

FULL PAPER

TNF and TNFR polymorphisms in severe sepsis and septic shock: a prospective multicentre study

AC Gordon¹, AL Lagan², E Aganna¹, L Cheung³, CJ Peters¹, MF McDermott¹, JL Millo⁴, KI Welsh², P Holloway⁴, GA Hitman¹, RD Piper³, CS Garrard⁴ and CJ Hinds¹

¹Institute of Cell and Molecular Science & William Harvey Research Institute, Barts and The London Queen Mary's School of Medicine and Dentistry, University of London, London, UK; ²Clinical Genomics Group, National Heart and Lung Institute, Imperial College, London, UK; ³Kolling Institute of Medical Research and Royal North Shore Hospital, University of Sydney, St Leonard's, NSW, Australia; ⁴Intensive Care Unit, John Radcliffe Hospital, Oxford, UK

Tumour necrosis factor (TNF) is an important pro-inflammatory cytokine produced in sepsis. Studies examining the association of individual TNF single nucleotide polymorphisms with sepsis have produced conflicting results. This study investigated whether common polymorphisms of the TNF locus and the two receptor genes, TNFRSF1A and TNFRSF1B, influence circulating levels of encoded proteins, and whether individual polymorphisms or extended haplotypes of these genes are associated with susceptibility, severity of illness or outcome in adult patients with severe sepsis or septic shock. A total of 213 Caucasian patients were recruited from eight intensive care units (ICU) in the UK and Australia. Plasma levels of TNF ($P=0.02$), sTNFRSF1A ($P=0.005$) and sTNFRSF1B ($P=0.01$) were significantly higher in those who died on ICU compared to those who survived. There was a positive correlation between increasing soluble receptor levels and organ dysfunction (increasing SOFA score) (sTNFRSF1A $R=0.51$, $P<0.001$; sTNFRSF1B $R=0.53$, $P<0.001$), and in particular with the degree of renal dysfunction. In this study, there were no significant associations between the selected candidate TNF or TNF receptor polymorphisms, or their haplotypes, and susceptibility to sepsis, illness severity or outcome. The influence of polymorphisms of the TNF locus on susceptibility to, and outcome from sepsis remains uncertain.

Genes and Immunity (2004) 5, 631–640. doi:10.1038/sj.gene.6364136

Published online 4 November 2004

Keywords: TNF; TNF receptors; polymorphisms; haplotypes; sepsis; septic shock

Introduction

Sepsis is the most common cause of death in adult intensive care units (ICU), and is being diagnosed with increasing frequency.^{1–3} Attempts to reduce the high mortality rates (20–60%) associated with sepsis, severe sepsis and septic shock by manipulating the inflammatory response have met with only limited success,^{4,5} at least in part due to our limited understanding of the complex mechanisms which regulate the innate immune response in these conditions. Also, such interventions have usually been applied unselectively to heterogeneous groups of patients, without considering the potential influence of their ethnic and genetic diversity on the response to treatment.

More than 10 years ago, it was clearly demonstrated that premature death in adults, especially when due to infectious and vascular causes, is a strongly heritable trait.⁶ Since then, a number of relatively small studies

have suggested that interindividual variations in susceptibility to, and outcome from, severe sepsis/septic shock can be partly explained by polymorphisms of the genes encoding proteins involved in mediating and controlling innate immunity and the inflammatory response.^{7–18}

Tumour necrosis factor (TNF) is a pivotal cytokine in the host response to infection. Systemic administration of TNF produces most of the symptoms and signs of sepsis,^{19,20} and previous studies have shown that high TNF levels are associated with a poor outcome from meningococcal infection.^{21,22} Several single nucleotide polymorphisms (SNPs) within the *TNF/LTA* locus are thought to influence TNF production, and have therefore been identified as candidate genes that might influence susceptibility to and/or outcome from infectious disease. In sepsis, interest has particularly focused on the promoter *TNF* –308 G/A SNP. Although several studies have found an association of the A allele with a predisposition to septic shock and/or outcome from sepsis, findings have been inconsistent (Table 1).^{7,8,23–26} One explanation for these contradictory observations may be the linkage disequilibrium (LD), which is known to exist between this SNP and other functionally important polymorphisms in the region, and it is notable that only one previous study has investigated TNF

Correspondence: Professor CJ Hinds, Department of Anaesthesia and Intensive Care Medicine, Barts and The London Queen Mary's School of Medicine and Dentistry, London EC1A 7BE, UK.
E-mail: c.j.hinds@qmul.ac.uk

Received 06 July 2004; revised 13 August 2004; accepted 16 August 2004; published online 4 November 2004

Table 1 Previous TNF -308 severe sepsis and septic shock association studies in adults

| Country and ethnic group | Sepsis case-control study | | | Outcome studies | | | Other TNF loci SNPs studied | |
|---|---------------------------|------------------------------------|-------------------|-----------------|--------------|------------------|-------------------------------------|---------|
| | Cases (n) | Frequency of G/A and A/A genotypes | | Survivors | Nonsurvivors | OR | | P-value |
| | | Cases | Conts | | | | | |
| German Caucasian ²³ | 80 | 0.31 | 0.33 ^a | 0.38 | 0.24 | — | NS* LTA +249** | |
| French Caucasian ⁷ | 89 | 0.39 | 0.18 ^b | 0.24 | 0.52 | 3.7 ^c | TNF -419, -376, -244, -238 (all NS) | |
| Belgian ⁸ (ethnicity not stated) | 34 | 0.26 | — | 0.11 | 0.47 | 12 ^b | Nil | |
| Taiwan ²⁵ | 42 | 0.31 | 0.19 ^c | 0.08 | 0.4 | — | Nil | |
| USA mixed ethnicity ²⁶ | 31 | 0.22 | 0.31 ^d | 0.25 | 0.2 | — | LTA +249*** | |
| USA mixed ethnicity ²⁴ | 37 | 0.43 | 0.17 ^e | — | — | 4.6 ^b | TNF -376, -238 (both NS) | |

^aHealthy local controls.^bAdjusted odds ratios.^cPost-op septic patients without shock.^dCommunity acquired pneumonia patients without septic shock.^eTrauma patients without severe sepsis.

*Allele frequencies used in calculation of P-value in publication.

**LTA +249 A/A higher mortality (P = 0.007).

***LTA +249 A/A higher incidence of septic shock (P = 0.01) but no mortality association.

haplotype associations in patients with sepsis.²⁶ Moreover, although the A allele of the -308 SNP has been shown to increase TNF transcription in some *in vitro* studies,²⁷ in others this has not been the case,²⁸ leading some to question the functional importance of this SNP.

