Studies of human immune responses to various antigenic proteins of *Chlamydia trachomatis*

John Mark Felton

Thesis submitted for the degree of
Doctor of Philosophy

London School of Hygiene & Tropical Medicine
(University of London)

Abstract

Chlamydiae are gram-negative bacteria which cause diverse diseases of humans and animals. *Chlamydia trachomatis*, the focus of this work, causes the blinding eye disease trachoma, and reproductive tract infections. Trachoma affects 500 million individuals of whom seven million are blind. *Chlamydia* is the most common reproductive tract infection and can cause pelvic inflammatory disease and tubal infertility. In both cases treatment with antibiotics is effective but they may not be available, recurrent infection is common and the long-term complications irreversible. A vaccine is needed but attempts at developing one have met with limited success. The recent discovery of a family of antigenic surface proteins, the polymorphic membrane proteins, has revitalised the search for potential vaccine candidates.

One member of this family, *pmpG*, was cloned in two fragments and the recombinant products expressed and purified. Humoral and cellular responses to these and other chlamydial antigens, and to common recall antigens, were examined in human subjects in The Gambia using immunoblotting, whole blood assay and cytokine ELISA techniques. Children with active trachoma, adults with trachomatous scarring and women with tubal infertility were examined, together with matched controls.

PMPG was shown to be a target of both humoral and cellular responses. These responses were more commonly directed towards the amino- (PMPGa), rather than carboxyl-terminal (PMPGc) fragment. Antibodies to PMPGa were associated with a reduced risk of active trachoma but antibodies to PMPGc were associated with conjunctival scarring. PMPGa stimulated the production of TNFα, IFNγ and TGFβ. Children with active trachoma produced higher levels of TNFα and IL-10 than controls in response to PMPGa. High levels of IL-10 in response to both chlamydial and common recall antigens were noted in children with active trachoma and this may be a mechanism by which *Chlamydia* survives intracellularly. Responses to other chlamydial proteins were characterised and an association between intestinal helminth infection and conjunctival scarring was noted.

Table of Contents

1 BACKGROUND 25

1.1 CHLAMYDIA, THE ORGANISM 25

1.2 EPIDEMIOLOGY AND DISEASE 25

1.2.1 Trachoma 25

1.2.2 *Chlamydia trachomatis* urogenital tract infection 26

1.2.3 Other chlamydial infections 27

1.3 IMMUNE RESPONSES 28

1.3.1 Innate responses 28

1.3.2 Humoral responses 29

1.3.3 T-cell responses 30

1.3.4 Cytokine responses and the relative importance of T_H1- and T_H2-type responses 31

1.4 CHLAMYDIAL ANTIGENS 34

1.4.1 Major Outer Membrane Protein (MOMP) 34

1.4.2 Outer Membrane Protein 2 (OMP2) 35

1.4.3 Heat-shock protein 60 (HSP60) 36

1.4.4 The polymorphic membrane proteins (PMPs) 37

1.4.5 Other antigens 45

1.5 PATHOGENESIS 46

1.6 PREVIOUS VACCINE WORK 49

1.7 LOCATIONS 50

1.7.1 London School of Hygiene & Tropical Medicine 50

1.7.2 Medical Research Council Laboratories, Fajara and Farafenni, The Gambia 50

1.7.3 Study villages, The Gambia 50

1.8 AIMS OF THE PROJECT 52

2 MATERIALS AND METHODS 54

2.1 CLONING 54

2.1.1 Preparation of *Chlamydia trachomatis* serovar A DNA 54

2.1.1.1 *Purification of Chlamydia trachomatis serovar A DNA 54*

2.1.1.2 *Spectrophotometric estimation of concentration, yield and purity of DNA 55*

2.1.1.3 Digestion of chlamydial DNA with restriction enzymes.....55
2.1.2 PCR Amplification.....56
2.1.2.1 Clontech Advantage® PCR.....56
2.1.2.2 Hi-fidelity Pfu amplification.....57
2.1.3 Purification of PCR products.....57
2.1.3.1 Ethidium bromide gel electrophoresis.....57
2.1.3.2 Crystal violet gel electrophoresis.....58
2.1.3.3 Qiaex II DNA extraction kit.....58
2.1.3.4 Invitrogen Snap Columns®.....58
2.1.4 Preparation of vectors and inserts for ligation.....59
2.1.4.1 pGEM-T®.....59
2.1.4.2 pTrcHis®.....59
2.1.4.3 pBluescript® II phagemid (SKII).....60
2.1.4.4 pBAD-TOPO TA®.....60
2.1.4.5 pCR-II-TOPO®.....60
2.1.4.6 PET-30®.....60
2.1.4.7 Isolation of restriction fragments for use as inserts.....61
2.1.4.8 De-phosphorylation of vector or insert termini prior to cloning.....61
2.1.4.9 Filling in 5' single stranded extensions with Klenow fragment of DNA polymerase.....62
2.1.5 Ligation.....62
2.1.5.1 Calculation of vector/insert ratio.....62
2.1.5.2 pGEM-T®.....63
2.1.5.3 pTrcHis®.....63
2.1.5.4 pBluescript II®.....63
2.1.5.5 pBAD-TOPO®.....63
2.1.5.6 pCR-II-TOPO®.....63
2.1.5.7 pPET®.....63
2.1.5.8 Preparation of competent cells.....64
2.1.6 Transformation into competent cells.....64
2.1.6.1 Competent cells used.....64
2.1.6.2 Transformation into XL1-Blue.....65
2.1.6.3 Transformation into Top10 One-Shot® competent cells.....65
2.1.6.4 Transformation into BL21.....66
2.1.7 Screening of transformants.....66

2.1.7.1 Screening by antibiotic selection....66
2.1.7.2 Blue/white screening for lacZ....66
2.1.7.3 Quick plasmid preparation....67
2.1.7.4 Restriction enzyme analysis of plasmid DNA....67
2.1.8 Purification and archiving of plasmid constructs and plasmid-bearing bacterial strains....68
2.1.8.1 Plasmid purification – QIAwell 8® kit....68
2.1.8.2 Plasmid purification – Plasmid Midi Kit®....69
2.1.8.3 Freezing of plasmid-bearing bacterial strains....69
2.1.9 Individual cloning strategies....70
2.1.9.1 Cloning of pTrcHis/pmpG-1410....70
2.1.9.2 Cloning of pBluescript/pmpD-1218....70
2.1.9.3 Cloning of pBAD/pmpG....71
2.1.9.4 Cloning of pPET/pmpGc....71
2.1.9.5 Cloning of pCR-II/pmpG....72
2.1.9.6 Cloning of pPET/pmpGa....72
2.2 EXPRESSION....73
2.2.1 Basic techniques....73
2.2.1.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)....73
2.2.1.2 Western blotting & immuno-detection....74
2.2.2 pTrcHis constructs....75
2.2.3 pBAD constructs....76
2.2.4 pPET constructs....77
2.2.5 rMOMP construct....77
2.3 RECOMBINANT PROTEIN PURIFICATION....78
2.3.1 General methods....78
2.3.1.1 Determination of the solubility of recombinant proteins in PBS....78
2.3.1.2 Purification of inclusion bodies....78
2.3.2 Purification of rMOMP....79
2.3.3 Ni-NTA Protein Affinity Purification....80
2.3.4 ProBond® Column Resin Affinity Purification....80
2.3.5 TALON® Metal Affinity Resin Column....82
2.4 WHOLE BLOOD ASSAY....84
2.4.1 Preparation of antigens....84
2.4.1.1 Phytohaemagglutinin....84

2.4.1.2 Purified protein derivative 84
2.4.1.3 Candida albicans 84
2.4.1.4 Induced pPET without insert 84
2.4.1.5 Chlamydia trachomatis elementary bodies 85
2.4.1.6 MOMP 85
2.4.1.7 OMP2 85
2.4.1.8 HSP60 85
2.4.1.9 PMPGc 86
2.4.1.10 PMPGa 86
2.4.2 Preparation of antigen plates 86
2.4.3 Setting up whole blood assays 87
2.5 CYTOKINE ELISA 88
2.5.1 Standard ELISA protocol 88
2.5.2 Variations on the standard method for individual cytokine ELISA 89
2.5.2.1 TNFα 89
2.5.2.2 IFNγ 89
2.5.2.3 TGFβ 90
2.5.2.4 IL-2 90
2.5.2.5 IL-5 90
2.5.2.6 IL-10 90
2.5.3 Preparation of whole blood assay control supernatants 90
2.6 IMMUNOBLOTTING 91
2.7 FULL BLOOD COUNT 92
2.8 EXTRACTION OF DNA FROM WHOLE BLOOD 92
2.9 CHLAMYDIA PCR 92
2.9.1 Extraction of DNA from eye swabs 92
2.9.2 Amplicor CT® PCR for *Chlamydia* 93
2.9.3 Stool microscopy 93
2.10 RECRUITMENT 93
2.10.1 Sample size calculation 94
2.10.2 WHO classification of trachoma 94
2.10.3 Active trachoma 95
2.10.4 Trachomatous scarring 96
2.10.5 Infertile women 96
2.11 SUMMARY OF RECRUITMENT, INVESTIGATIONS AND DATA SETS GENERATED 97

2.12 STATISTICAL ANALYSIS 98
2.12.1 General data management 98
2.12.2 Statistical analysis 98

3 RESULTS 1 – GENERATION OF PLASMID CONSTRUCTS AND EXPRESSION OF RECOMBINANT PROTEIN 101

3.1 CLONING 101
3.1.1 Purification of *Chlamydia trachomatis* serovar A DNA 101
3.1.2 PCR Amplification 101
3.1.3 Restriction enzyme analysis of PCR products 102
3.1.3.1 pmpG 102
3.1.3.2 pmpD 103
3.1.4 Preparation of competent cells 105
3.1.5 Individual cloning strategies 105
3.1.5.1 Cloning of pTrcHis/pmpG-1410 105
3.1.5.2 Cloning of pBluescript/pmpD-1218 108
3.1.5.3 Cloning of pBAD/pmpG 109
3.1.5.4 Cloning of pPET/pmpGc 111
3.1.5.5 Cloning of pCR-II/pmpG 112
3.1.5.6 Cloning of pPET/pmpGa 113
3.2 EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS 114
3.2.1 pTrcHis/pmpG 114
3.2.1.1 Expression of pTrcHis/pmpG 114
3.2.1.2 Determination of solubility of recombinant proteins from pTrcHis/pmpG 115
3.2.1.3 Purification of recombinant proteins from pTrcHis/pmpG 115
3.2.2 pBAD/pmpG 117
3.2.2.1 Expression of pBAD/pmpG 117
3.2.2.2 Purification of recombinant proteins from pBAD/pmpG 118
3.2.3 pPET/pmpGc 119
3.2.3.1 Expression of pPET/pmpGc 119
3.2.3.2 Purification of recombinant proteins from pPET/pmpGc 120
3.2.4 pPET/pmpGa 122
3.2.4.1 Expression of pPET/pmpGa 122
3.2.4.2 Purification of recombinant proteins from pPET/pmpGa 123

3.2.5 pPET without insert 125
3.2.5.1 Expression of pPET without insert and purification of induced products 125
3.2.6 MOMP 126
3.2.6.1 Expression and purification of rMOMP 126
4 RESULTS 2 – RESULTS OF FIELD WORK IN THE GAMBIA 128
4.1 QUALITY CONTROL ISSUES 128
4.1.1 Quality control issues for immunoblots 128
4.1.2 Quality control issues for cytokine ELISAs 128
4.2 DEMOGRAPHY 132
4.3 RESULTS OF STUDIES ON INDIVIDUALS WITH TRACHOMA 135
4.3.1 Immunoblot results 135
4.3.1.1 Active trachoma 135
4.3.1.2 Scarred trachoma 139
4.3.2 Cytokine ELISAs from whole blood assays 142
4.3.2.1 Active trachoma 142
4.3.2.1.1 TNF-α 142
4.3.2.1.2 IFNγ 147
4.3.2.1.3 IL-2 151
4.3.2.1.4 IL-5 154
4.3.2.1.5 IL-10 157
4.3.2.1.6 TGF-β 162
4.3.2.2 Scarred trachoma 165
4.3.2.2.1 TNF-α 165
4.3.2.2.2 IFNγ 168
4.3.2.2.3 IL-2 171
4.3.2.2.4 IL-5 174
4.3.2.2.5 IL-10 177
4.3.2.2.6 TGFβ 181
4.4 RESULTS OF STUDIES IN INFERTILE WOMEN 184
4.4.1 Immunoblot results 184
4.4.2 Cytokine ELISAs from whole blood assays 187
4.4.2.1 TNF-α 187
4.4.2.2 IFNγ 190

4.4.2.3 IL-2 ..... *193*
4.4.2.4 IL-5 ..... *196*
4.4.2.5 IL-10 ..... *199*
4.4.2.6 TGF-β *202*
4.5 MISCELLANEOUS RESULTS 205
4.5.1 Effect of gender 205
4.5.2 Effect of ethnicity 206
4.5.3 Chlamydial PCR results 209
4.5.4 Effect of BCG vaccination 210
4.5.5 Effects of helminth and parasitic infestation 212
4.6 SUMMARY OF RESULTS 214
5 DISCUSSION 220
5.1 PRINCIPAL FINDINGS 220
5.2 LIMITATIONS OF THIS STUDY 233
5.3 CONCLUSIONS AND IMPLICATIONS 240
5.4 UNANSWERED QUESTIONS AND FUTURE RESEARCH 243
BIBLIOGRAPHY 248
APPENDIX - IMMUNOBLOT SCORES BY OBSERVER 280

