*, A-G-G/G-G-G). Of the five infants, only one was ambiguous with respect to the high/low diplootype assignment, because the ambiguity was removed for four infants when simplifying the haplotypes. This infant with ambiguous H/L diplootype did not have relevant data on BPD and fetal chorioamnionitis and thus, did not influence the outcome of subsequent analyses.

Sequential changes in actual and normalized TNF- $\alpha$  protein levels plotted according to infants' diplotypes (LL, HL, and HH) are depicted in Table 4 and Figure 2, respectively. All three diplotypes demonstrate a rise in TNF- $\alpha$  protein levels from day 1 to 14. The HH diplootype demonstrates the most rapid rate of increase, the HL diplootype, an intermediate and the LL diplootype the slowest rate of increase. Interestingly,

TAF levels of TNF- $\alpha$  for HL and HH diplotypes peaked at 14 d, compared with the LL diplootype for which TNF- $\alpha$  levels peaked at 21 d. Levels of TNF- $\alpha$  for all three diplotypes returned to very-near baseline levels at 28 d.

Finally, we found no correlation between infants' diplootype status and the development of BPD ( $p = 0.54$ ); however, fetal chorioamnionitis was significantly associated with the diplootype status (Table 5,  $p < 0.005$ ). The latter association was maintained when the linear-by-linear association model was applied ( $p < 0.001$ ) indicating that the association was consistent with an additive genetic model in which the risk of fetal chorioamnionitis increases with the number of copies of H haplotypes in each diplootype. Because fetal chorioamnionitis may affect levels of TNF- $\alpha$ , and as a result an infant's diplootype assignment, we estimated haplotype frequencies after adjusting for fetal chorioamnionitis in the linear model. The frequencies estimate, and the betas, change very slightly, not affecting the results.

Because TNF- $\alpha$  measurements were available only for those infants who were intubated and survived the first 28 d of life, there was a potential that data would be missing selectively by genotype or diplootype. We therefore modeled survival using Cox proportional hazard regression with diplootype as covariate and found no significant effect of diplootype on survival ( $p = 0.76$ ).

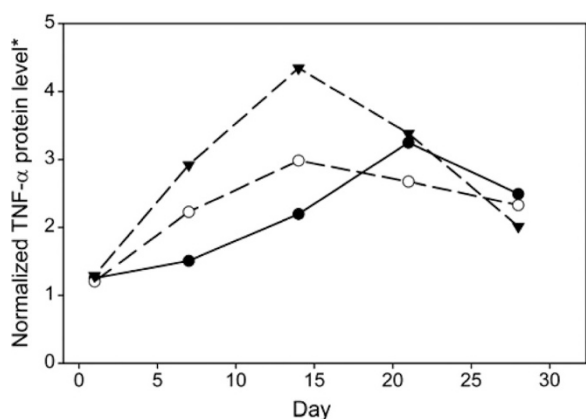
## DISCUSSION

Reports of variability in the production of TNF- $\alpha$  in association with three alleles of the TNF gene (LT- $\alpha$ +250, TNF- $\alpha$ -308, and TNF- $\alpha$ -238) (10–13) instigated our study of determining whether haplotypes of these alleles affect TAF levels of TNF- $\alpha$  among ventilated preterm infants at risk of BPD. We speculated that a diplootype of "high secretor" haplotypes of TNF- $\alpha$  might increase an infant's risk of developing BPD. We selected the above-mentioned alleles of TNF and LT- $\alpha$  because they are in linkage disequilibrium and are inherited as haplotypes (11,21). By using diplotypes of haplotypes, we aimed to capture the sum effect of both copies of TNF gene on TAF levels of TNF- $\alpha$ . Finally, we measured TAF rather than plasma concentrations of TNF- $\alpha$  because they are better reflective of lung tissue inflammation.