In order to recover from an infectious insult, the pro-inflammatory response to infection must be balanced and controlled by anti-inflammatory mechanisms. In the case of TNF, regulatory mechanisms include shedding into the circulation of two membrane-bound TNF receptors, TNFRSF1A and TNFRSF1B (previously known as TNFR1 and TNFR2 and both members of the TNF receptor superfamily). Cleavage of the extracellular portion of these receptors produces soluble molecules (sTNFRSF1A and sTNFRSF1B) in the blood that retain the ability to bind TNF and inhibit its acute activity.²⁹ Evidence is gradually emerging that genetic variations within the TNF receptor gene loci may be important in the pathogenesis of various inflammatory conditions. A rare, dominantly inherited autoinflammatory syndrome, formerly known as familial Hibernian fever but renamed TRAPS (TNF Receptor-Associated Periodic Syndrome), is caused by germline mutations, mostly within the extracellular domain of the *TNFRSF1A* gene.³⁰ Some of these mutations (eg C52F and T50M) result in impaired shedding of the extracellular portion of TNFRSF1A, such that these patients have high levels of membrane-bound TNFRSF1A (mTNFRSF1A) on leucocytes and low circulating sTNFRSF1A levels in plasma. The characteristic fevers, skin rashes and arthritis in this condition are therefore believed to be due to the unrestrained pro-inflammatory actions of TNF. Polymorphisms within the *TNFRSF1B* locus have also been associated with other conditions in which TNF is believed to play an important role.³¹⁻³³ We therefore hypothesised that such polymorphisms might influence plasma levels of TNF receptors, and hence the extent of the inflammatory response and outcome in patients with severe sepsis/septic shock. To date, no studies have investigated the role of TNF receptor polymorphisms in sepsis.

The aim of this study was to recruit a larger number of patients with severe sepsis and septic shock than has been previously studied, in order to investigate whether common polymorphisms or haplotypes of the *TNF* locus and the two receptor genes, *TNFRSF1A* and *TNFRSF1B* (on chromosomes 6, 12 and 1 respectively) influence circulating levels of encoded proteins, susceptibility to sepsis, severity of illness or outcome.

Results

Patients

A total of 213 Caucasian patients were recruited, 75 from London, 82 from Oxford and 56 from Sydney. In all, 60% were male; the median age was 64 (19-80) years. The median admission APACHE II score was 19 (4-42). The APACHE II predicted hospital mortality was 39.4%. The overall ICU mortality rate was 24.4% (52/213) and hospital mortality 34.3% (73/213). There were no significant differences in patient characteristics between recruiting centres, except in the proportion of medical admissions (Table 2).

Table 2 Patient characteristics according to recruiting centre

| | All patients (n = 213) | London (n = 75) | Sydney (n = 56) | Oxford (n = 82) | P |
|----------------------------------|------------------------|-----------------|-----------------|-----------------|--------|
| Age | 64 (19–80) | 62 (22–80) | 65.5 (19–80) | 66 (19–80) | 0.92 |
| Male (%) | 128 (60) | 44 (58.7) | 29 (51.8) | 55 (67.1) | 0.18 |
| Medical admissions (%) | 128 (60) | 41 (54.7) | 47 (83.9) | 40 (48.8) | <0.001 |
| APACHE II | 19 (4–42) | 17 (9–37) | 22 (4–42) | 18 (4–39) | 0.10 |
| ICU mortality (%) | 52 (24.4) | 22 (29.3) | 13 (23.2) | 17 (20.7) | 0.47 |
| Hospital mortality (%) | 73 (34.3) | 28 (37.3) | 19 (33.9) | 26 (31.7) | 0.76 |
| Predicted hospital mortality (%) | 39.4 | 37.2 | 45.5 | 37.4 | — |

Cytokine and receptor levels

Plasma samples were collected on the day of recruitment from 53 of the patients recruited in London. Plasma levels of TNF were significantly higher in those who died on the ICU compared to those who survived (19, 0–72 pg/ml vs 6.4, 0–74.6 pg/ml, $P=0.02$, Figure 1). Similarly, plasma levels of sTNFRSF1A and sTNFRSF1B were both significantly higher in the nonsurvivors (11.3, 3–42.8 ng/ml vs 5.5, 1–27 ng/ml, $P=0.005$ and 19, 2.9–48.8 ng/ml vs 7.7, 2.8–35.1 ng/ml, $P=0.01$, respectively, Figure 1).

There was no relationship between the ratio of plasma TNF to sTNFRSF1A ($P=0.19$) or TNF to sTNFRSF1B ($P=0.19$) and survival. Similarly, there was no relationship between the ratio of plasma levels of soluble receptors 1A and 1B and survival ($P=0.27$).

There was a positive correlation between increasing soluble receptor levels and SOFA score, and in particular with the degree of renal dysfunction (Figures 2 and 3).

Genotype and plasma protein levels

There was no association between TNF genotype and plasma TNF levels ($P=0.71$, 0.69, 0.55, 0.64 for *TNF* –308, –238, *LTA* +249, +365, respectively). Similarly, there was no association between sTNFRSF1A and 1B plasma levels and their respective polymorphisms ($P=0.58$, 0.45, 0.86 for *TNFRSF1A* –609, +36, +1135 and $P=0.77$ and 0.76 for *TNFRSF1B* +676, +1663, respectively).

Genotype and outcome

All genotype frequencies were in Hardy–Weinberg equilibrium. There were no significant differences between genotype or allele frequencies in the patients with severe sepsis/septic shock when compared to normal health controls (Table 3).

The genotype frequencies in ICU survivors and nonsurvivors are shown in Table 4. None of the individual genotypes or alleles was significantly associated with ICU outcome. Only increasing age (odds ratio 1.03, 95% CI 1.00–1.05, for each year) and medical rather than surgical admission (odds ratio 2.47, 95% CI 1.22–5.00) were statistically associated with ICU mortality. After adjusting for these two variables, the lack of a statistically significant association between genotype and outcomes remained. Similarly, there was no association between genotype or allele frequency and hospital outcome (data not shown).

There was no association between illness severity (assessed by the maximum SOFA score attained during the ICU stay³⁴), and genotype of any of the SNPs studied.

Extended haplotypes and outcome

There was no association between any of the estimated haplotypes in the three genetic loci and outcome. The estimated haplotype frequencies are shown in Table 5.

Discussion

In this study, there were no significant associations between the selected candidate TNF or TNF receptor polymorphisms, or their haplotypes, and susceptibility to sepsis, illness severity or outcome. In particular, we were unable to confirm previous findings of significant associations between the *TNF* –308 and the *LTA* +249 SNPs and susceptibility to, or outcome from severe sepsis and septic shock.^{7–9,24–26} Also, in keeping with one earlier study,⁷ we were unable to confirm previous findings of an association between either the *TNF* –308 SNP^{8,25} or the *LTA* +249 SNP⁹ and circulating levels of TNF. Such inconsistency has been a common experience with candidate gene association studies in many diseases,³⁵ and in particular with those which have examined TNF polymorphisms.³⁶ Possible explanations have included limited statistical power (sometimes compounded by multiple testing and overestimation of marginal results in small populations), heterogeneous patient populations with unrecognised confounding factors, stratification of population substructure, imprecise definition of phenotype and variable quality control of genotyping techniques.³⁷ Moreover, the functional importance of many of these polymorphisms is uncertain;³⁶ indeed many SNPs may simply represent genomic markers for other more functionally relevant genetic variants with which they are in linkage disequilibrium. Of particular relevance to this study, *TNF*/*LTA* SNPs are located in the major histocompatibility complex (MHC) class III region on chromosome 6p21 and are therefore in strong LD with the MHC extended haplotypes.³⁸ Lastly, sepsis is the epitome of a complex polygenic disorder and it is possible that in this condition the inconsistent findings can be explained, at least in part by differences in case mix between the various studies.