Table of Figures

Figure 1-1: Clinical progression of trachoma.....26

Figure 1-2: Putative model of the envelope of chlamydial elementary bodies. Adapted from Everett et al[128] and Hatch.[129].....35

Figure 1-3: pmp gene arrangement within Chlamydia trachomatis genome 40

Figure 1-4: Map of The Gambia showing study villages 51

Figure 2-1: PET-30a expression vector.....61

Figure 2-2: Whole blood assay plate layout.....87

Figure 2-3: WHO classification of trachoma (from Dawson et al)[6].....95

Figure 2-4: Summary of recruitment, investigations and data sets generated.....97

Figure 3-1: Restriction enzyme analysis of *Chlamydia trachomatis* genomic DNA....101

Figure 3-2: *pmpC, pmpD* and *pmpG* PCR products amplified using the Clontech Advantage® PCR kit and visualised on an ethidium bromide-stained agarose gel under 254nm UV illumination 102

Figure 3-3: *pmpC* and *pmpG* PCR products amplified using Pfu DNA polymerase and visualised on an ethidium bromide-stained agarose gel under 254nm UV illumination 102

Figure 3-4: Predicted *pmpG* PCR product restriction map 102

Figure 3-5: Fragments obtained on restriction enzyme analysis of *pmpG* PCR product and visualised under 254nm UV illumination after agarose gel electrophoresis ..102

Figure 3-6: *pmpD* PCR product. Predicted restriction map 104

Figure 3-7: pmpD PCR product. Results of restriction enzyme analysis (*Bam*H1 + *Bgl*II digestion not shown) 104

Figure 3-8: Predicted restriction map of pGEM-T/pmpG.....105

Figure 3-9: Restriction enzyme analysis of pGEM-T/pmpG 106

Figure 3-10: Predicted restriction map of pTrcHis/pmpG-1410 107

Figure 3-11: Restriction enzyme analysis of pTrcHis/pmpG-1410 107

Figure 3-12: Predicted restriction map of pBluescript/pmpD-Bam-1218.....108

Figure 3-13: Restriction enzyme analysis of pBluecript/ pmpD-Bam-1218 (*Kpn*I + *Eag*I digestion not shown) 108

Figure 3-14: Potential point mutations leading to a novel *Sac*I site in *pmpD*.....109

Figure 3-15: Predicted restriction map of pBAD/pmpG 110

Figure 3-16: Restriction enzyme analysis of pBAD/pmpG 110

Figure 3-17: *Xho*/*Sac* fragment of pmpG used for cloning pPET/pmpGc 111

Figure 3-18: Predicted restriction map of pPET/pmpGc.....111
Figure 3-19: Restriction enzyme analysis of pPET/pmpGc.....111
Figure 3-20: Predicted restriction maps of pCRII/pmpG showing both forward and reverse orientations of insert.....112
Figure 3-21: Restriction enzyme analysis of pCRII/pmpG.....112
Figure 3-22: Predicted restriction map of pPET/pmpGa.....113
Figure 3-23: Restriction enzyme analysis of pPET/pmpGa.....113
Figure 3-24: Expression of recombinant protein from pTrcHis/pmpG - SDS-PAGE..114
Figure 3-25: Expression of recombinant protein from pTrcHis/pmpG - Western blot.115
Figure 3-26: Solubility of recombinant protein from pTrcHis/pmpG - Western blot...115
Figure 3-27: Ni-NTP column purification of recombinant protein from pTrcHis/pmpG - SDS-PAGE.....116
Figure 3-28: Ni-NTP column purification of recombinant protein from pTrcHis/pmpG - Western blot.....116
Figure 3-29: ProBond resin column purification of recombinant protein from pTrcHis/pmpG - SDS-PAGE.....117
Figure 3-30: ProBond resin column purification of recombinant proteins from pTrcHis/pmpG - Western blot.....117
Figure 3-31: Expression of recombinant protein from pBAD/pmpG- SDS-PAGE.....118
Figure 3-32: Expression of recombinant protein from pBAD/pmpG- Western blot 118
Figure 3-33: Purification of recombinant protein from pBAD/pmpG using Clontech TALON and Qiagen Ni-NTA methods - SDS-PAGE.....119
Figure 3-34: Purification of recombinant protein from pBAD/pmpG using Clontech TALON and Qiagen Ni-NTA methods - Western blot.....119
Figure 3-35: Expression of recombinant protein from pPET/pmpGc - SDS-PAGE 120
Figure 3-36: Expression of recombinant protein from pPET/pmpGc - Western blot...120
Figure 3-37: TALON spin-column purification of recombinant protein from pPET/pmpGc - SDS-PAGE.....121
Figure 3-38: TALON spin-column purification of recombinant protein from pPET/pmpGc - Western blot.....121
Figure 3-39: Pro-Bond purification of recombinant protein from pPET/pmpGc - SDS-PAGE.....121
Figure 3-40: Pro-Bond purification of recombinant protein from pPET/pmpGc - Western blot.....122
Figure 3-41: Expression of recombinant protein from pPET/pmpGa - SDS-PAGE 122

Figure 3-42: Expression of recombinant protein from pPET/pmpGa - Western blot ...123
Figure 3-43: TALON spin-column purification of recombinant protein from pPET/pmpGa - SDS-PAGE ...123
Figure 3-44: TALON spin-column purification of recombinant protein from pPET/pmpGa - Western blot ...123
Figure 3-45: Pro-Bond purification of recombinant protein from pPET/pmpGa - SDS-PAGE ...124
Figure 3-46: Pro-Bond purification of recombinant protein from pPET/pmpGa - Western blot ...124
Figure 3-47: Pro-Bond purification of products of induction of pPET vector without an insert - SDS-PAGE ...125
Figure 3-48: Pro-Bond purification of products of induction of pPET vector without an insert - Western blot ...125
Figure 3-49: Purification of recombinant MOMP - SDS-PAGE ...126
Figure 4-1: Two typical immunoblots ...128
Figure 4-2: Distribution of values for cytokine positive control wells ...129
Figure 4-3: Spread of values for TGFβ by ELISA, for each antigen or control ...131
Figure 4-4: Age distribution graph of all cases and controls ...132
Figure 4-5: Distribution of study participants by ethnicity ...133
Figure 4-6: Frequency-distribution graphs of immunoblot density scores for children with active trachoma and their controls ...135
Figure 4-7: Mean immunoblot density scores and 95% confidence intervals for children with active trachoma and their controls ...137
Figure 4-8: Frequency distribution of immunoblot scores for individuals with scarring and their controls ...139
Figure 4-9: Mean immunoblot density scores for scarred individuals and their controls ...140
Figure 4-10: Frequency distribution of TNFα responses in children with active trachoma and their controls ...142
Figure 4-11: Mean TNFα concentrations in children with active trachoma and their controls ...144
Figure 4-12: Frequency-distribution graphs of IFNγ responses in children with active trachoma and their controls ...147

Figure 4-13: Mean IFNγ concentrations in children with active trachoma and their controls 149
Figure 4-14: Frequency-distribution graphs of IL-2 responses in children with active trachoma and their controls 151
Figure 4-15: Mean IL-2 concentrations in children with active trachoma and their controls 153
Figure 4-16: Frequency-distribution graphs of IL-5 responses in children with active trachoma and their controls 154
Figure 4-17: Mean IL-5 concentrations in children with active trachoma and their controls 155
Figure 4-18: Frequency-distribution graphs of IL-10 responses in children with active trachoma and their controls 157
Figure 4-19: Mean IL-10 concentrations in children with active trachoma and their controls 158
Figure 4-20: Frequency-distribution graphs of TGFβ responses in children with active trachoma and their controls 162
Figure 4-21: Mean TGFβ concentrations in children with active trachoma and their controls 163
Figure 4-22: Frequency-distribution graphs of TNFα responses in individuals with trachomatous scarring and their controls.....165
Figure 4-23: Mean TNFα concentrations in individuals with trachomatous scarring and their controls.....167
Figure 4-24: Frequency-distribution graphs of IFNγ responses in individuals with trachomatous scarring and their controls.....168
Figure 4-25: Mean IFNγ concentrations in individuals with trachomatous scarring and their controls.....169
Figure 4-26: Frequency-distribution graphs of IL-2 responses in individuals with trachomatous scarring and their controls.....171
Figure 4-27: Mean IL-2 concentrations in individuals with trachomatous scarring and their controls.....172
Figure 4-28: Frequency-distribution graphs of IL-5 responses in individuals with trachomatous scarring and their controls.....174
Figure 4-29: Mean IL-5 concentrations in individuals with trachomatous scarring and their controls.....176

Figure 4-30: Frequency-distribution graphs of IL-10 responses in individuals with trachomatous scarring and their controls.....177
Figure 4-31: Mean IL-10 concentrations in individuals with trachomatous scarring and their controls.....178
Figure 4-32: Frequency-distribution graphs of TGFβ responses in individuals with trachomatous scarring and their controls.....181
Figure 4-33: Mean TGFβ concentrations in individuals with trachomatous scarring and their controls.....182
Figure 4-34: Frequency-distribution graphs of immunoblot density scores from infertile women and their controls 184
Figure 4-35: Mean immunoblot density scores from infertile women and their controls 185
Figure 4-36: Frequency-distribution graphs of TNFα responses in infertile women and their controls.....187
Figure 4-37: Mean TNFα concentrations in infertile women and their controls.....188
Figure 4-38: Frequency-distribution graphs of IFNγ responses in infertile women and their controls.....190
Figure 4-39: Mean IFNγ concentrations in infertile women and their controls.....191
Figure 4-40: Frequency-distribution graphs of IL-2 responses in infertile women and their controls.....193
Figure 4-41: Mean IL-2 concentrations in infertile women and their controls.....194
Figure 4-42: Frequency-distribution graphs of IL-5 responses in infertile women and their controls.....196
Figure 4-43: Mean IL-5 concentrations in infertile women and their controls.....197
Figure 4-44: Frequency-distribution graphs of IL-10 responses in infertile women and their controls.....199
Figure 4-45: Mean IL-10 concentrations in infertile women and their controls.....200
Figure 4-46: Frequency-distribution graphs of TGFβ responses in infertile women and their controls.....202
Figure 4-47: Mean TGFβ concentrations in infertile women and their controls 203
Figure 4-48: Likelihood of having received BCG vaccination by age group 210
Figure 4-49: Summary of results - cases vs. controls for all groups 214

Table of Tables

Table 1-1: Characteristics of the *Chlamydia trachomatis pmps* 38

Table 2-1: Primer sequences used for PCR.....56

Table 2-2: Plasmid antibiotic resistance.....66

Table 2-3: SDS-PAGE gel composition.....73

Table 2-4: IPTG concentrations used to induce expression in pTrcHis constructs.....75

Table 2-5: Arabinose concentrations used to induce expression in pBAD constructs....76

Table 2-6: IPTG concentrations used to induce expression in pPET constructs.....77

Table 3-1: Predicted and observed fragments of the *pmpG* PCR product after restriction enzyme analysis.....103

Table 3-2: Predicted and observed fragments of the pmpD PCR product after restriction enzyme analysis.....104

Table 3-3: Transformation efficiencies of competent cells.....105

Table 3-4: Predicted and observed fragments of pGEM-T/pmpG after restriction enzyme analysis.....106

Table 3-5: Predicted and observed restriction fragments of pTrcHis/pmpG-1410 107

Table 3-6: Predicted and observed fragments of pBluescript/pmpD-Bam-1218 after restriction enzyme analysis 108

Table 3-7: Predicted and observed fragments of pBAD/pmpG after restriction enzyme analysis 110

Table 3-8: Predicted and observed fragments of pPET/pmpGc after restriction enzyme analysis 111

Table 3-9: Predicted and observed fragments of pCRII/pmpG after restriction enzyme analysis 112

Table 3-10: Predicted and observed fragments of pPET/pmpGa after restriction enzyme analysis 113

Table 4-1: Correlation coefficients between observers for immunoblot scores.....128

Table 4-2: Quality control statistics for cytokine ELISAs 129

Table 4-3: Correlation coefficients between values for the positive control wells and those for patient samples on the same plate 130