**Table 4.** TAF TNF- $\alpha$  levels by diplotype status\*

	Actual (untransformed)					Transformed (power = -0.06)				
	D1	D7	D14	D21	D28	D1	D7	D14	D21	D28
<b>LL</b>										
<i>n</i>	24	12	9	13	9	24	12	9	13	9
Min	0.11	0.44	1.11	1.23	4.12	-2.36	-0.84	0.10	0.21	1.36
25%	0.94	2.56	3.85	3.58	7.44	-0.07	0.91	1.29	1.23	1.89
Median	2.85	4.40	10.04	24.84	15.52	1.01	1.41	2.15	2.92	2.53
75%	6.45	8.91	44.09	88.55	43.93	1.76	2.01	3.39	3.93	3.38
Max	45.13	47.74	117.93	231.33	885.02	3.41	3.45	4.15	4.64	5.57
<b>LH</b>										
<i>n</i>	26	19	19	18	15	26	19	19	18	15
Min	0.19	1.03	0.17	1.68	1.11	-1.75	0.03	-1.87	0.51	0.10
25%	1.65	4.54	4.78	3.98	7.85	0.49	1.44	1.49	1.28	1.93
Median	3.86	10.74	11.65	17.64	17.32	1.29	2.21	2.28	2.59	2.62
75%	16.92	27.99	40.28	38.14	55.33	2.60	3.02	3.30	3.27	3.57
Max	111.95	78.05	200.64	94.63	163.40	4.11	3.83	4.54	3.98	4.39
<b>HH</b>										
<i>n</i>	22	16	12	13	6	22	16	12	13	6
Min	0.25	2.78	3.27	3.52	4.35	-1.45	0.99	1.14	1.21	1.41
25%	1.25	13.71	12.16	8.73	5.77	0.20	2.42	2.29	2.03	1.66
Median	4.93	19.92	38.01	17.59	7.93	1.52	2.73	3.23	2.63	1.93
75%	15.67	43.34	73.17	42.72	11.03	2.52	3.37	3.77	3.36	2.23
Max	53.02	147.30	219.88	122.83	56.18	3.53	4.31	4.61	4.18	3.58

\* TNF- $\alpha$  protein levels in units of pg TNF- $\alpha$ /mg total protein. The Box and Cox power transformation is identical to log transformation when the power is 0. Here we have a power close to 0. The difference between the usual log transformation and the power transformation is that the power transformation reduces both the standard deviations and kurtosis at the expense of a little more skew.



**Figure 2.** Sequential TAF levels of TNF- $\alpha$  protein among African American infants according to TNF diplotypes: high (HH,  $\blacktriangledown$ ,  $n = 34$ ), intermediate (HL,  $\circ$ ,  $n = 48$ ), and low (LL,  $\bullet$ ,  $n = 33$ ) secreting. \*Data were transformed using Box and Cox power transformation with an exponent of 0.05 [ $(\chi^{0.05} - 1)/0.05$ ].

**Table 5.** Fetal chorioamnionitis and TNF diplotypes among African American infants\*

	TNF diplotype		
	LL (%)	HL (%)	HH (%)
Chorioamnionitis present ( $n = 38$ )	5 (13)	15 (40)	18 (47)
Chorioamnionitis absent ( $n = 58$ )	23 (40)	23 (40)	12 (20)

Values are counts (percentage) of infants.

\* Fisher's exact test,  $p < 0.005$ ; linear by linear association model,  $p < 0.001$ .

We found significant variability in the frequency of haplotypes of TNF among African American and white infants. The most common TNF haplotype among all infants was AGG (A

allele of LT- $\alpha$ +250, G allele of TNF- $\alpha$ -308, and G allele of TNF- $\alpha$ -238, respectively). Among African American infants, significantly lower TAF TNF- $\alpha$  levels were noted on day 7 in association with GGG haplotype compared with AGG haplotype (reference). Both AGA and AGG haplotypes were associated with higher TAF levels of TNF- $\alpha$  on day 7. TAF TNF- $\alpha$  levels increased during the first 2 wk of life among African American infants with the rate of rise and peak levels of TNF- $\alpha$  being highest among infants with a diplotype of "high secreting" haplotypes (HH), followed by a diplotype of "intermediate secreting" (HL), and "low secreting" haplotypes (LL). Interestingly, infants with the LL diplotype had the slowest rate of rise in TAF TNF- $\alpha$  levels reaching a delayed and lower peak at 21 d compared with infants with HH diplotype. All TAF TNF- $\alpha$  levels returned to near baseline by 28 d of the study. Despite significantly affecting TAF levels of TNF- $\alpha$ , infants' diplotype status did not seem to affect the risk of BPD. In contrast, the risk of fetal chorioamnionitis increased with increasing number of H haplotypes in each diplotype.