In view of these conflicting results, the present study was designed to achieve more complete genotyping, including haplotype analysis. In addition to the

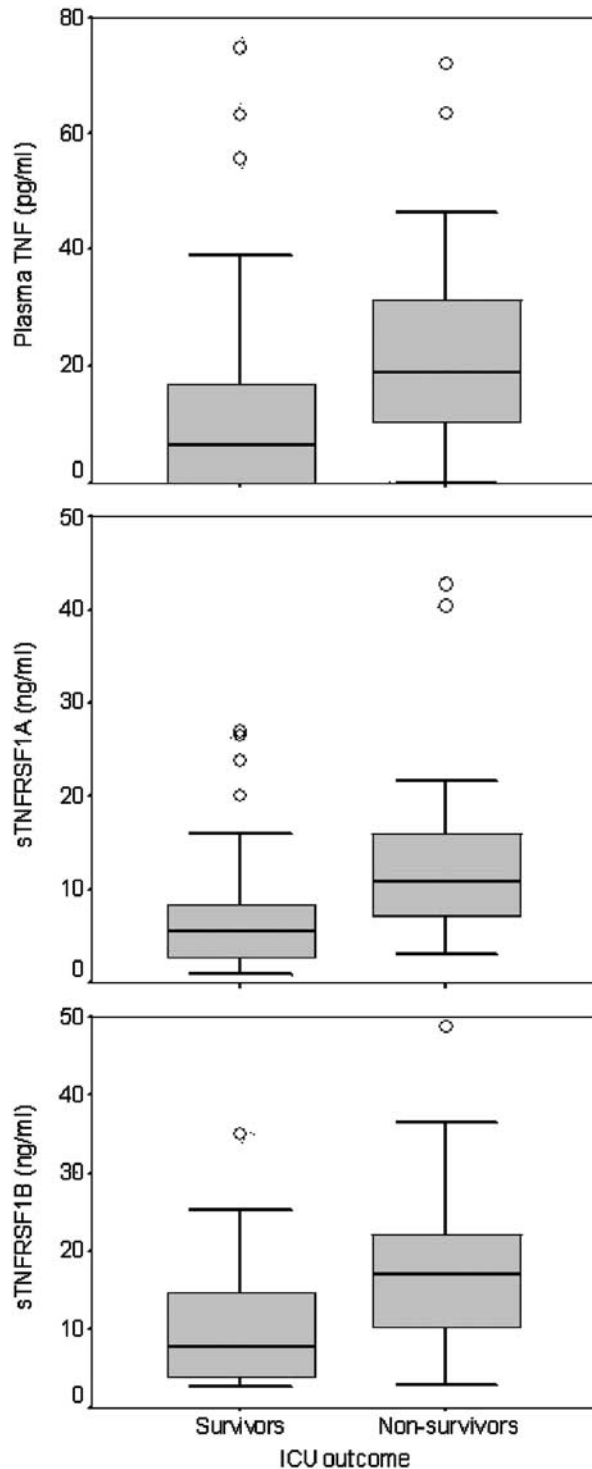


Figure 1 Plasma levels of TNF, sTNFRSF1A and sTNFRSF1B in ICU survivors and nonsurvivors, $P=0.02$, 0.005 and 0.01 , respectively (line = median, box = interquartile range, O = outliers).

commonly studied *TNF* -308 polymorphism, we also examined the G/A SNP at -238 in the *TNF* promoter region (another SNP associated with inflammatory conditions and TNF production^{39,40}) and the *LTA* +365 G/C SNP⁴¹ as well as the *LTA* +249 polymorphism (which influences RNA polymerase loading,⁴² has been

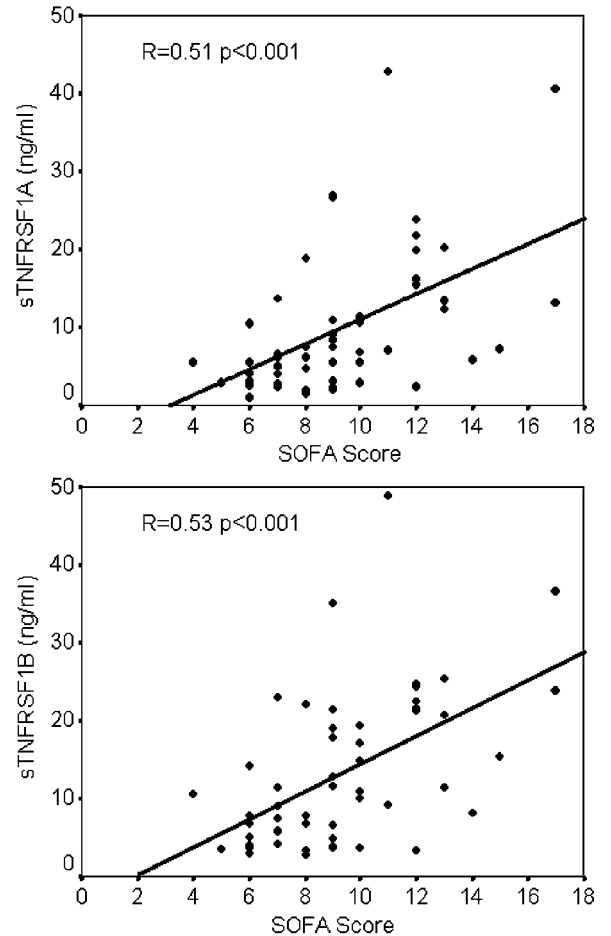


Figure 2 Plasma levels of sTNFRSF1A and sTNFRSF1B compared to SOFA score.

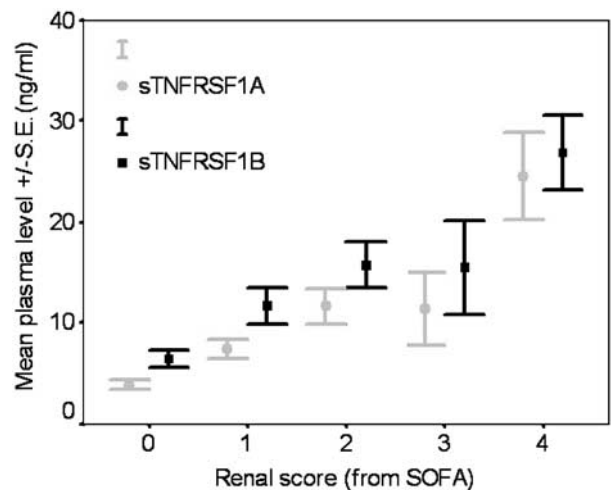


Figure 3 Mean plasma levels of sTNFR with increasing renal failure.

associated with susceptibility to myocardial infarction,⁴³ has been found to have some positive associations in sepsis^{9,26} and recently has been shown to be the most informative SNP for estimating haplotypes across the *TNF/LTA* locus⁴⁴).