Table 4-4: Distribution of study participants by ethnicity 132

Table 4-5: Distribution of study participants by village of residence 133

Table 4-6: Clinical classification of trachoma in study participants 134

Table 4-7: Individuals with negative immunoblot responses to chlamydial antigens (numbers are unique identifiers for individual study participants) 136
Table 4-8: Mean immunoblot density scores for children with active trachoma and their controls 136
Table 4-9: Conditional logistic regression of immunoblot scores from children with active trachoma and their controls.....137
Table 4-10: χ^2analysis for trend for immunoblot scores to MOMP in children with active trachoma and their controls.....138
Table 4-11: χ^2 analysis for trend for immunoblot scores to PMPGa in children with active trachoma and their controls.....138
Table 4-12: Mean immunoblot density scores for scarred individuals and their controls 140
Table 4-13: Conditional logistic regression of immunoblot scores for scarred individuals and their controls 141
Table 4-14: χ^2analysis for trend for immunoblot scores to PMPGc in scarred individuals and their controls 141
Table 4-15: Mean TNF concentrations in children with active trachoma and their controls 143
Table 4-16: Conditional logistic regression of TNFα concentrations in children with active trachoma and their controls.....144
Table 4-17: χ^2 test for trend for TNFα responses to *Candida* in children with active trachoma and their controls 145
Table 4-18: χ^2 test for trend for TNFα responses to chlamydial EBs in children with active trachoma and their controls.....145
Table 4-19: test for trend for TNFα responses to OMP2 in children with active trachoma and their controls 145
Table 4-20: χ^2 test for trend for TNFα responses to HSP60 in children with active trachoma and their controls 146
Table 4-21: χ^2 test for trend for TNFα responses to PMPGa in children with active trachoma and their controls 146
Table 4-22: Mean IFNγ concentrations in children with active trachoma and their controls 148
Table 4-23: Conditional logistic regression of IFNγ concentrations for children with active trachoma and their controls.....149

Table 4-24: χ^2 test for trend for IFNγ responses to PHA in children with active trachoma and their controls 150
Table 4-25: χ^2 test for trend for IFNγ responses to chlamydial EBs in children with active trachoma and their controls.....150
Table 4-26: Mean IL-2 concentrations in children with active trachoma and their controls 152
Table 4-27: Conditional logistic regression of IL-2 concentrations for children with active trachoma and their controls.....153
Table 4-28: Mean IL-5 concentrations in children with active trachoma and their controls 155
Table 4-29: Conditional logistic regression of IL-5 concentrations for children with active trachoma and their controls.....156
Table 4-30: Mean IL-10 concentrations in children with active trachoma and their controls 158
Table 4-31: Conditional logistic regression of IL-10 concentrations for children with active trachoma and their controls.....159
Table 4-32: χ^2 test for trend for IL-10 responses to *Candida* in children with active trachoma and their controls 159
Table 4-33: χ^2 test for trend for IL-10 responses to PPD in children with active trachoma and their controls 159
Table 4-34: χ^2 test for trend for IL-10 responses to chlamydial EBs in children with active trachoma and their controls.....160
Table 4-35: χ^2 test for trend for IL-10 responses to OMP2 in children with active trachoma and their controls 160
Table 4-36: χ^2 test for trend for IL-10 responses to HSP60 in children with active trachoma and their controls 160
Table 4-37: χ^2 test for trend for IL-10 responses to PMPGa in children with active trachoma and their controls 161
Table 4-38: χ^2 test for trend for IL-10 responses to PMPGc in children with active trachoma and their controls 161
Table 4-39: Mean TGFβ concentrations in children with active trachoma and their controls 163
Table 4-40: Conditional logistic regression of TGFβ concentrations for children with active trachoma and their controls.....164

Table 4-41: Mean TNFα concentrations in individuals with trachomatous scarring and their controls 166
Table 4-42: Conditional logistic regression of TNFα concentrations for individuals with trachomatous scarring and their controls 167
Table 4-43: Mean IFNγ concentrations in individuals with trachomatous scarring and their controls 169
Table 4-44: Conditional logistic regression of IFNγ concentrations for individuals with trachomatous scarring and their controls 170
Table 4-45: Mean IL-2 concentrations in individuals with trachomatous scarring and their controls 172
Table 4-46: Conditional logistic regression of IL-2 concentrations for individuals with trachomatous scarring and their controls 173
Table 4-47: Mean IL-5 concentrations in individuals with trachomatous scarring and their controls 175
Table 4-48: Conditional logistic regression of IL-5 concentrations for individuals with trachomatous scarring and their controls 176
Table 4-49: Mean IL-10 concentrations in individuals with trachomatous scarring and their controls 178
Table 4-50: Conditional logistic regression of IL-10 concentrations for individuals with trachomatous scarring and their controls 179
Table 4-51: χ^2 test for trend for IL-10 responses to chlamydial EBs in individuals with trachomatous scarring and their controls 179
Table 4-52: χ^2 test for trend for IL-10 responses to pmpGc in individuals with trachomatous scarring and their controls 180
Table 4-53: Mean TGFβ concentrations in individuals with trachomatous scarring and their controls 182
Table 4-54: Conditional logistic regression of TGFβ concentrations for individuals with trachomatous scarring and their controls 183
Table 4-55: Mean immunoblot density scores from infertile women and their controls 185
Table 4-56: Conditional logistic regression of immunoblot density scores for infertile women and their controls 186
Table 4-57: χ^2 test for trend for immunoblot density scores in response to MOMP in infertile women and their controls 186

Table 4-58: Mean TNFα concentrations in infertile women and their controls 188
Table 4-59: Conditional logistic regression of TNFα concentrations for infertile women and their controls 189
Table 4-60: Mean IFNγ concentrations in infertile women and their controls 191
Table 4-61: Conditional logistic regression of IFNγ concentrations for infertile women and their controls 192
Table 4-62: χ^2 test for trend for IFNγ responses to PHA in infertile women and their controls 192
Table 4-63: Mean IL-2 concentrations in infertile women and their controls 194
Table 4-64: Conditional logistic regression of IL-2 concentrations for infertile women and their controls 195
Table 4-65: χ^2 test for trend for IL-2 responses to PHA in infertile women and their controls 195
Table 4-66: Mean Il-5 concentrations in infertile women and their controls.....197
Table 4-67: Conditional logistic regression of IL-5 concentrations for infertile women and their controls 198
Table 4-68: Mean IL-10 concentrations in infertile women and their controls 200
Table 4-69: Conditional logistic regression of IL-10 concentrations for infertile women and their controls 201
Table 4-70: Mean TGFβ concentrations in infertile women and their controls.....203
Table 4-71: Conditional logistic regression of TGFβ concentrations for infertile women and their controls 204
Table 4-72: Effects of gender 205
Table 4-73: Effects of ethnicity - Mandinka 206
Table 4-74: Effects of ethnicity – Fula.....207
Table 4-75: Effects of ethnicity - Wolof 207
Table 4-76: Effects of ethnicity - Jola 208
Table 4-77: Chlamydial PCR results.....209
Table 4-78: Effects of positive chlamydial PCR.....209
Table 4-79: Likelihood of having received BCG vaccination by age group.....210
Table 4-80: Effects of prior BCG vaccination 211
Table 4-81: Prevalence of helminth and protozoan infection, and comparison with previous studies in The Gambia 212
Table 4-82: Effects of intestinal helminth infection.....213

Table 4-83: Effects of intestinal protozoan infection 213
Table 5-1: Stimulation of TNFα, IL-10, IFNγ and TGFβ by PMPG recombinant proteins 222
Table 5-2: Number of cases required to give adequate power to determine significant differences between cases and controls 239

Abbreviations

µg	microgram
µl	microlitre
µM	micromolar
10X	ten-times strength
BCG	bacillus Calmette-Guérin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
C.I.	confidence interval
CO_2	carbon dioxide
COMC	chlamydial outer-membrane complex
CTL	cytotoxic T lymphocyte
dH_2O	distilled/deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EB	elementary body
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay
FHAB	filamentous haemagglutinin
g	unit of gravity
HIV	human immuno-deficiency virus
HLA	human leukocyte antigen
hsp	heat-shock protein
ICAM-1	intercellular adhesion molecule 1
IFNγ	interferon-γ
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-	interleukin -
Inc	inclusion membrane protein
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
kHz	kilohertz
LB	Luria-Bertani
LPS	lipopolysaccharide
M	molar
MAdCAM-1	mucosal addressin cell adhesion molecule 1
mg	milligram
MIF	microimmunofluorescence assay
MIP	macrophage infectivity potentiators
ml	millilitre
mM	millimolar
MOMP	major outer membrane protein
MRC	Medical Research Council

mRNA	messenger ribonucleic acid
NBT	nitro-blue tetrazolium
OD	optical density
OEA	ovine enzootic abortion strain (of *Chlamydia psittaci*)
OMP2	outer membrane protein 2
OMP4	outer membrane protein 4
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pgp3	polypeptide encoded by ORF3 of the *Chlamydia*
PHA	phytohaemagglutinin
PID	pelvic inflammatory disease
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMP	polymorphic membrane protein
pmp	gene encoding polymorphic membrane protein
POMP	polymorphic (outer) membrane protein (≡ PMP)
PorB	porin B
PPD	purified protein derivative
RNAse	ribonuclease
RompA	rickettsial outer-membrane protein A
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFS	simple frozen storage
STD	sexually transmitted disease
STI	sexually transmitted infection
TBS	tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	follicular trachoma
TGFβ	transforming growth factor β
TI	intense trachoma
TLR2	Toll-like receptor 2
TNFα	tumour necrosis factor α
Tris	tris(hydroxymethyl)aminomethane
TS	scarred trachoma
TT	trichiasis
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volts
v/v	volume/volume
VCAM-1	vascular cell adhesion molecule 1
w/v	weight/volume
w/w	weight/weight
x-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Acknowledgments

As a result of undertaking this piece of work I have learnt many things, some academic and some not. I have had the privilege of working with many fellow scientists and clinicians in London, Uganda and The Gambia and now count many of them as friends.

I would especially like to thank David Mabey for helping to develop the original project proposal, for supervising my PhD studies, for his critical reading of the manuscript and for being my academic mentor for many years. Patrik Bavoil, and Ru-ching Hsia were also instrumental in developing the project proposal and in the molecular biology aspects of this work. I am also grateful to Robin Bailey for his input into the project development and for facilitating and coordinating the field work.

I am grateful to Alison Elliott for familiarising me with the whole blood assay, for introducing me to field-work in Africa and for her great hospitality in Entebbe; and to Angela Hunt-Cooke for coming to The Gambia to diagnose helminth and protozoal carriage. I am indebted to many colleagues in The Gambia, especially to Hassan Joof for his untiring and good-natured help with the field-work and for much sound advice; to Roland Roberts for his enthusiastic and conscientious processing of the immunoblots; to Gijs Walraven and Ros Coleman for their hospitality in Farafenni and to them and to Gloria Ekpo for help in recruiting the infertile women and controls; to Esther Aryee for carrying out the Amplicor PCRs on the conjunctival swabs; to Beryl West for carrying out the original PCRs, for always being happy to help with logistical problems, and for her friendship; to Nkoyo Faal for preliminary data analysis and to all the staff of the MRC laboratories in The Gambia for all their assistance and kindness.

This study would not have been possible without the generous gifts of *Chlamydia trachomatis* elementary bodies from Rosanna Peeling, Mike Ward and Sylvia Everson, and of OMP2 and HSP60 from Professor Hill Gaston.

My wholehearted thanks goes to a few people whose help has gone far beyond the call of duty. Jim Todd stepped in to provide expert and very practical statistical advice; Sowsan Atabani demonstrated perfect African hospitality whilst at the same time having a solution to every problem both in the laboratory and outside it; Mike Kidd provided scientific help and advice, friendship and a roof over my head in The Gambia and indispensable critical advice on the manuscript; and Tamara Hurst who was involved in developing the original project proposal, provided scientific input into the whole blood assay and cytokine ELISAs, provided logistical support throughout the project, critically read the manuscript and whose scientific and pastoral support to all Fellows of the Wellcome Bloomsbury Centre for Tropical Medicine cannot be underestimated.

Finally I would like to thank The Wellcome Trust Tropical Interest Panel for supporting this project through a Training Fellowship in Clinical Tropical Medicine, all the villagers in The Gambia who took part in the study, and my parents whose support for my endeavours has never wavered, and to whom this work is dedicated.

Chapter One

Background

1 Background

1.1 Chlamydia, the organism

Chlamydiae are a phylogenetically unique group of gram-negative bacteria responsible for a large variety of diseases in humans, animals and birds. Four species are currently recognised: *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci* and *Chlamydia pecorum*. *Chlamydia trachomatis* and *Chlamydia pneumoniae* are principally pathogens of humans however *Chlamydia trachomatis* can also infect rodents and pigs[1] and *Chlamydia pneumoniae* can infect horses, koalas and frogs[2]. *Chlamydia psittaci* is primarily a pathogen of lower vertebrate mammals, koalas and birds with humans being accidental hosts. *Chlamydia trachomatis* is the focus of this work. There has recently been some discussion regarding radical changes to the taxonomy of Chlamydiacae[3], however this remains contentious[4] and for the purposes of this thesis the current classification will be used.

Within the host, *Chlamydia* exist in a non-acidified vacuole (inclusion) which does not fuse with the cell's lysosomes. Here the bacteria sustain a unique intracellular developmental cycle. Shortly after entry, the infectious but metabolically inert elementary bodies (EBs) differentiate to the metabolically active reticulate bodies (RBs) which undergo several rounds of multiplication before differentiating back to EBs. The host cell is then lysed to release the infectious EBs.