The frequencies of TNF gene haplotypes in our population are comparable with those reported by other investigators (22,23). We found no report in the literature relating TAF levels of TNF- $\alpha$  to haplotypes or diplotypes of the TNF gene. Investigators who explored associations between haplotypes of TNF gene, levels of TNF- $\alpha$  and disease outcome reported variable results, some of which could be attributed to differences in pathogenetic mechanisms (23-29). Some, similar to our findings with BPD, noted an effect on levels of TNF without an association to underlying disease risk (24-26). Increased beryllium-stimulated BAL cell TNF- $\alpha$  levels were observed among patients with chronic beryllium disease who

had TNF haplotype -1031T, -863C, -857C, -307G, -237G without a significant association with measures of disease severity (24). Elevated levels of TNF- $\alpha$  secretion by activated mononuclear cells were associated with TNF haplotype [-238G, -308A, LTA (AspHI) C, LTA *NcoI* G] without a difference in TNF- $\alpha$  or LT- $\alpha$  production between patients with inflammatory bowel disease and healthy controls (25). Three TNF haplotypes defined by eight SNPs within the TNF gene region were associated with lower levels of expression of TNF- $\alpha$  without affecting the risk of developing schizophrenia (26). Other investigators reported effects of TNF haplotypes on levels of TNF and disease risk and or outcome (27–29). Increased serum IgE and TNF- $\alpha$  levels were associated with an increased risk for asthma among Indian individuals homozygous for TNF haplotype GATCCG [LT- $\alpha$  (His51Pro), LT- $\alpha$  (Thr60Asn), TNF- $\alpha$ -1031C, -863A, -857C, -308G] (27). A better response to chemotherapy and a greater progression-free and overall survival were reported among patients with non-Hodgkin's lymphoma carrying a low-risk TNF haplotype, *i.e.*, containing less than two adenine alleles of TNF- $\alpha$ -308 or LT- $\alpha$ +250 that were associated with high plasma levels of TNF- $\alpha$  or LT- $\alpha$ , respectively (28). Higher nasal levels of TNF- $\alpha$  were observed during acute wheezing episodes among infants with a TNF haplotype containing one or two LT- $\alpha$ +252A alleles (29). In our study, we found that the risk of fetal chorioamnionitis increased with increasing number of high secreting TNF haplotype. Finally, a multicenter study among adult patients with severe sepsis and septic shock failed to demonstrate an association between TNF genotypes (TNF- $\alpha$ -308, TNF- $\alpha$ -238, LTA + 249, and LTA + 365), plasma levels of TNF- $\alpha$  and risk, severity or outcome of sepsis (23).

Our findings of a lack of association between diplotypes of TNF and BPD suggest that mechanisms other than prolonged inflammation may be important in the development of BPD. In support of this argument is our observation that, following an initial rise, tracheal TNF- $\alpha$  levels returned to baseline by 28 d. It is likely that, beyond the first month of life, the extent of disrupted lung development, airway and vascular damage is the main determinant for the development of BPD (30).

Using genotype analysis of individual alleles of TNF, our group previously reported a decreased risk and severity of BPD in association with the adenine allele of TNF- $\alpha$ -238 (rs361525) (31). In this study, we examined the relationship between diplotypes of haplotypes defined by level of TNF- $\alpha$  secretion and BPD. The diplotypes did not replicate the association of SNP (rs361525) with BPD. The comparisons and results are different. Either or both may be true associations. We speculate that the previous association of BPD with rs361525 may be due to a different mechanism, not dependent on the secretion level of TNF- $\alpha$ . Replication studies are essential to validate the results.

On the other hand, our diplotype analysis confirmed a rising risk of fetal chorioamnionitis with increasing number of high secretor TNF haplotypes. These findings are in agreement with an earlier report in which we described an increased risk of fetal chorioamnionitis in the presence of A allele of LT- $\alpha$ +250, a fixed component of high secretor diplotype as defined in this study (17). Other investigators reported a

similar association between severity of fetal chorioamnionitis and levels of TNF- $\alpha$  (32).

In summary, by using an analysis of genotypes of haplotypes, we were able to demonstrate the presence of different genotypes of TNF in association with various levels of production of TNF- $\alpha$ . Specifically, genotypes of TNF consisting of haplotypes containing the A allele of LT- $\alpha$ +250 and G allele of TNF- $\alpha$ -308 were associated with higher levels of secretion of TNF- $\alpha$ . The absence of an association between genotypes of TNF and risk of BPD highlights the difficulties encountered in clinical practice in delineating the contribution of individual risk factors to multifactorial diseases.

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