Table 3 Genotype and allele frequencies for each SNP in normal healthy controls and patients with severe sepsis and septic shock

| | | % Controls (n) | % Patients (n) | P |
|----------------|----------|----------------|----------------|------|
| TNF -308 | G/G | 65.8 (233) | 63.7 (135) | 0.88 |
| | G/A | 31.1 (110) | 32.5 (69) | |
| | A/A | 3.1 (11) | 3.8 (8) | |
| | G allele | 81.4 (576) | 80 (339) | |
| | A allele | 18.6 (132) | 20 (85) | |
| TNF -238 | G/G | 90.7 (321) | 85.9 (176) | 0.17 |
| | G/A | 9.3 (33) | 13.7 (28) | |
| | A/A | 0 (0) | 0.5 (1) | |
| | G allele | 95.3 (675) | 92.7 (380) | |
| | A allele | 4.7 (33) | 7.3 (30) | |
| LTA +249 | G/G | 14.4 (50) | 12.7 (27) | 0.46 |
| | G/A | 47.7 (166) | 44.1 (94) | |
| | A/A | 37.9 (132) | 43.2 (92) | |
| | G allele | 38.2 (266) | 34.7 (148) | |
| | A allele | 61.8 (430) | 65.3 (278) | |
| LTA +365 | C/C | 13.8 (48) | 11.7 (24) | 0.79 |
| | C/G | 49.6 (173) | 50.7 (104) | |
| | G/G | 36.7 (128) | 37.6 (77) | |
| | C allele | 38.5 (269) | 37.1 (152) | |
| | G allele | 61.5 (429) | 62.9 (258) | |
| TNFRSF1A -609 | G/G | 39.5 (47) | 35.6 (74) | 0.49 |
| | G/T | 46.2 (55) | 52.9 (110) | |
| | T/T | 14.3 (17) | 11.5 (24) | |
| | G allele | 62.6 (149) | 62.0 (258) | |
| | T allele | 37.4 (89) | 38.0 (158) | |
| TNFRSF1A +36 | A/A | 37.8 (45) | 33.2 (69) | 0.59 |
| | A/G | 42.9 (51) | 48.6 (101) | |
| | G/G | 19.3 (23) | 18.3 (38) | |
| | A allele | 59.2 (141) | 57.5 (239) | |
| | G allele | 40.8 (97) | 42.5 (177) | |
| TNFRSF1A +1135 | C/C | 34.1 (45) | 36.1 (75) | 0.33 |
| | C/T | 49.2 (65) | 52.9 (110) | |
| | T/T | 16.7 (22) | 11.1 (23) | |
| | C allele | 58.7 (155) | 62.5 (260) | |
| | T allele | 41.3 (109) | 37.5 (156) | |
| TNFRSF1B +676 | T/T | 60.9 (117) | 59.6 (124) | 0.84 |
| | T/G | 32.8 (63) | 35.1 (73) | |
| | G/G | 6.3 (12) | 5.3 (11) | |
| | T allele | 77.3 (297) | 77.2 (321) | |
| | G allele | 22.7 (87) | 22.8 (95) | |
| TNFRSF1B +1663 | A/A | 26.0 (50) | 24.6 (51) | 0.93 |
| | A/G | 45.3 (87) | 45.4 (94) | |
| | G/G | 28.6 (55) | 30.0 (62) | |
| | A allele | 48.7 (187) | 47.3 (196) | |
| | G allele | 51.3 (197) | 52.7 (218) | |

Small variation in 'n' is due to a small number of persistent failures in genotyping.

Table 4 Genotype and allele frequencies for each SNP, in ICU survivors and nonsurvivors

| | | % Survivors (n) | % Nonsurvivors (n) | P |
|----------------|----------|-----------------|--------------------|------|
| TNF -308 | G/G | 60.0 (96) | 75.0 (39) | 0.14 |
| | G/A | 35.6 (57) | 23.1 (12) | |
| | A/A | 4.4 (7) | 1.9 (1) | |
| | G allele | 77.8 (249) | 86.5 (90) | |
| | A allele | 22.2 (71) | 13.5 (14) | |
| TNF -238 | G/G | 86.5 (135) | 83.7 (41) | 0.72 |
| | G/A | 12.8 (20) | 16.3 (8) | |
| | A/A | 0.6 (1) | 0 (0) | |
| | G allele | 92.9 (290) | 91.8 (90) | |
| | A allele | 7.1 (22) | 8.2 (8) | |
| LTA +249 | G/G | 13.7 (22) | 9.6 (5) | 0.66 |
| | G/A | 44.7 (72) | 42.3 (22) | |
| | A/A | 41.6 (67) | 48.1 (25) | |
| | G allele | 36.0 (116) | 30.8 (32) | |
| | A allele | 64.0 (206) | 69.2 (72) | |
| LTA +365 | C/C | 11.5 (18) | 12.2 (6) | 0.89 |
| | C/G | 50.0 (78) | 53.1 (26) | |
| | G/G | 38.5 (60) | 34.7 (17) | |
| | C allele | 36.5 (114) | 38.8 (38) | |
| | G allele | 63.5 (198) | 61.2 (60) | |
| TNFRSF1A -609 | G/G | 35.0 (55) | 37.3 (19) | 0.14 |
| | G/T | 51.0 (80) | 58.8 (30) | |
| | T/T | 14.0 (22) | 3.9 (2) | |
| | G allele | 60.5 (190) | 66.7 (68) | |
| | T allele | 39.5 (124) | 33.3 (34) | |
| TNFRSF1A +36 | A/A | 36.3 (57) | 23.5 (12) | 0.19 |
| | A/G | 45.2 (71) | 58.8 (30) | |
| | G/G | 18.5 (29) | 17.6 (9) | |
| | A allele | 58.9 (185) | 52.9 (54) | |
| | G allele | 41.1 (129) | 47.1 (48) | |
| TNFRSF1A +1135 | C/C | 35.0 (55) | 39.2 (20) | 0.17 |
| | C/T | 51.6 (81) | 56.9 (29) | |
| | T/T | 13.4 (21) | 3.9 (2) | |
| | C allele | 60.8 (191) | 67.6 (69) | |
| | T allele | 39.2 (123) | 32.4 (33) | |
| TNFRSF1B +676 | T/T | 60.5 (95) | 56.9 (29) | 0.63 |
| | T/G | 35.0 (55) | 35.3 (18) | |
| | G/G | 4.5 (7) | 7.8 (4) | |
| | T allele | 78.0 (245) | 74.5 (76) | |
| | G allele | 22.0 (69) | 25.5 (26) | |
| TNFRSF1B +1663 | A/A | 23.7 (37) | 27.5 (14) | 0.88 |
| | A/G | 46.2 (72) | 43.1 (22) | |
| | G/G | 30.1 (47) | 29.4 (15) | |
| | A allele | 46.8 (146) | 49.0 (50) | |
| | G allele | 53.2 (166) | 51.0 (52) | |

Small variation in 'n' is due to a small number of persistent failures in genotyping.