1.2 Epidemiology and disease

1.2.1 Trachoma

Chlamydia trachomatis is the causative agent of trachoma, the commonest infectious cause of blindness and, some consider, the most common preventable cause of blindness. It is thought to account for an average of 15.5 per cent of blindness in developing countries, with figures ranging from 6.8% in Latin America and the Caribbean, to 19.4% in sub-Saharan Africa and 25.7% in the Middle East and central Asia.[5] Some 500 million individuals are affected by trachoma of whom 7 million are blind[6]. Although not restricted to the tropics, trachoma is most prevalent in these regions through which it is widely distributed. Active disease is usually seen in children

with transmission occurring either through close contact or by flies, and thus it is associated with overcrowding, poor sanitation, substandard levels of hygiene and medical care and poor standards of living.

Infection begins in early childhood with conjunctivitis. Repeated cycles of trachomatous conjunctivitis and secondary bacterial infections follow. Over a number of years this leads to scarring of the conjunctiva and the eyelids (Figure 1-1). Scarring of the underside of the eyelids causes them to turn inwards so that the eyelashes abrade the cornea causing pain, inflammation, further bacterial infections and ultimately blindness. Antibiotic treatment is effective, though often not available, but the infection recurs rapidly leading to progressive eye damage.

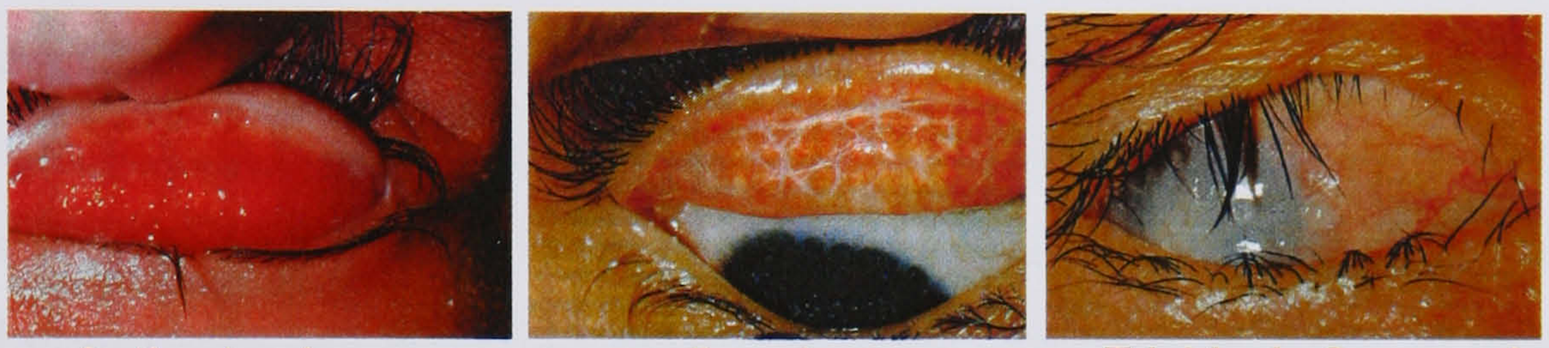


Figure 1-1: Clinical progression of trachoma

The field-work for this study took place in The Gambia. Here the second National Survey of Low Vision and Blindness in 1996 showed that 5.9% of children under 10 years of age had active trachoma whilst 3.3% of adults had trichiasis. The situation is improving due to an excellent national eye care program and these are significant improvements from the initial study some 10 years earlier, for example the prevalence of active trachoma has decreased by 54%.[7] However recent reports of the emergence of drug-resistant mutants are cause for concern.[8]

1.2.2 *Chlamydia trachomatis* urogenital tract infection

Chlamydia trachomatis infections are the most common bacterial sexually-transmitted infections (STIs) causing mostly urethritis and cervicitis. In United States there are an estimated 4 million cases occurring each year.[9,10] The cost of treating these infections and their sequelae approached $1.4 billion a year in 1987 and must be considerably more now. Estimates of the prevalence of chlamydial infection vary widely and most studies tend to collect data from individuals attending STD or ante-natal clinics where the prevalence is clearly very different form that in the general population. One recent study investigated the prevalence of infection in a variety of venues including Chinese

food markets, Peruvian barrios, Zimbabwean villages and student dormitories in Russia. Overall the prevalence was 7.4%. The prevalence in women ranged from 13.9% in Russia to 3.7% in Zimbabwean villages. In men the prevalence ranged from 6.9% in Peruvian barrios to 1.6% in Zimbabwean villages.[11] In the United Kingdom, as part of a national survey of sexual attitudes and lifestyles (Natsal 2000), 11,161 randomly selected, sexually active men and women aged 18-44 were screened for *Chlamydia trachomatis* by a ligase chain reaction (LCR) test on urine. Overall 2.2% of men and 1.5% of women tested positive for *Chlamydia trachomatis* with the highest prevalence being in the 25-34 year olds where 3% of men and 3.1% of women had a positive antigen test for *Chlamydia trachomatis*.[12]

Approximately 10% of women who acquire *Chlamydia trachomatis* urogenital tract infections develop upper genital tract complications, such as salpingitis and pelvic inflammatory disease (PID), chronic inflammation and subsequent fallopian tube scarring which greatly increases the risk of ectopic pregnancy and tubal infertility.[13] Epididymitis and prostatitis may complicate urogenital infection in men and *Chlamydia trachomatis* has been implicated in male infertility.[14,15] Chlamydial urogenital infection may also be associated with a reactive arthritis.

The L1,L2 and L3 strains of *Chlamydia trachomatis* cause another sexually transmitted disease, lymphogranuloma venereum, characterised by inguinal and sometimes generalised lymphadenopathy, and systemic symptoms such as fever, headache and myalgia.

1.2.3 Other chlamydial infections

In addition to trachoma and urogenital tract infections which are the focus of this work, *Chlamydia trachomatis* also causes inclusion conjunctivitis in both adults and neonates, and pneumonia in infants.

Chlamydia psittaci is primarily a pathogen of lower vertebrate mammals and birds but humans are sometimes infected and suffer a pneumonic illness, psittacosis, which may be severe.

Chlamydia pneumoniae is an important cause of community-acquired pneumonia[16] and is currently the source of much interest due to its association with atherosclerosis.[17] Saikku first demonstrated higher titres of anti-*Chlamydia pneumoniae* antibodies in patients with atherosclerosis,[18] and subsequently the organism has been detected in

atherosclerotic lesions by a immunohistochemistry, electron microscopy, polymerase chain reaction (PCR) and culture.[19,20]. Animal models have shown that *Chlamydia pneumoniae* infection initiates vascular atheromatous changes in rabbits,[21] promotes atheroma development in mice,[22] and that antibiotic treatment of infected rabbits inhibits these atheromatous changes.[23] Large studies are now underway in humans to see if antibiotic treatment of patients with coronary heart disease may be of benefit.[24]

1.3 Immune responses

There is clear evidence of a degree of immunity developing in individuals with chlamydial infection. Pathology is often due to repeated or recurrent infections, with epidemiological evidence suggesting that solid immunity is acquired slowly and may be strain specific[25]. For example, active trachoma and the isolation of live organisms from the conjunctiva is seen principally in childhood[26], and isolation rates in genital disease fall with age[27] or increasing duration of exposure[25]. This immunity appears to operate through both humoral (antibody mediated) and cellular mechanisms (in which antigen specific T-cells play the principal role), and there appear to be differences in the response to a primary and secondary infection.

1.3.1 Innate responses

The first line of defence against most bacterial infections are the phagocytes of the innate immune system, the neutrophils and the macrophages.

Neutrophils appear to be important in controlling primary infection with *Chlamydia* where depletion of neutrophils results in a much higher bacterial load and more severe disease[28,29] but are of lesser importance in secondary infection where mice depleted of neutrophils are as able as a wild type mice to eradicate infection.[30]

Chlamydia are effectively phagocytosed by macrophages and monocytes[31] and induce the differentiation of monocytes into macrophages,[32] which then act as antigen presenting cells and release interleukin-12 (IL-12) thus activating T-cells and triggering the adaptive immune response. However *Chlamydia* may persist within macrophages,[31,33] and macrophages activated by *Chlamydia* produce interferon γ (IFNγ),[34] nitric oxide[35] and matrix metalloproteinases.[36] *Chlamydia* may also replicate inside monocytes or macrophages triggering the production of pro-inflammatory

cytokines such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6).[37]

1.3.2 Humoral responses

The importance of humoral responses to chlamydial infection has been the subject of much contention. Initially it was felt that humoral responses were of primary importance in defence against chlamydial infection. Certainly immature plasmablasts, or antibody secreting cells, responsive to chlamydial major outer membrane protein (MOMP), heat-shock protein-60 (HSP60) and whole EBs have been detected in the blood of patients with chlamydial urogenital disease and individuals with both active and scarred trachoma,[38] and *Chlamydia*-specific immunoglobulin-G (IgG) and immunoglobulin-A (IgA) are detected in urethral swabs from men with *Chlamydia trachomatis* urethritis.[39] Women with cervical infection and high anti-chlamydial IgG levels are protected against subsequent salpingitis[40] and mice immunised intra-nasally with *Chlamydia* organisms developed antibody responses and were protected against subsequent genital infection and infertility[41,42]. In the guinea pig model of genital tract infection also, *Chlamydia*-specific antibody seems to play a significant role in immunity as guinea-pigs given pooled immunoglobulins from immune animals had a lower organism load on subsequent genital infection with *Chlamydia*[43].

However, humoral responses are not essential in combating chlamydial infection. The presence of anti-chlamydial antibodies in mice correlates poorly with protection from re-infection,[44] indeed mice deficient in B-cells and antibody develop strong immunity to re-infection with *Chlamydia trachomatis* both in short and long-term.[45]

The evidence seems to suggest that humoral factors may be more important in resistance to re-infection than in combating primary infection. A study in B-cell knockout mice showed that cell-mediated immunity alone was sufficient to clear primary infection but that the B-cell deficient mice were then more susceptible to re-infection[46], whilst Fc-receptor knockout mice were able to clear primary infection as well as wild-type mice but tended to have a more severe secondary infection.[47] A similar study showed that B-cell deficient mice demonstrate no difference in immunity to re-infection to wild type mice to *Chlamydia trachomatis* 45 days after primary infection,[48] but after 70 days B-cell deficient mice are more susceptible to colonisation and re-infection.[46] In another study, following resolution of primary infection, mice were depleted of both CD_4 and CD_8 T-cells and then re-challenged with *Chlamydia*.

Depletion of both CD_4 and CD_8 T-cells had only a very limited effect on the course of secondary infection suggesting that B-cell and/or antibody play an important role in immunity to secondary infection.[49] Thus the evidence appears to suggest that B-cells and humoral responses may be more important for long-term memory.

1.3.3 T-cell responses

A rapidly expanding body of evidence points to the importance of T-cell responses in immunity to chlamydial infection. Strong lymphoproliferative responses, to a number of chlamydial antigens, are associated with resolution of chronic infection in trachoma[50]. The fact that *Chlamydiae* reside within a vacuole inside the host cell makes it unclear how chlamydial antigens could gain access to either the major histocompatibility complex (MHC) class I or class II processing systems. Despite this around 30 human leukocyte antigen (HLA) class II-restricted T-cell epitopes[51,52] and eight HLA class I-restricted cytotoxic T-lymphocyte (CTL) epitopes in MOMP have been described.[53,54]

Evidence regarding the relative contributions of CD_4 and CD_8 T-cells is not clearly defined and at times conflicting. Approximately equal numbers of CD_4 and CD_8 T-cells are seen in acute chlamydial infection of the human cervix[55], the guinea-pig genital tract and eye[56] and the genital tract and eyes of non-human primates[57,58]. Increased MHC class I and class II expression has been seen in infected epithelial cells in trachoma,[59] though MHC class II restricted T-cell responses, but not MHC class I, were shown to be important in resolving genital *Chlamydia trachomatis* in mice[60].

Depletion of both CD_4 and CD_8 T cells in mice leads to increased pulmonary organism load in *Chlamydia trachomatis* pneumonia, but only CD_4 T cell deletion leads to increased mortality[61]. In further studies, both CD_4 T cells (producing both T_H1 and T_H2 cytokines on stimulation)[62] and CD_8 cells[63,64] were able to transfer adoptive immunity. Depletion of CD_4 but not CD_8 T-cells leads to a much prolonged course of secondary infection in B-cell deficient mice but has no effect on the ability of animals to resolve that infection,[65] and in humans the risk of pelvic inflammatory disease (PID) is independently associated with falling CD_4 T cell levels in human immunodeficiency virus (HIV) infection[66].