This study was designed to achieve greater statistical power than previous studies (Table 1), with a patient population nearly two-and-a-half times the size of the previous largest published report.⁷ However, if the relative risk attributable to these polymorphisms is in fact substantially less than that used in our power calculations, we may still not have the power to answer the relevant questions. This is particularly the case for outcome from sepsis in a cohort of 213 subjects among whom only 52

were nonsurvivors. We would therefore suggest that future studies should incorporate much larger numbers and be powered for a relative risk of around 1.5. If this were to be applied to the outcome from septic shock for the TNF -308 A allele, for example, 2000 patients would be required to achieve 90% power to obtain a P-value of 0.01. An alternative approach would be to perform a meta-analysis, although the combined number of patients from previous studies and our own would still be less than 1000.

Table 5 Estimated haplotype frequencies for *TNF/LTA*, *TNFRSF1A* and *TNFRSF1B* loci, comparing ICU survivors and nonsurvivors, $P = 0.48, 0.44, 0.22$, respectively

| <i>TNF</i> -238 | <i>TNF</i> -308 | <i>LTA</i> +249 | <i>LTA</i> +365 | Survivors (%) | Nonsurvivors (%) |
|-----------------|-----------------|-----------------|-----------------|---------------|------------------|
| A | G | A | G | 6.3 | 8.2 |
| G | A | G | G | 22.1 | 13.3 |
| G | G | A | G | 21.2 | 23.5 |
| G | G | A | C | 36.5 | 38.8 |
| G | G | G | G | 12.7 | 16.3 |
| A | G | G | G | 0.7 | 0 |
| G | A | A | G | 0.4 | 0 |

| <i>TNFRSF1A</i> -609 | <i>TNFRSF1A</i> +36 | <i>TNFRSF1A</i> +1135 | Survivors (%) | Nonsurvivors (%) |
|----------------------|---------------------|-----------------------|---------------|------------------|
| G | A | C | 18.2 | 19.6 |
| G | A | T | 1.6 | 0 |
| G | G | C | 40.7 | 47.1 |
| T | A | C | 1.6 | 1.0 |
| T | A | T | 37.5 | 32.3 |
| T | G | C | 0.4 | 0 |

| <i>TNFRSF1B</i> +676 | <i>TNFRSF1B</i> +1663 | Survivors (%) | Nonsurvivors (%) |
|----------------------|-----------------------|---------------|------------------|
| G | A | 6.2 | 15.2 |
| G | G | 15.3 | 10.3 |
| T | A | 40.6 | 33.9 |
| T | G | 37.9 | 40.7 |

In this study, patients were recruited from eight different ICUs in two different countries; we cannot exclude therefore the possibility of population stratification. As in many other studies, only Caucasian adults were included to avoid spurious findings attributable to ethnic differences, and, except for the proportion of medical admissions, patient characteristics in the three regions were remarkably similar (Table 2). In reality, the risk of population stratification is likely to be small.⁴⁵ On the other hand, although the consensus definitions⁴⁶ were strictly applied and the patients recruited were typical of a population with severe sepsis/septic shock in terms of age, illness severity and outcome (Table 2), it is now well recognised that in practice these definitions identify a very heterogeneous group of patients with, for example, different co-morbidities and infecting organisms. In this respect, it is striking that the background mortality has varied considerably between the studies published to date.^{7,24,25} It is possible, therefore, that some of these polymorphisms may be important in certain, more homogeneous subsets of patients, as may be the case, for example, in relation to the development of septic shock and consequent mortality in patients with acute severe pancreatitis.⁴⁷

The potential influence of polymorphisms of the TNF receptors on susceptibility to, and outcome from, severe sepsis/septic shock was also examined. The shed portions of these receptors (sTNFRSF1A and sTNFRSF1B) are thought to bind and 'neutralise' circulating TNF,²⁹ although it has also been suggested that the TNF receptors may act as a reservoir of TNF, thus prolonging its activity.⁴⁸ We also attempted to assess the possible functionality of the polymorphisms studied. As well as confirming the association between increased circulating levels of TNF and a poor outcome from sepsis,^{21,22} we found that plasma levels of both soluble

receptors were significantly higher in those who died compared to those who survived. It is not clear whether in this situation high sTNFR levels contribute directly to a worse outcome by prolonging the inflammatory response or whether the increased circulating levels simply reflect an appropriate compensatory response to a more severe initial insult. There was also a strong positive correlation between increasing soluble receptor levels and SOFA score, and in particular with renal impairment. Given that soluble receptors are known to be excreted by the kidney, this is not surprising and is consistent with the findings of an earlier smaller study.⁴⁹ There was, however, no association between TNF receptor genotype and plasma levels of circulating receptor or outcome. The functional role of the *TNFRSF1A* SNPs has not yet been established, whereas the *TNFRSF1B* +676 SNP has been associated with sTNFRSF1B levels⁵⁰ and has been implicated in the pathogenesis of SLE,^{31,32} while the +1663 SNP has been associated with Crohn's disease³³ (another condition in which TNF is believed to play a pivotal role). It would seem, therefore, that although circulating sTNFR levels are related to outcome in sepsis any possible influence of genotype on plasma levels is obscured by changes caused by the nature and severity of the acute illness, and in particular the presence of renal impairment.

In this study, there were no associations between outcome and the ratios of plasma TNF concentrations to levels of the soluble receptors 1A or 1B. A high TNF/sTNFR ratio has been associated with a poor outcome from meningococcal disease in children,⁵¹ and burns and trauma in adults.⁵² In the study by Girardin *et al*, this ratio seemed to be important on hospital admission but not 6 h later.⁵¹ The patients in our study were recruited on the ICU after obtaining informed consent/

assent, and there was inevitably some delay between hospital admission and study inclusion. Additionally, because TNF has a short half-life and is known to be released in pulses, peak TNF concentrations are frequently missed. Pellegrini *et al*⁵² measured membrane-associated TNF (mTNF) on stimulated monocytes and compared this to sTNFR levels; they found that an increased mTNF/TNFR ratio correlated with the development of organ failure and mortality. It is likely that the role of mTNF is very different from that of TNF in the circulation.