The role of MHC Class I-restricted CD_8 T-cells in protective immunity remains controversial. CD_8 cells from the synovial fluid of patients with Reiter's syndrome proliferate in response to chlamydial antigens,[67] and *Chlamydia*-specific cytotoxic T cells have been found in the blood of patients with trachoma,[68] but the significance of

these remains uncertain. *In vitro* studies suggest that CD_8 T-cells are cytotoxic for *Chlamydia*-infected cells,[69-71] but *in vivo* studies suggest only a minor role. *Chlamydia*-specific CD_8 cells, isolated from mice following infection with *Chlamydia trachomatis*, were able to lyse *Chlamydia*-infected cells and adoptively transfer protection to naive mice in some experiments[63], but not in others,[62] and in the murine model, depletion of CD_8 cells had no effect on clearance of infection.[65] CD_8 T-cells may also have a role to play through the production of IFNγ,[72] which appears to mediate cytotoxicity[73], and also inhibits chlamydial activity.[74,75] *Chlamydia* suppresses IFNγ-inducible MHC class I and II expression.[76,77] The suppression of MHC class I synthesis would prevent the processing and presentation of chlamydial epitopes on the infected cell surface and therefore preclude recognition and subsequent lysis by CTLs.[76] Human peripheral blood mononuclear cells (PBMCs), infected with *Chlamydia pneumoniae*, secrete IL-10 which down-regulates expression of MHC class I molecules. One consequence of this may be a reduction in the presentation of bacterial epitopes by MHC. This would decrease the ability of CD8 T-cells to eliminate infected cells.[77]

1.3.4 Cytokine responses and the relative importance of T_H1- and T_H2-type responses

Much of the field work in this study concentrates on the cytokine responses to a number of chlamydial antigens. The inter-relationships between the various cytokines produced in response to infection is hugely complex. One useful way to look at cytokine responses is in terms of the T_H1/T_H2 dichotomy.

Mosmann et al first identified two sub-populations of CD_4 T-cells in mice in 1986 on the basis of their cytokine secretion patterns. He described T_H1-type T-cells which produce interleukin-2 (IL-2) and IFNγ and a T_H2-type population which produce interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-10 (IL-10). Subsequently it was felt that these two subsets were derived from T_H0 cells that secrete both T_H1 and T_H2 cytokines.[78,79] There was some controversy as to whether a similar dichotomy existed in humans until the first evidence was presented in 1991.[80] A T_H1-type response is now defined as a strong cellular response with normal or increased levels of IL-2, IFNγ, TNFα and/or IL-12, while a T_H2-type response is characterised by a predominantly humoral response with an increase in the levels of IL-4, IL-5, IL-6, IL-10 and interleukin-13 (IL-13) together with an increase in one or more B-cell activities (hypergammaglobulinaemia, autoantibody production or hyper-immunoglobulin E).

The classification is based on the relative predominance of either a cell-mediated or humoral-type response and not on an absolute dichotomy of either type of cytokine response. The definition of T_H1 and T_H2-type responses is now generally expanded to include not only responses by T-cells, but also those by monocytes/macrophages, natural killer cells, B cells, mast cells and eosinophils, all of which are now known to produce cytokines originally attributed only to CD_4+ T-cells.

Differing cytokine profiles have been described for T-cell responses to various microbial pathogens. T_H1 responses have characteristically been associated with the resistance to, and elimination of, intracellular pathogens such as *Mycobacterium tuberculosis* [81,82], *Mycobacterium leprae* [83] and *Leishmania major* [84]. T_H2 responses, on the other hand, are elicited by helminth infection and correlate with protective immunity for several species of parasitic nematode [85,86].

A study of the progress of chlamydial genital infection in mice showed that cells producing the T_H1 pro-inflammatory cytokines IL-12 or TNFα were the first to be detected, with cells producing the T_H2 anti-inflammatory cytokines IL-4 and IL-10 being present in greatest numbers at three weeks post infection, a time when the infection was nearing resolution. They were unable to convincingly demonstrate the presence of either IL-5 or IFNγ using immunohistochemical staining.[87] T_H1-type responses however appear to be essential for the resolution of chlamydial infection. T_H1-deficient mice fail to clear primary genital infection with *Chlamydia* despite generating high levels of a specific antibody, whilst T_H2-knockout mice clear infection as well as wild type mice.[44] T_H1-type responses are associated with resolution of *Chlamydia trachomatis* infection in mice[314] and T_H2-type responses with scarring in trachoma[315]. Interestingly, mice mounted a predominantly T_H1-type response to primary genital chlamydial infection but a more mixed picture on subsequent infection[89]. Lymphocytes isolated from *Chlamydia*-infected genital tracts demonstrate a predominance of T_H1-type cytokine and messenger ribonucleic acid (mRNA) responses[90,91]. Additionally anti-cytokine antibodies that diminished T_H2-type responses are beneficial and those that inhibit T_H1-type responses are detrimental to the host response to infection[92].

Much recent work has focused on *Chlamydia pneumoniae* due to its association with atherosclerosis. Due to the differences in the structure, biology and pathogenesis of *Chlamydia pneumoniae* and *Chlamydia trachomatis* extrapolating the results of experiments with *Chlamydia pneumoniae* to other chlamydial species should be done

with caution. However, infection with *Chlamydia pneumoniae* appears to be a potent inducer of pro-inflammatory cytokine production. The growth of *Chlamydia pneumoniae* inside monocytes or macrophages triggers the production of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 [37] and PBMCs stimulated with sonicated *Chlamydia pneumoniae* produce TNFα, IL-1, IL-6, interleukin-8 (IL-8), monocyte chemoattractant protein-1 and macrophage inflammatory protein-1.[93]. This appears to be mediated through the Toll-like receptor-2 (TLR2), an innate immune receptor involved in the pattern recognition of bacterial pathogens.[94] IL-12 is an important pro-inflammatory cytokine which augments natural killer cell and T-cell cytotoxic activity, promotes T_H1 differentiation and induces the production of IFNγ and other cytokines.[95] IL-12 is critical for the development of T_H1-type responses, and IFNγ production induced by chlamydial infection is mediated largely in an IL-12 dependent fashion. Mice with pulmonary *Chlamydia pneumoniae* infection produced IL-12, IFNγ, TNFα and IL-10. Depletion of IL-12 resulted in markedly reduced levels of IFNγ production and a much higher organism load. Clearance of the organism was delayed but it did occur. [96]

IFNγ appears to play a very important role in clearing chlamydial infection. IFNγ has been shown to directly inhibit the growth of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae*.[75] Neutralisation of IFNγ resulted in an increase in both in the number of organisms seen in the lung and also in the severity of pneumonia in the murine *Chlamydia pneumoniae* model,[97] whilst mice deficient in IFNγ are unable to completely resolve genital tract infection, and chlamydial infection in those mice disseminates to systemic sites[98]. In other experiments IFNγ-receptor knockout mice were able to clear primary genital infection but were not able to resist re-infection suggesting that they failed to develop protection, whilst IFNγ knockout mice were unable to clear even primary infection.[44]

TNFα is another cytokine which has been extensively studied in the context of chlamydial infection. TNFα is a pro-inflammatory cytokine released primarily from monocytes and macrophages upon invasion of the host by a wide variety of pathogens. Mouse spleen cells infected with *Chlamydia trachomatis* generate TNFα[99] and high levels of TNFα are found in genital tract secretions during the first week of primary chlamydial infection.[100] In humans TNFα is found in the genital tract of women with chlamydial salpingitis[101] and levels of TNFα in the tears[102] and conjunctivae[103] are

higher in individuals with trachoma than in controls. In a murine model of chlamydial pneumonia, exogenous administration of anti-TNFα increased the bacterial load in the lungs and significantly accelerated mortality.[104] TNFα also appears to directly inhibit the growth of Chlamydia, at least *in vitro*.[105] However TNFα is not essential for clearance of *Chlamydia*: mice deficient in the TNFα receptor show only a marginal delay in the rate of clearance of *Chlamydia* from the genital tract,[106] whilst local blockade of TNFα in the genital tract had no effect on the course of the ascending infection or on the resultant chronic tissue pathology.[107]

1.4 Chlamydial antigens

1.4.1 Major Outer Membrane Protein (MOMP)

The major outer membrane protein of *Chlamydia trachomatis* (MOMP) is the dominant surface protein (~60% of outer membrane content) which functions as a structural protein[108] but also as a porin[109,110] and possibly an adhesin[111]. It is the serologically variant antigen that defines the serovars.[112,113] MOMP is encoded by the *ompA* gene which consists of five conserved sequence regions alternating with four variable regions.[114] Serovar-specific antibodies are directed towards amino-acids sequences encoded in these variable segments.[115] Over 15 serovariants, or serovars, of *Chlamydia trachomatis* have been described. The tendency for successive infections to be with different serovars of *Chlamydia trachomatis* suggests that protective immunity is in part associated with sequences that vary among serovars[25] and this, together with the relative abundance of MOMP has meant that it has been extensively studied.

61% of individuals with chlamydial urethritis or cervicitis were found to be positive for anti-*Chlamydia trachomatis* MOMP IgG compared to 16% of 'healthy' blood donors.[116] A number of B-cell epitopes have been determined, and neutralising antibodies have been produced to a number of these[117-119]. T-cell responses to epitopes in both the constant and variable regions of MOMP have been described in *Chlamydia trachomatis* infected subjects.[52]

There have been some successes using MOMP as a potential vaccine candidate. Rabbit anti-MOMP IgG has been shown to neutralise infectivity despite not affecting internalisation[120], and passive immunisation of pregnant mice with anti-MOMP antibodies prevented infection and foetal loss[121]. One anti-MOMP monoclonal antibody

was able to protect mice from intravenous challenge with EBs and prevent infection of monkey's conjunctivae[122] The T-cell epitope, TINKP, elicited recall responses and protected against salpingitis,[123] and MOMP, enclosed within 'lipophilic immune response stimulating complexes' and injected intramuscularly, has been shown to induce a local genital mucosal T_H1 response which protected against subsequent *Chlamydia trachomatis* infection and which could be adoptively transferred to naive syngeneic animals.[124]

In general, however, attempts at generating protection using peptide and denatured *Chlamydia trachomatis* MOMP protein have failed (review[125]). Intramuscular immunization of mice with chlamydial deoxyribonucleic acid (DNA) encoding the chlamydial MOMP induced both cellular and humoral immune responses suggestive of a T_H1-biased immunity, and the immunized mice suffered less weight loss and had a 100-fold reduction in pulmonary organism load.[126] In another experiment however, MOMP DNA vaccination failed to protect mice against subsequent genital challenge.[127] Though initially the most promising sub-unit vaccine candidate, MOMP has so far failed to deliver on that promise.

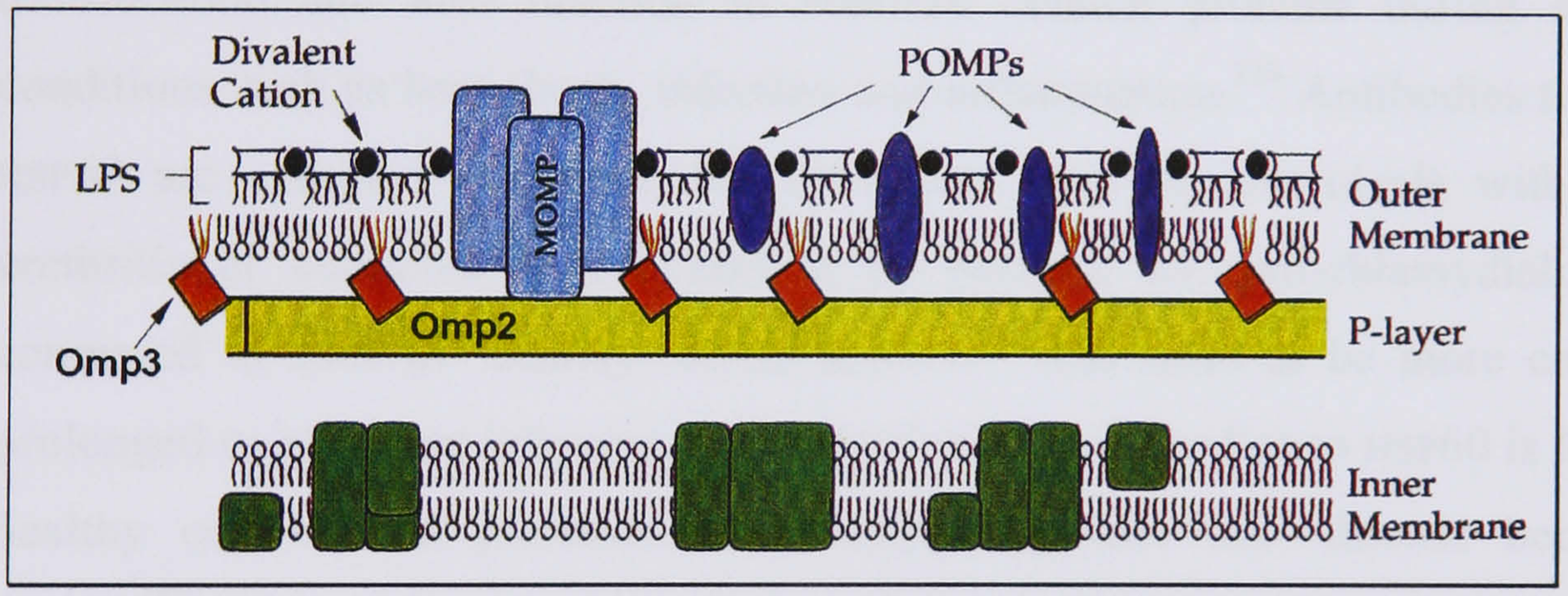


Figure 1-2: Putative model of the envelope of chlamydial elementary bodies. Adapted from Everett et al[128] and Hatch.[129]