In conclusion, in patients with severe sepsis and septic shock, plasma levels of TNF and its two soluble receptors, sTNFRSF1A and sTNFRSF1B, were higher in nonsurvivors than in survivors. There was no association between individual genotype of the three gene loci and plasma levels of the encoded proteins, illness severity or outcome. Similarly, no association was seen between extended haplotypes of these genes and outcome. These findings add to the uncertainty regarding the influence of polymorphisms of the *TNF* locus on susceptibility to and outcome from severe sepsis and highlight the difficulties associated with performing candidate gene association studies in complex polygenic diseases.

Methods

This prospective multi-centre study was conducted in three centres: London and Oxford in the UK and Sydney, Australia. Patients were recruited from a total of eight different ICU (see appendix for individual centres). The study was approved by the local ethics committees in both countries and written consent was obtained from patients, or written assent from next of kin as appropriate.

Adult Caucasian patients (18–80 years) with severe sepsis or septic shock, as defined by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) Consensus Conference 1992,⁴⁶ were recruited. Patients who were immunocompromised prior to developing sepsis were excluded, including those with known HIV, haematological malignancies or chronic liver failure (according to the APACHE II definition⁵³) and patients who had recently received chemotherapy, immunosuppressants or systemic steroids.

Clinical information recorded for each patient included demographic details (age, sex), past medical history,⁵⁴ details of acute illness, including ICU admission details, Acute Physiology And Chronic Health Evaluation (APACHE II) score⁵³ on admission to ICU and daily Sequential Organ Failure Assessment (SOFA) score.⁵⁵ In view of the difficulty of assessing the Glasgow Coma Score in sedated patients, the central nervous system component of the SOFA score was excluded, thus giving a maximum score of 20. Outcome was assessed at ICU and hospital discharge.

Data from normal healthy Caucasian controls ($n = 354$ for *TNF/LTA*, 132 for *TNFRSF1A* and 192 for *TNFRSF1B*), collected as part of previous genetic association studies,^{56,57} were used for comparison with recruited patients in order to assess susceptibility to severe sepsis and septic shock.

Blood sampling

Whole blood was collected in ethylene diamine tetraacetic acid (EDTA) tubes for DNA extraction from all patients enrolled in the study. In the London arm of the study, blood was also collected in EDTA on the day of recruitment for measurement of plasma levels of TNF, sTNFRSF1A and sTNFRSF1B. This was spun immediately and frozen at -40°C until analysis by immunoassay (Quantikine™, R&D Systems).

Genotyping

Nine individual polymorphisms were studied, four within the *TNF* locus, three in *TNFRSF1A* and two in *TNFRSF1B*, as shown in Figure 4. In London and Sydney, PCR-RFLP was used for all SNP genotyping except *TNF* -238, which was typed by end-labelled allele-specific probe hybridisation. The various primers and conditions used for each SNP are available in the online appendix. After enzyme digestion, the PCR products were size separated and visualised on 1.0% agarose gels. In Oxford, analysis of these same SNPs was carried out by PCR with sequence-specific primers (PCR-SSP). The methods for identifying the polymorphisms of the *TNF* locus and *TNFRSF1B* were as described previously.^{56,58} Similar methods were used for the *TNFRSF1A* SNPs, and the primers used are shown in Table 6. Genotypes were assigned by investigators blinded to outcome data and were subjected to quality checks by another blinded investigator. Equivocal results were repeated.

Haplotype analysis

Haplotypes of the polymorphisms of the three different gene loci were estimated using the EH+ programme,⁵⁹ an extension of the estimating haplotypes (EH) programme. We investigated the possible association of haplotypes with disease outcome using EH+'s

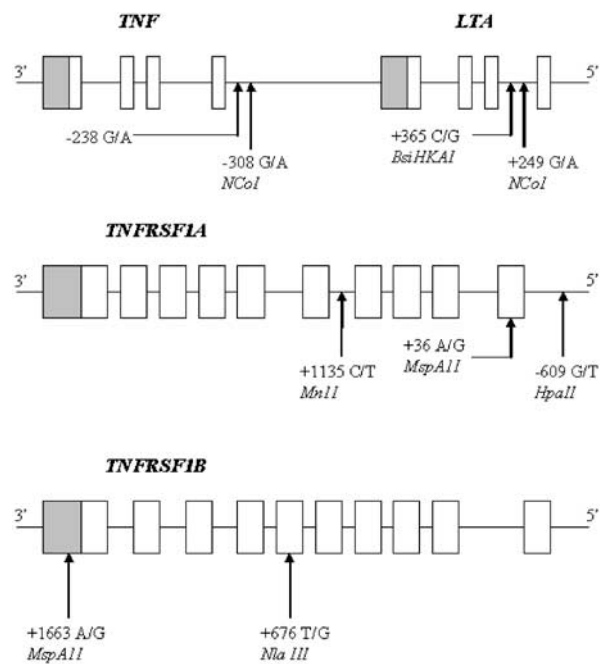


Figure 4 Position of SNPs studied in the three gene loci, including restriction endonucleases used in RFLP assays. Clear boxes represent exons, shaded boxes untranslated regions.

Table 6 Primers used for PCR-SSP to detect *TNFRSF1A* SNPs

| Primer/SNP | | Conc (μ M) |
|----------------|-------------------------------|--------------------|
| -609G | 5'-TGGAAAACAGATCCAGACAGG-3' | 0.82 |
| -609T | 5'-ATTGGAAAACAGATCCAGACAGT-3' | 0.76 |
| +36G | 5'-TCCCTGGTCTCACCAGC-3' | 1.05 |
| +36A | 5'-GTCCTGGTCTCACCAGT-3' | 0.98 |
| 211 | 5'-TTCATCAGTTGCTGCCCTC-3' | 0.64 |
| 210 | 5'-ATGATGTTGACCTTCCAGGG-3' | 0.59 |
| +1135C | 5'-CGGCACAGCTAAAGGAGG-3' | 0.60 |
| +1135T | 5'-GCGGCACAGCTAAAGGAGA-3' | 0.60 |
| +1135consensus | 5'-TCTTCTTGCACAGTGGACCG/A-3' | 0.56 |
| 63 | 5'-TGCCAAGTGGAGACCCAA-3' | 0.20 |
| 64 | 5'-GCATCTTGCTCTGTGCAGAT-3' | 0.19 |

Primers -609 G/T (forward) and +36 G/A (reverse) were used in four combinations to genotype the SNPs and were multiplexed with a control PCR amplified by primers 210 and 211. SSP-PCR primers for detecting SNP 1135 C/T were multiplexed with control primers 63/64.

model-free T4 statistic. This assesses the likelihood of association under a disease model estimated over a range of disease models varying from Mendelian recessive to null effect and from null effect to Mendelian dominant. Significance was assessed with a *P*-value obtained by Monte-Carlo permutation methods.

Sample size and statistical analysis

Power calculations were informed by previous *TNF* -308 studies^{7,8,23-26} with regard to estimating allele frequency and previous audits in the participating hospitals in order to estimate mortality rates. It was calculated that in a case-control study with a relative risk for mortality of 2.5 (overall ICU mortality of 30%) and a -308 A allele frequency of 0.2 (frequency of G/A and A/A 0.4) 200 patients would provide >80% power to detect a difference between the two groups with *P*<0.05 (two-sided *P*-value). (<http://calculators.stat.ucla.edu/powercalc/>).

Genotype data were analysed using Fisher's exact test. A multiple logistic regression analysis was then performed with independent variables that might influence the outcome, that is, age, sex, type of admission, past medical history and recruiting centre. Genotype associations were reanalysed, adjusting for those variables found to be independently associated with outcome.

Continuous data are expressed as median (range) and analysed using Mann-Whitney *U* or Kruskal-Wallis *H* tests, as appropriate. SPSS (version 11) for Windows was used for analysis.

Acknowledgements

We thank Dr B Norman for his advice regarding statistical analysis. This study was funded by the Joint Research Board (JRB), St Bartholomew's Hospital, London and GlaxoSmithKline, UK. ACG was in receipt of an Aylwen Bursary from the JRB.

References

- 1 Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001; **29**: 1303-1310.
- 2 Sands KE, Bates DW, Lanken PN *et al*. Epidemiology of sepsis syndrome in 8 academic medical centers. Academic Medical Center Consortium Sepsis Project Working Group. *JAMA* 1997; **278**: 234-240.
- 3 Brun-Buisson C, Doyon F, Carlet J *et al*. Incidence, risk factors, and outcome of severe sepsis and septic shock in adults. A multicenter prospective study in intensive care units. French ICU Group for Severe Sepsis. *JAMA* 1995; **274**: 968-974.
- 4 Fisher CJ, Agosti JM, Opal SM *et al*. Treatment of septic shock with the tumor necrosis factor receptor: Fc fusion protein. *N Engl J Med* 1996; **334**: 1697-1702.
- 5 Bernard GR, Vincent JL, Laterre PF *et al*. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med* 2001; **344**: 699-709.
- 6 Sorensen TI, Nielsen GG, Andersen PK, Teasdale TW. Genetic and environmental influences on premature death in adult adoptees. *N Engl J Med* 1988; **318**: 727-732.
- 7 Mira JP, Cariou A, Grall F *et al*. Association of *TNF2*, a *TNF*-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study. *JAMA* 1999; **282**: 561-568.
- 8 Appoloni O, Dupont E, Vandercruys M, Andriens M, Duchateau J, Vincent JL. Association of tumor necrosis factor-2 allele with plasma tumor necrosis factor-alpha levels and mortality from septic shock. *Am J Med* 2001; **110**: 486-488.
- 9 Stuber F, Petersen M, Bokelmann F, Schade U. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. *Crit Care Med* 1996; **24**: 381-384.
- 10 Majetschak M, Flohe S, Obertacke U *et al*. Relation of a *TNF* gene polymorphism to severe sepsis in trauma patients. *Ann Surg* 1999; **230**: 207-214.
- 11 Lowe PR, Galley HF, Abdel-Fattah A, Webster NR. Influence of interleukin-10 polymorphisms on interleukin-10 expression and survival in critically ill patients. *Crit Care Med* 2003; **31**: 34-38.
- 12 Ma P, Chen D, Pan J, Du B. Genomic polymorphism within interleukin-1 family cytokines influences the outcome of septic patients. *Crit Care Med* 2002; **30**: 1046-1050.
- 13 Hubacek JA, Stuber F, Frohlich D *et al*. Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis patients: gender-specific genetic predisposition to sepsis. *Crit Care Med* 2001; **29**: 557-561.
- 14 Gibot S, Cariou A, Drouet L, Rossignol M, Ripoll L. Association between a genomic polymorphism within the *CD14* locus and septic shock susceptibility and mortality rate. *Crit Care Med* 2002; **30**: 969-973.
- 15 Hermans PW, Hibberd ML, Booy R *et al*. 4G/5G promoter polymorphism in the plasminogen-activator-inhibitor-1 gene and outcome of meningococcal disease. Meningococcal Research Group. *Lancet* 1999; **354**: 556-560.
- 16 Hibberd ML, Sumiya M, Summerfield JA, Booy R, Levin M. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group. *Lancet* 1999; **353**: 1049-1053.
- 17 Roy S, Hill AV, Knox K, Griffiths D, Crook D. Research pointers: association of common genetic variant with susceptibility to invasive pneumococcal disease. *BMJ* 2002; **324**: 1369.
- 18 Roy S, Knox K, Segal S *et al*. MBL genotype and risk of invasive pneumococcal disease: a case-control study. *Lancet* 2002; **359**: 1569-1573.