1.4.2 Outer Membrane Protein 2 (OMP2)

OMP2 is the second most abundant protein in chlamydial elementary bodies and is expressed when reticulate bodies differentiate into elementary bodies.[130] Antibodies to OMP2 have been reported to be present in the majority of patients infected with *Chlamydia*.[131,132] 17/17 patients with *Chlamydia trachomatis* urethritis had antibodies to OMP2[133] in one study whilst in another 89% of individuals with chlamydial urethritis or cervicitis were found to be positive for anti-OMP2 IgG compared to 43% of 'healthy' blood donors.[116] The authors suggested that the high level of false positives might be

due to cross reaction with *Chlamydia pneumoniae* OMP2. Another group tested seven different fragments of the OMP2 protein for immunoreactivity in the sera of patients with *Chlamydia* infection and all were found to be reactive.[134] The importance of these antibodies is uncertain however as OMP2 appears to be confined to the inner surface of the EB's outer membrane.[130,135]

T-cells responsive to OMP2 have been detected in both mice[136] and humans. *Chlamydia trachomatis*-specific CD_4 T-cells from patients with *Chlamydia trachomatis*-induced reactive arthritis and with chlamydial urethritis, have been shown to respond to both whole OMP2 and epitopes of OMP2, though the levels of response were lower than those to whole EBs, suggesting that, although OMP2 represents a major component of the protein composition of EBs, other less abundant proteins may be more important as targets of the immune response.[137]

1.4.3 Heat-shock protein 60 (HSP60)

Heat shock proteins are highly conserved across species, chaperoning newly synthesised or damaged proteins during their intracellular folding or unfolding, assembly and translocation and also function to stabilise cellular proteins during a variety of conditions such as heat shock, infection and inflammation.[138] Antibodies to chlamydial HSP60 are common in chlamydial infections, 61% of individuals with chlamydial urethritis or cervicitis were found to be positive for anti-chlamydial HSP60 IgG compared to 23% of 'healthy' blood donors,[116] and seem to be more common with prolonged or recurrent infection - the prevalence of antibodies to HSP60 is low amongst healthy controls but increases as chlamydial genital tract disease becomes more severe.[139] HSP60 antibody responses were found in 32% of Gambians with trachomatous scarring compared to 16% of controls ($P < .001$)[140] and anti-HSP60 antibodies were found in tears of Nepalese subjects with trachoma.[141] T-cell proliferative responses are seen in trachoma patients who resolve their infection[50].

HSP60 has been shown to be a potent stimulant of immune and inflammatory responses. Both human and chlamydial HSP60 activate human PBMCs, monocyte-derived macrophages and endothelial cells through CD_{14} signalling and p38 mitogen-activated protein kinase via NF-κB through the Toll-like receptor 4,[142] in the same way as bacterial lipopolysaccharide (LPS).[143] Chlamydial HSP60 has been shown to induce the expression of TNFα, IL-6, matrix metalloproteases, E-selectin, intercellular adhesion molecule-1(ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1).[144-147]

Mice immunised intra-nasally with a DNA vaccine encoding *Chlamydia pneumoniae* HSP60 demonstrated a reduced bacterial load and decreased severity of disease after challenge though no specific antibodies were detected. In contrast mice immunised intradermally developed *Chlamydia*-specific IgG but were not protected against challenge with *Chlamydia pneumoniae*.[148]

Because of the highly conserved nature of HSP60, responses directed against microbial hsp may also be deployed against autologous HSP. These cross-reactive responses could convert the protective immune responses into pathological ones. This is discussed further in section 1.5.

1.4.4 The polymorphic membrane proteins (PMPs)

The family of polymorphic membrane proteins, known as PMPs or POMPs , were first identified, by Longbottom et al, in 1996 on screening of a *Chlamydia psittaci* λgt11 genomic expression library with post-abortion sera from sheep infected with *C. psittaci* ovine enzootic abortion strain (OEA). They identified four members of a multigene family encoding proteins of approximately 90 kilodaltons (kDa) which appeared to be localized to the chlamydial surface membrane[149]. A number of other groups also began looking at this gene family and the Chlamydia Genome Project (http://chlamydia-www.berkeley.edu:4231) later reported 9 members of the *pmp* family in *Chlamydia trachomatis* and 21 in *C. pneumoniae*. There are at least 6 *pmp* genes in *Chlamydia psittaci*.[150]

Comparison of the *pmp* genes encoded within isolates of *Chlamydia pneumoniae* from the United States and from Japan demonstrated truncation of two of the *pmp* genes in the Japanese strain. This appeared to be due to frameshift mutation. The remaining *pmp* genes were very highly conserved between the two strains.[151] Similarly, comparison of the *pmp* genes encoded within isolates of *Chlamydia pneumoniae* from the United States and from Denmark demonstrated only one nucleotide difference between the two strains and this also resulted in a truncated PMP protein due to the deletion of a single residue which gave rise to a premature stop codon.[152] In a restriction fragment length polymorphism analysis (RFLP) all the *pmp*s (except *pmpB*) were compared from isolates of *Chlamydia trachomatis* serovars B and K and the previously sequenced serovar D. Between these serovars, *pmpH* varied by almost 4% and *pmpE* by 2%. Serovar K had a 6 base pair (bp) deletion at the 5' end and serovar B had a 3 bp

insertion at the 5' end. No 'marked' variation was noted in the *pmpA, pmpC, pmpF, pmpG, pmpH* or *pmpI* genes.[153]

The PMPs appear to be surface-localised proteins. The predicted secondary structure of these proteins is predominantly of β-pleated sheets, typical of bacterial surface proteins whose membrane spanning regions are formed by β-strands. Also typical of bacterial outer membranes is the consistently present carboxyl-terminal phenylalanine[154]. These proteins are relatively cysteine-rich, having between 9 and 26 cysteine residues in the case of *Chlamydia trachomatis* PMPs, representing between 0.9 and 2.1% of the proteins' amino acid composition; this is similar to the figure of 1.9% for MOMP. These cysteine residues may be important for disulphide cross-linkage of the surface proteins as has been shown for MOMP – which does not migrate on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) unless a reducing agent is added to break these disulphide bonds. The 98 kDa proteins do migrate on SDS-PAGE in the absence of a reducing agent which is more suggestive of intra-molecular rather than inter-molecular bonding[155]. More recent computer modelling has predicted parallel β-helices in the amino-terminal regions of most *pmp* genes in *Chlamydia pneumoniae*.[156] Similar structures are found in a number of pathogenic organisms and are often associated with virulence, such as the filamentous haemagglutinin adhesin of *Bordetella pertussis*.[157]

	Amino acids	Mol wt. kDa	pI	Cysteine	
				No	%
pmpA	975	106	8.58	9	0.9
pmpB	1751	183	5.52	19	1.1
pmpC	1770	187	4.52	14	0.8
pmpD	1531	161	4.75	26	1.7
pmpE	964	105	6.80	15	1.6
pmpF	1034	112	8.88	13	1.3
pmpG	1002	106	5.26	15	1.5
pmpH	1016	108	6.08	14	1.4
pmpI	878	96	6.09	18	2.1

Table 1-1: Characteristics of the *Chlamydia trachomatis pmps*

Apart from these predictions based on the amino acid sequence, a considerable body of evidence pointing towards a surface localisation comes from studies of the chlamydial outer membrane complex (COMC) which can be isolated and purified[108]. *Chlamydia psittaci* COMCs contain an immunogenic 89 kDa protein which is synthesised early in

the life cycle and is not particularly strongly cross-linked by disulphide bonds[158]. Further immunoblotting studies of *Chlamydia psittaci* COMCs with post-abortion sera from infected ewes demonstrated a triplet of bands at 90-95 kDa which, on Coomassie staining of SDS-PAGE gels, were shown to correspond to proteins present only in very small amounts, suggesting that these proteins are highly immunogenic[159]. Immune sera from convalescent animals recognises the 98 kDa antigen on SDS-PAGE separated COMC's of *Chlamydia pneumoniae*[155]. Evidence that these proteins are indeed the PMPs has been facilitated by the cloning of a number of *pmp* genes as this has allowed the production of recombinant proteins for immunoblotting, and specific monoclonal antibodies which can then be used for immuno-staining of whole *Chlamydia* and COMCs. In one study a recombinant 98.9 kDa PMP protein from *Chlamydia pneumoniae* was recognised by polyclonal antibody to *Chlamydia pneumoniae* COMCs whilst conversely monoclonal antibody against this PMP recognised a double band of proteins from *Chlamydia pneumoniae* EB's at 96-97 kDa[160]. Interestingly this group found that recognition of their recombinant proteins was greater when samples were not boiled before SDS-PAGE (when the 97/99 kDa proteins migrate as if 65 kDa) suggesting that conformational, non-linear epitopes may be important[161]. One of their PMPs, outer membrane protein 4 (OMP4), was shown to be exposed on *Chlamydia pneumoniae* organisms in the lungs of infected mice by immuno-staining with polyclonal anti-OMP4 antibody whilst another, outer membrane protein 5 (OMP5) was shown to be exposed on *Chlamydia pneumoniae* in tissue culture but was not seen *in vivo*, suggesting that perhaps not all the PMPs are expressed in vivo[161]. Immuno-electron microscopy of *Chlamydia psittaci* demonstrated surface localisation of the PMP proteins on RBs but not on EBs where it appeared to be located periplasmicaly[162]. This was in contradiction to other findings, a similar study, using different antibodies does appear to convincingly demonstrate surface localisation of the *Chlamydia psittaci* PMPs on both EBs and RBs[163]. A recent study, using a combination of electrophoresis and mass spectroscopy has clearly demonstrated that at least PMPG and PMPH are located on the outer membrane of *Chlamydia trachomatis*,[164] whilst another study, using both immuno-staining and detergent solubility, showed that PMPD of *Chlamydia trachomatis* L2 is also located on the surface.[165]

It has been suggested that the *Chlamydia psittaci* PMPs might have two domains separated by the short central polyglycine region, and that the carboxyl-terminal domain might be hidden. Supporting evidence comes from the fact that in natural infection in

sheep, antibodies are not produced to the carboxyl-terminal, even though it has been shown to be immunogenic, whilst antibodies are generated to the amino-terminal 'domain'[159]. Further evidence is provided by the paucity of immuno-staining of whole EBs with antibodies to the carboxyl-terminal compared to marked staining by amino-terminal antibodies[163]. Further studies are required to confirm or refute this hypothesis. In *Chlamydia trachomatis* only one of the PMPs, PMPH, has a polyglycine region.

Figure 1-3: pmp gene arrangement within Chlamydia trachomatis genome

In *Chlamydia trachomatis* the nine PMPs have predicted molecular weights ranging from 96 to 187 kDa, with a mean molecular weight of 129 kDa. Despite the small genome of *Chlamydia* these genes comprise 3.2% of the *Chlamydia trachomatis* genome. In an organism with such a small genome and so little redundant DNA it seems unlikely that a multigene family such as this would be present if it did not fulfil an essential role. The amino acid identity within the family varies from 2 – 26%[166]. This figure hides the fact that within the genes are regions of high homology separated by much more variable regions. Structurally the *pmp* genes are encoded within three clusters, one containing *pmpA, pmpB* and *pmpC*, one containing only *pmpD* and finally one containing *pmps E* to *I* (Figure 1-3). Phylogenetically they also fall into three major groups but these do not appear to have any relationship to their localisation within the genome. These groups can be further subdivided into 6 smaller groups which, if analysed together with the *Chlamydia pneumoniae pmp*s, each contain at least one *Chlamydia trachomatis* and one *Chlamydia pneumoniae pmp*, suggesting that perhaps each of these sub-groups has a specific function[167]. The exception to this is *Chlamydia trachomatis pmpG* which clusters with 11 *Chlamydia pneumoniae pmp*s which appear

to have arisen through gene duplication. The presence of the repetitive GGAI element has been noted to be a feature of the *pmp* family of genes[167] in *Chlamydia pneumoniae* and are seen here in all the *Chlamydia trachomatis pmp* genes with the exception of *pmpH* which co-incidentally is the only *Chlamydia trachomatis pmp* which does possess the polyglycine central region which is a feature of the *Chlamydia psittaci pmp*s identified to date. As noted for *Chlamydia pneumoniae* this GGAI motif is confined to the amino-terminal of the proteins.

There is some evidence that the PMP proteins may undergo some post-translational modification. Certainly there are glycosylation and phosphorylation sites present in the *pmp* genes which may be involved in post-translational modification.[168]

Work on the functional biology of the PMPs is still at an early stage. Reverse-transcriptase polymerase chain reaction (RT-PCR) studies suggests that all the *pmp*s are transcribed and at least four are transcribed as early as 10 hours[166] although immunofluorescent staining did not detect PMPs until 30-36 hours[159,169]. This discrepancy may be explained by the low abundancy of the proteins and the lesser sensitivity of the method. If they are all transcribed throughout the life cycle this implies that they are functionally important in both RBs and EBs. The function of these proteins remains unknown although it is tempting to speculate that such a family of homologous genes might be involved in antigenic variation, in adaptation to various host environments, or in tissue tropism, though the number of genes in *Chlamydia trachomatis* is low compared to those involved in antigenic variation in trypanosomes and plasmodia for example. A search of genome databases for proteins with homology to the *Chlamydia* PMP proteins, looking for the GGAI or the GGA[I/L/V] and FxxN motifs, revealed homology to membrane proteins of four other bacterial species: the rickettsial outer membrane protein A (RompA) of several *Rickettsia* species which appear to be involved in adhesion, *Bordetella pertussis* adhesin FHAB, *Helicobacter pylori* HP1288 and *Eschericia coli* YFAL proteins, the functions of which are unknown.[152]

Homology searches also showed that the *Chlamydia pmp* genes were frequently identified with members of the autotransporter family, leading one group of researchers to suggest that the chlamydial PMP proteins may indeed be members of this family.[170] The autotransporter family, sometimes known as the Type V secretion system, is a highly efficient mechanism of protein export found amongst gram-negative bacteria. Proteins secreted through this pathway all have a similar configuration consisting of a

signal sequence, a passenger domain and a carboxyl-terminal translocating unit. The secreted protein crosses the inner membrane by a sec-dependent mechanism but then the carboxyl terminus assembles into a β-barrel pore in the outer-membrane and the passenger domain is transported through this to the bacterial surface. At the surface the passenger domain and the β-domain either remain intact or are cleaved into separate subunits. Autotransporters may be the primary mechanism of protein transport in many gram negative pathogens with autotransporter proteins being discovered in *Neisseria*, *Haemophilus*, *Bordetella* and *Helicobacter* as well as several members of the Enterobacteriacae including *Shigella* and *Eschericia coli*.[171] It is suggested that the size of the proteins (90 – 187 kDa) greatly exceeds that expected for membrane proteins which are typically 30 to 90 kDa but would be typical sizes for autotransporter proteins which range from 50 to 300 kDa.[171] One 144 kDa PMP protein has been shown to migrate on SDS-PAGE as a 100 kDa protein which would be consistent with a processed protein having had the ~40 kDa carboxyl-β barrel cleaved from the passenger domain. They also argue that the arrangement of motifs within the gene would be typical of autotransporters with a signal sequence being followed by functional motifs and then the carboxyl terminal unit. Certainly there are interesting functional motifs encoded within the *pmp* genes including serine proteases, leucine zippers and RGD adhesion motifs. Additionally the PMP proteins all have a predicted carboxyl-terminal amphipathic β-sheet which might be capable of forming a β-barrel translocation unit. The GG[A/L/V/I][I/L/V/Y] motif followed at a variable distance by the FxxN motif, found throughout the PMP proteins, is a common feature of many autotransporter proteins.[170] The authors find themselves drawn to the conclusion that the chlamydial PMP proteins really are autotransporters. Clearly this is speculative but the evidence is fascinating and certainly plausible.

Evidence of the immunogenicity and importance of these proteins has been building over the last decade, even though the identity of the 90-98 kDa proteins was not known. In 1989 it was noted on SDS-PAGE analysis of *Chlamydia psittaci* that invasive strains had a protein band present at 98 kDa which were not seen with non-invasive strains[172]. Later these virulent, abortion-inducing, strains were noted to be mostly of serotype 1 whilst the non-invasive intestinal forms belonged to two other serotypes[173]. This raised the possibility that the 98 kDa strains might somehow be involved in virulence an/or be serotype specific. Antibodies to this virulent strain, raised in rabbits and mice, were shown to recognise *Chlamydia psittaci* proteins of 96, 90 and 88 kDa on

immunoblotting[174]. Later the same group noted the presence of highly reactive antibodies to 80-90 kDa proteins on immunoblots of *Chlamydia psittaci* EB's when screened with sera from post-abortion ewes infected with *Chlamydia psittaci* OEA. These antibodies appeared to be serotype-specific suggesting variability in the relevant antigens between serovars[175].

Although antibodies to a number of chlamydial proteins, such as MOMP demonstrate considerable cross-reactivity between the species, antibody responses to the 90-98 kDa proteins show considerable species and even serovar specificity. This has been noted both in rabbits, using *Chlamydia pneumoniae* 98 kDa protein[155], and in humans. Immunoblotting convalescent sera from patients with *Chlamydia pneumoniae* infection against *Chlamydia pneumoniae*, *Chlamydia trachomatis* and *Chlamydia psittaci* EB proteins, the 98 kDa proteins of *Chlamydia pneumoniae* but not those of *Chlamydia trachomatis* or *Chlamydia psittaci* were recognized by the sera whereas there was considerable cross-reactivity of other proteins (68, 60, 30 kDa proteins and MOMP). Reaction to the 98 kDa proteins appeared to correlate with high titres of circulating anti-*Chlamydia pneumoniae* antibody when followed over time in individual patients. No sera from patients negative for anti-chlamydial antibodies recognized the 98 kDa proteins but all patients from whom *Chlamydia pneumoniae* was isolated, did[176]. In another series, protein from the EBs of *Chlamydia trachomatis, Chlamydia pneumoniae* and *Chlamydia psittaci* was immunoblotted with sera from patients infected with *Chlamydia trachomatis*, *Chlamydia pneumoniae* or both. They found that antigens of 170, 155, 145, 120, 115, 100, 57 and 38 kDa were specific for *Chlamydia trachomatis*, antigens of 175, 130, 110, 98 and 30 kDa were specific for *Chlamydia pneumoniae* and antigens of 90, 80, 75, 62, 60 and 55 kDa were recognized by sera from patients infected with either of the species. Only 39% of their *Chlamydia pneumoniae* patients' sera recognised *Chlamydia pneumoniae* 98 kDa protein[177]. In one other similar study sera from the majority of patients with *Chlamydia pneumoniae* infection recognised the 98 kDa *Chlamydia pneumoniae* protein but it was not recognized by sera from patients with *Chlamydia trachomatis* infection, who recognised only *Chlamydia pneumoniae* proteins of 73, 68, 65 and 62 kDa[178]. Since the formal identification of the PMP family, work has been ongoing to demonstrate specific responses to these proteins. PMP10 (of the PMPG family) from *Chlamydia pneumoniae* has been shown to be an immunodominant antigen, with antibodies against it being generated during natural disease.[179] In further, recently reported work, an indirect enzyme-linked immunosorbent

assay (ELISA) test was used to determine antibody responses to both MOMP and to a recombinant POMP-91B from *Chlamydia psittaci* OEA, in sheep with experimentally induced abortion. The POMP-based ELISA successfully detected all cases of *Chlamydia psittaci* OEA infection and antibodies became detectable early in the infection. In contrast antibodies to MOMP developed later with a small number of animals never having detectable anti-MOMP antibodies. They concluded that a POMP-based ELISA could be a highly sensitive and specific test of *Chlamydia psittaci* OEA infection in sheep flocks.[180]

It seems clear then that antibodies to the 90-98 kDa antigens appear to be produced in response to chlamydial infection, they have even been implicated in the association between *Chlamydia pneumoniae* and coronary heart disease. Patients with circulating antibodies to the *Chlamydia pneumoniae* 98 kDa antigens appear to have an increased incidence of coronary heart disease with an odds ratio of 2.3 (95% confidence interval 1.3-3.9)[181], whilst investigation of the antibodies bound to circulating immune complexes in coronary heart disease showed them to be specific for 98 and 42 kDa *Chlamydia pneumoniae* proteins[182]. Even more controversially 55% of patients with sarcoidosis were noted to have antibodies to *Chlamydia pneumoniae* 98 kDa antigen compared to 30% of patients with acute respiratory infection. Unfortunately there was no control group and the differences here are not significant[183]. One difficulty of these immunoblotting studies is that there is no standardisation of the antigen quantities used or the dilution of sera, the latter ranging from 1 in 20 to 1 in 200 in the studies quoted here. The results, therefore, must be interpreted with some caution.

Evidence of the importance of the *pmp* gene products in cell-mediated immunity is scarce. pmpD was identified as the target of *Chlamydia*-reactive human T-cell clones from an expression library and the recombinant protein was later shown to stimulate the proliferation of synovial fluid mononuclear cells from individuals with *Chlamydia*-associated reactive arthritis.[184]

COMCs, of which PMPs are a constituent, have already been shown capable of inducing protective immunity. Inoculation of pregnant ewes with a COMC preparation of *Chlamydia psittaci* provided good protection against subsequent abortion due to *C. psittaci* OEA[185] and mice were similarly protected against infection and foetal loss by a similar *Chlamydia trachomatis* COMC preparation, though not by recombinant MOMP[186].

In summary then the *pmp*s compose a multigene family encoding highly immunogenic, surface-localised proteins. These demonstrate species and serovar specific epitopes, making them useful diagnostically, and may generate protective antibodies and hence have potential as vaccine candidates.

1.4.5 Other antigens

The antigens described above are those which have been subjected to the most scrutiny, however a number of others appear to stimulate immune responses and may be potential vaccine candidates though much further work is required.

Two other chlamydial heat shock proteins are recognised by the immune system. Chlamydial HSP10 appears to be associated with chronic chlamydial genital tract infection[187] and is also recognised by antibodies from women with tubal infertility, where serological response correlates with the severity of genital tract disease.[188] Chlamydial HSP70 is thought not to be surface-exposed on chlamydial EBs but is thought to be important in the early stages of infection.[189,190] Despite not being surface-localised, antibodies to HSP70 were found in 65% of 17 patients with chlamydial genital tract disease,[133] and HSP70 has been shown *in vitro* to be a target of neutralising antibodies [119,191]. At least one T-cell epitope capable of stimulating T-cell proliferative responses has been determined[192].

Rockey et al have been working on a group of chlamydial proteins for some time. They have now identified over 40 inclusion membrane proteins (INCs) from each chlamydial species. Five out of six of these from *Chlamydia trachomatis* were shown to reside in the inclusion membrane. Some members of this family of proteins have been shown to be immunogenic in patients recovering from chlamydial genital infection and *Chlamydia pneumoniae* infection[193]. Although several INC proteins are localized to the inclusion membrane, virtually nothing is known of their function. INCA from *Chlamydia psittaci* is a serine-threonine phospho-protein that contacts the cytosol of the host cell. It is possible that the INC proteins may be effector molecules transported out of the chlamydial RB through the Type-III secretion pathway.[194]

Like MOMP, the 38 kDa outer membrane protein PORB appears to function as a porin but is more highly conserved[195]. Antibodies to PORB are capable of neutralising chlamydial infectivity.[196] PorB is predicted to consist of 16 trans-membrane anti-parallel β-sheets with the antigenic regions protruding into the extracellular matrix.

Unlike MOMP, PorB is highly conserved between species and serovars and thus warrants further investigation as a possible vaccine candidate.

CAP1, an inclusion membrane-localised chlamydial protein of unknown function, stimulates the generation of the *Chlamydia*-specific CD_8 T-cells during natural infection of mice with *Chlamydia trachomatis*. A Vaccinia construct including a portion of the CAP1 molecule was able to generate a level of protection from subsequent chlamydial genital infection.[197]

PGP3 is a protein constituent of the elementary body which is encoded on the chlamydial plasmid. 81% of patients with urogenital chlamydial infection had antibodies to PGP3, compared to none in healthy controls with a negative *Chlamydia trachomatis* microimmunofluorescence test (MIF).[198]

A further antigen, macrophage infectivity potentiator (MIP), has recently been identified with similarity to the MIPs of *Legionella pneumophila* and *Coxiella burnetii*. It appears to be localised to the outer membrane, and polyclonal anti-MIP antibodies were able to reduce infectivity of cell cultures in the presence of complement.[199,200]

Chlamydial protease-like activity factor (CPAF) is currently the subject of much interest. CPAF is secreted into the host cytosol by both *Chlamydia trachomatis* and *Chlamydia pneumoniae,*[318,317] where it selectively degrades host transcription factors including RFX5 (a critical component of the RFX transcription complexes required for MHC antigen expression). CPAF seems to be exclusively secreted into the host cytosol with none detectable in either RBs or EBs,[316,317] suggesting that its prime function is to manipulate host cells. Although this poses the risk of CPAF being processed and presented to T-cells, CPAF appears highly stable in the cytosol suggesting poor degradation by host proteosomes.[316] The usefulness of CPAF as a diagnostic or therapeutic target remains to be determined.

1.5 Pathogenesis

A common feature of many chlamydial infections is that they are often asymptomatic and may persist for long periods of time if left untreated. It may be that this inability of the host to clear the chlamydial infection enables the organism to establish a chronic state which eventually leads to the resultant adverse immuno-pathology. It seems that the host responses to that infection may be the primary cause of the long-term sequelae.