- 19 Tracey KJ, Lowry SF, Fahey III TJ *et al*. Cachectin/tumor necrosis factor induces lethal shock and stress hormone responses in the dog. *Surg Gynecol Obstetr* 1987; **164**: 415–422.
- 20 Tracey KJ, Beutler B, Lowry SF *et al*. Shock and tissue injury induced by recombinant human cachectin. *Science* 1986; **234**: 470–474.
- 21 Girardin E, Grau GE, Dayer JM, Roux-Lombard P, Lambert PH. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N Engl J Med* 1988; **319**: 397–400.
- 22 Waage A, Halstensen A, Espevik T. Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet* 1987; **1**: 355–357.
- 23 Stuber F, Udalova IA, Book M *et al*. –308 tumor necrosis factor (TNF) polymorphism is not associated with survival in severe sepsis and is unrelated to lipopolysaccharide inducibility of the human TNF promoter. *J Inflamm* 1996; **46**: 42–50.
- 24 O’Keefe GE, Hybki DL, Munford RS. The G→A single nucleotide polymorphism at the –308 position in the tumor necrosis factor- α promoter increases the risk for severe sepsis after trauma. *J Trauma* 2002; **52**: 817–825.
- 25 Tang GJ, Huang SL, Yien HW *et al*. Tumor necrosis factor gene polymorphism and septic shock in surgical infection. *Crit Care Med* 2000; **28**: 2733–2736.
- 26 Waterer GW, Quasney MW, Cantor RM, Wunderink RG. Septic shock and respiratory failure in community-acquired pneumonia have different TNF polymorphism associations. *Am J Resp Crit Care Med* 2001; **163**: 1599–1604.
- 27 Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor α promoter on transcriptional activation. *Proc Natl Acad Sci USA* 1997; **94**: 3195–3199.
- 28 Brinkman BM, Zuijdeest D, Kaijzel EL, Breedveld FC, Verweij CL. Relevance of the tumor necrosis factor α (TNF α) –308 promoter polymorphism in TNF α gene regulation. *J Inflamm* 1995; **46**: 32–41.
- 29 Van Zee KJ, Kohno T, Fischer E, Rock CS, Moldawer LL, Lowry SF. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor α *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 1992; **89**: 4845–4849.
- 30 McDermott MF, Aksentjevich I, Galon J *et al*. Germline mutations in the extracellular domains of the 55 kDa receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndrome. *Cell* 1999; **97**: 133–144.
- 31 Komata T, Tsuchiya N, Matsushita M, Hagiwara K, Tokunaga K. Association of tumor necrosis factor receptor 2 (TNFR2) polymorphism with susceptibility to systemic lupus erythematosus. *Tissue Antigens* 1999; **53**: 527–533.
- 32 Morita C, Horiuchi T, Tsukamoto H *et al*. Association of tumor necrosis factor receptor type II polymorphism 196R with systemic lupus erythematosus in the Japanese: molecular and functional analysis. *Arthritis Rheum* 2001; **44**: 2819–2827.
- 33 Sashio H, Tamura K, Ito R *et al*. Polymorphisms of the TNF gene and the TNF receptor superfamily member 1B gene are associated with susceptibility to ulcerative colitis and Crohn’s disease, respectively. *Immunogenetics* 2002; **53**: 1020–1027.
- 34 Moreno R, Vincent JL, Matos R *et al*. The use of maximum SOFA score to quantify organ dysfunction/failure in intensive care. Results of a prospective, multicentre study. Working Group on Sepsis related Problems of the ESICM. *Intensive Care Med* 1999; **25**: 686–696.
- 35 Colhoun HM, McKeigue PM, Davey SG. Problems of reporting genetic associations with complex outcomes. *Lancet* 2003; **361**: 865–872.
- 36 Bayley J-P, Ottenhoff THM, Verweij CL. Is there a future for TNF promoter polymorphisms? *Genes Immun* 2004, advance online publication—doi:10.1038/sj.gene.6364055.
- 37 Peters DL, Barber RC, Flood EM, Garner HR, O’Keefe GE. Methodologic quality and genotyping reproducibility in studies of tumor necrosis factor –308 G→A single nucleotide polymorphism and bacterial sepsis: implications for studies of complex traits. *Crit Care Med* 2003; **31**: 1691–1696.
- 38 Carroll MC, Katzman P, Alicot EM *et al*. Linkage map of the human major histocompatibility complex including the tumor necrosis factor genes. *Proc Natl Acad Sci USA* 1987; **84**: 8535–8539.
- 39 Fabris M, Di PE, D’Elia A, Damante G, Sinigaglia L, Ferraccioli G. Tumor necrosis factor- α gene polymorphism in severe and mild-moderate rheumatoid arthritis. *J Rheumatol* 2002; **29**: 29–33.
- 40 Huizinga TW, Westendorp RG, Bollen EL *et al*. TNF- α promoter polymorphisms, production and susceptibility to multiple sclerosis in different groups of patients. *J Neuroimmunol* 1997; **72**: 149–153.
- 41 Ferencik S, Lindemann M, Horsthemke B, Grosse-Wilde H. A new restriction fragment length polymorphism of the human TNF-B gene detected by AspHI digest. *Eur J Immunogenet* 1992; **19**: 425–430.
- 42 Knight JC, Keating BJ, Rockett KA, Kwiatkowski DP. *In vivo* characterization of regulatory polymorphisms by allele-specific quantification of RNA polymerase loading. *Nat Genet* 2003; **33**: 469–475.
- 43 Ozaki K, Ohnishi Y, Iida A *et al*. Functional SNPs in the lymphotoxin- α gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 2002; **32**: 650–654.
- 44 Ackerman HC, Ribas G, Jallow M *et al*. Complex haplotypic structure of the central MHC region flanking TNF in a West African population. *Genes Immun* 2003; **4**: 476–486.
- 45 Cardon LR, Palmer LJ. Population stratification and spurious allelic association. *Lancet* 2003; **361**: 598–604.
- 46 American College of Chest Physicians/Society of Critical Care Medicine. Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Crit Care Med* 1992; **20**: 864–874.
- 47 Zhang DL, Li JS, Jiang ZW, Yu BJ, Tang XM. Association of two polymorphisms of tumor necrosis factor gene with acute severe pancreatitis. *J Surg Res* 2003; **112**: 138–143.
- 48 Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J Exp Med* 1992; **175**: 323–329.
- 49 Froom AH, Bemelmans MH, Greve JW, van der Linden CJ, Buurman WA. Increased plasma concentrations of soluble tumor necrosis factor receptors in sepsis syndrome: correlation with plasma creatinine values. *Crit Care Med* 1994; **22**: 803–809.
- 50 Geurts JM, Janssen RG, van Greevenbroek MM *et al*. Identification of TNFRSF1B as a novel modifier gene in familial combined hyperlipidemia. *Hum Mol Genet* 2000; **9**: 2067–2074.
- 51 Girardin E, Roux-Lombard P, Grau GE, Suter P, Gallati H, Dayer JM. Imbalance between tumour necrosis factor- α and soluble TNF receptor concentrations in severe meningococcaemia. The J5 Study Group. *Immunology* 1992; **76**: 20–23.
- 52 Pellegrini JD, Puyana JC, Lapchak PH, Kodys K, Miller-Graziano CL. A membrane TNF- α /TNFR ratio correlates to MODS score and mortality. *Shock* 1996; **6**: 389–396.
- 53 Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med* 1985; **13**: 818–829.
- 54 McCabe WR, Jackson GG. Gram-negative bacteremia. *Arch Intern Med* 1962; **110**: 847–855.
- 55 Vincent J-L, Moreno R, Takala J *et al*. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. *Intensive Care Med* 1996; **22**: 710.
- 56 Pantelidis P, Lympany PA, Foley PJ, Fanning GC, Welsh KI, Du Bois RM. Polymorphic analysis of the high-affinity tumor necrosis factor receptor 2. *Tissue Antigens* 1999; **54**: 585–591.
- 57 Grutters JC, Sato H, Pantelidis P *et al*. Increased frequency of the uncommon tumor necrosis factor –857T allele in British

- and Dutch patients with sarcoidosis. *Am J Resp Crit Care Med* 2002; **165**: 1119–1124.
- 58 Fanning GC, Bunce M, Black CM, Welsh KI. Polymerase chain reaction haplotyping using 3' mismatches in the forward and reverse primers: application to the biallelic polymorphisms of tumor necrosis factor and lymphotoxin alpha. *Tissue Antigens* 1997; **50**: 23–31.
- 59 Zhao JH, Curtis D, Sham PC. Model-free analysis and permutation tests for allelic associations. *Hum Hered* 2000; **50**: 133–139.

Appendix

Recruiting centres and personnel

UK – Barts and The London NHS Trust, JD Watson, S Withington; Colchester General Hospital, A Timmins; Homerton Hospital, JH Coakley; John Radcliffe Hospital, P Hutton, P Parsons and A Smith; Southend General Hospital, D Higgins; Whipps Cross Hospital, A Morris.
Australia – Royal North Shore Hospital, Sydney, A Marich.