Cell-mediated immunity appears to be essential in resolving infection and developing protective immunity to *Chlamydia*, however, it is also thought to be involved in the immunopathogenesis of chlamydial diseases. This view was first mooted after animal experiments which showed that sensitisation of guinea pigs with an extract of chlamydial EBs accelerated ocular inflammation.[201] In a further study with a non-human primate model, follicular conjunctival responses to chlamydial antigens were seen only in previously infected monkeys and not in naive animals, demonstrating the importance of hypersensitivity responses in repeated chlamydial infection.[202] Chlamydial HSP60 has been strongly implicated as being at least one of the antigens responsible for this effect.[203]

Because of the highly conserved nature of HSP60, responses directed against microbial hsp may also be deployed against autologous hsp. The expression of chlamydial *hsp60* is enhanced during persistent infection[33] and this is marked by the appearance of chlamydial HSP60-specific antibodies in the patient serum. Later the development antibodies to human HSP60 may be seen.[204] These cross-reactive responses could convert the protective immune responses into pathological ones.[138] Responses to chlamydial HSP60 have been associated with the sequelae of upper genital tract disease including ectopic pregnancy[205], pelvic inflammatory disease and chronic pelvic pain[204,206,207], perihepatitis[208] and tubal infertility.[209-211] Some, however, argue that the enhanced humoral response to chlamydial HSP60 is simply a consequence of the intense stimulation of the immune system by continuous or recurrent infections. Certainly chlamydial HSP60 has been shown to elicit a severe inflammatory response, almost identical to that seen in trachoma, when inoculated into the conjunctivae of previously immunised guinea pigs and monkeys.[203,212,213]

Chlamydia appear to be potent inducers of inflammatory responses. The infection of both murine and human reproductive tract epithelial cells with *Chlamydia trachomatis* results in the expression of the adhesion molecules Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) and VCAM-1/CD106 which then recruit effector T-cells to the site of inflammation[214,215] and PBMCs infected with *Chlamydia trachomatis* have been shown to produce reactive oxygen species which oxidise cellular membrane lipids.[216]

Whole EBs, MOMP, OMP2 and HSP60 from *Chlamydia pneumoniae*, and probably other *Chlamydia*, stimulate the expression of matrix metalloproteinases and these may also be important in the aetiology of trachomatous scarring. These enzymes are important both

for normal extracellular matrix turnover and for the exaggerated extracellular matrix breakdown associated with pathological conditions including tumour invasion and metastasis, angiogenesis, inflammatory reactions, wound healing and scar formation. The major members of this family include collagenases, gelatinases A and B, and stromolysins [217]. Many chronic inflammatory conditions which result in a restructuring of connective tissue are characterised by a local accumulation of macrophages. These bring about connective tissue destruction either directly, by the secretion of matrix metalloproteinases [218], or indirectly, by the production of pro-inflammatory cytokines, including IL-1 or and TNFα which themselves induce matrix metalloproteinase gene expression. Increased numbers of macrophages have been demonstrated in the conjunctiva of patients with active trachoma and these macrophages were shown to be expressing IL-1, TNFα and platelet-derived growth factor [103]. The production of gelatinase B is also stimulated by bacterial LPS [218]. Gelatinase B, produced by macrophages, monocytes and neutrophils, has been found to be present at increased levels at in the conjunctiva of patients with trachoma [219]. Gelatinase B degrades denatured collagen, collagen types IV, V, VII and XI, elastin and fibronectin so this may be one of the contributing factors towards conjunctival damage and scarring in trachoma.

The nature of the cytokine response to chlamydial infection appears to be important in the development of long term sequelae. Studies using mice that display different levels of TNFα production and correspondingly different pathological outcomes of chlamydial genital infection suggest that whilst TNFα and other inflammatory cytokines may aid in the eradication of *Chlamydia* infection, they may also promote long-term tissue damage.[220] Polymorphisms in the TNFα promoter also appear to be important. These polymorphisms have previously been associated with severe malaria.[221] The TNFα-308A allele (associated with greater TNFα production)[222] is also associated with trachomatous scarring in the Gambia and there is a trend towards association with the TNFα-238A allele[102] but no association with the TNFα-376A allele.[223] The importance of a T_H1 vs. T_H2 response is discussed in section 1.3.4.

The importance of CD_8 T-cells in immunopathology remains controversial. Associations between HLA-A31 and PID[66] and between HLA-A*6802 and scarring trachoma suggest that CD_8 T-cells may be involved in immunopathology. HLA-A28, subtype HLA-A*6802, is significantly more common in those with trachomatous scarring than in controls, suggesting that HLA-A*6802-restricted responses may play a

role in the pathogenesis of trachomatous scarring.[224] Follow-up studies to investigate CTL responses to a number of peptides with the predicted binding motifs for HLA-A*6802, however, failed to demonstrate CTL activity or IFNγ release, suggesting that either HLA-A*6802 CD_8+ T cells may not be important in ocular infections or that the peptides chosen did not represent epitopes.[225]

In summary, it appears that the long-term sequelae associated with chlamydial infection are a combination of direct effects of chronic infection, and 'collateral damage' sustained during the immune response to that infection. The mechanisms of damage are multiple and host genetic factors probably play a significant role.

1.6 Previous vaccine work

Although antibiotics are effective at treating individual episodes, regular treatment is often not available in endemic areas, early infection is often asymptomatic and re-infection occurs rapidly after treatment. Regular face washing has proved moderately effective in reducing the incidence of blinding trachoma[226] but is poorly sustainable. Clearly an effective vaccine would be of enormous benefit in the control of trachoma and would have the potential to prevent millions of cases of blindness in the future from this painful, disabling disease. A number of trachoma vaccine studies took place in the '60s and '70s with mixed results. Preliminary experiments in a variety of primate models demonstrated some short lived protection which was often, though not always, strain specific. In certain cases, however, vaccination appeared to induce hypersensitivity which was not strain specific, and which outlived the protective immunity[227-229]. Results of vaccination trials in children, using whole killed organisms, mirrored these results. Protection lasted less than two years, was partial, and again associated with hypersensitivity responses to infection in some case[230,231]. Laboratory testing of a variety of antigens as potential vaccines has continued and is discussed under the various antigens, however no human trials have taken place since the 1970s due to the risk of hypersensitivity. This combination of protective and damaging immune responses has so far continued to confuse and confound developments towards an effective human vaccine. In contrast a highly successful live vaccine against the *C. psittaci* OEA abortion strain is available in the form of a temperature sensitive mutant[232].

1.7 Locations

1.7.1 London School of Hygiene & Tropical Medicine

Molecular biology work was undertaken at The London School of Hygiene and Tropical Medicine.

1.7.2 Medical Research Council Laboratories, Fajara and Farafenni, The Gambia

Field work was undertaken at the Medical Research Council's main laboratories at Fajara and at the field station at Farafenni.

Since 1981 the MRC has operated a continuous demographic surveillance system in 40 villages and hamlets in the Farafenni area, on the north bank of the river Gambia and a number of studies are being undertaken in the field of reproductive health. Women for the infertility arm of the study and their controls were recruited from this population.

1.7.3 Study villages, The Gambia

Individuals were recruited from a number of villages. These are listed in detail in section 4.2. Long-term studies of trachoma have been ongoing in the villages of Berending (in south-western Gambia) and in Jali (close to the Kiang West National Park in central Gambia). Ninety-three individuals were recruited from these two villages. The remainder were recruited from other villages in the central region. These villages are underlined on the map overleaf.

Figure 1-4: Map of The Gambia showing study villages

1.8 Aims of the project

- To clone and express PMP protein antigens from *Chlamydia trachomatis* serovar A.
- To assess the immunogenicity of these proteins and develop a whole blood cytokine assay for field use.
- To assess the immune responses of selected groups of trachoma patients and controls in an endemic area to determine those responses associated with protection from blinding trachoma.
- To assess the immune responses of women with secondary tubal infertility and controls in order to determine if responses are similar to those with scarring trachoma
- To compare responses in those with and without current helminth infection.

The following hypotheses will be addressed:

- That individuals with chlamydial salpingitis and with ocular trachoma will demonstrate humoral or cell mediated responses, or both, to the PMP proteins.
- That trachoma patients with a predominantly T_H1-type response will suffer less severe pathology than those with either a reduced T_H1 or a predominantly T_H2-type response.
- That individuals with concurrent helminth infection will demonstrate either a reduced T_H1 or a predominantly T_H2-type response.

The long term benefits of this project are:

- That the characterisation of immune responses to these proteins may give some insight into their cell biology and may identify those antigens which may be useful diagnostically or as new vaccine candidates.

Chapter Two

Materials and Methods

2 Materials and Methods

2.1 Cloning

2.1.1 Preparation of *Chlamydia trachomatis* serovar A DNA

2.1.1.1 Purification of *Chlamydia trachomatis* serovar A DNA

Chlamydia trachomatis serovar A EBs were kindly provided by Dr Rosanna Peeling (Laboratory Centre for Disease Control, Winnipeg, Manitoba, Canada). This strain had been isolated from the eye of a patient in The Gambia with trachoma, and propagated in tissue culture in Dr Peeling's laboratory. The EB's were provided in a suspension of 700 micrograms per millilitre (µg/ml) EB protein and stored at –70^{O}C.

One millilitre EB suspension was transferred to a 14ml round-bottom polyethylene tube and centrifuged at 7840g for 30 minutes at 4^{O}C. The supernatant was removed and the pellet re-suspended in 200 microlitres (µl) TES (20% w/v sucrose, 50 millimolar (mM) Tris-HCl pH 7.6, 50mM ethylenediaminetetracetic acid (EDTA) – Sigma, Poole, UK) and transferred to a 2ml microfuge tube. 20µl 10% sodium dodecyl sulphate (SDS) and 4µl proteinase K (20 milligrams per millilitres (mg/ml) - Promega, Southampton, UK) was added to the suspension and incubated for one hour at 65^{O}C. 200µl distilled/deionised water (dH_2O) was added followed by 400µl of cold 100% isopropanol (Sigma). The solutions were mixed by gentle inversion and the precipitated DNA threads spooled onto a glass rod, transferred to 100µl TE buffer (10mM Tris.Cl, 1mM EDTA, pH 7.5) and solubilized by incubation at 65^{O}C for 15 minutes. The supernatant was then spun at 16,000g for 30 minutes at 4^{O}C after which a very small pellet was visible. This pellet was solubilized in 20µl TE buffer and the two DNA solutions combined.

Ribonucleic acid (RNA) was removed by the addition of 2µl ribonuclease A (RNAse A - Promega) and incubation for 45 minutes at 37^{O}C. 120µl phenol/chloroform (50:50 v/v) was then added and mixed by gentle inversion. The solution was centrifuged at 16,000g for 10 minutes at 4^{O}C and the aqueous phase transferred to a fresh microfuge tube. 120µl dH_2O was added to the organic phase and mixed by gentle inversion. The mixture was centrifuged at 16,000g for 10 minutes at 4^{O}C and the aqueous phase added to that

obtained with the first extraction. Finally, 260µl isopropanol was added and mixed by gentle inversion. The precipitated DNA strands were spooled onto a glass rod, transferred into 100µl TE buffer pH 8.5 and solubilized by incubation at 65^{O}C for 15 minutes.

The concentration and purity of the DNA was estimated as detailed below. Remaining organic solvents were then removed by ether extraction; 100µl water-saturated ether was added to the DNA solution and mixed by gentle inversion. The two phases were allowed to separate by standing for 5 minutes and the ether phase removed. This process was repeated three times. Spectrophotometry was repeated to estimate concentration and quality.

2.1.1.2 Spectrophotometric estimation of concentration, yield and purity of DNA

The concentration and purity of the DNA was estimated by spectrophotometry measuring the absorbance of a 100-fold dilution at 260 nanometre (nm) and 280nm wavelengths. The DNA concentration was calculated on the basis that an optical density (OD) of 1.0 at 260nm corresponds to approximately 50µg/ml of double-stranded DNA. The purity was estimated from the ratio of the OD at 260nm and at 280nm with pure DNA preparations being assumed to have an OD_{260}/OD_{280} ratio of 1.8 – 2.0.

2.1.1.3 Digestion of chlamydial DNA with restriction enzymes

In order to confirm that genomic DNA was present, digestion was performed with the restriction enzymes *Bam*H1, *Eco*R1 and *Pst*1. For each reaction, 2µl of the DNA solution prior to ether extraction (900 nanograms (ng)) was added to 15µl dH_2O, 2µl enzyme buffer and 20 units restriction enzyme (Promega) in a 500µl microfuge tube. The mixture was incubated overnight. 2µl ten-times strength (10X) agarose gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 25% Ficoll 400) was added to each tube and mixed thoroughly. The mixture was then electrophoresed in a 0.8% agarose (Promega) gel containing 0.5µg/ml ethidium bromide (Sigma) using TAE buffer (0.04M Tris.acetate, 0.001M EDTA) at 96 volts for 90 minutes with a λ/*Pst*1 marker, and the products visualised under 254nm ultraviolet light irradiation.

2.1.2 PCR Amplification

The gene sequences of *pmpC*, *pmpD* and *pmpG* as described on the *Chlamydia trachomatis* serovar D Sequencing Project at http://chlamydia-www.berkeley.edu:4231/ were examined (Genbank accession numbers: *pmpD* – 15605546, *pmpG* – 15605607). Signal P, available online at http://www.cbs.dtu.dk/services/SignalP and SMART, from the European Molecular Biology Laboratories at http://smart.embl-heidleberg.de, were used to identify signal sequences in the translated amino acid sequence. Corresponding DNA sequences were then input to the Primer 3 program, available online through the Whitehead Institute for Biomedical Research/MIT Centre for Genome Research at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi, to suggest potential primers. Primer sequences were selected to exclude the signal sequences and examined to exclude any potential secondary structures, which might lead to amplification artefacts or errors. The final choice of primers is detailed in the table.

Primer	Oligonucleotide Sequence (5'-3')	Target	Position	Description
DpmpD-3'	TCG CAA TCC AGT ATT CGC CTC A	pmpD	4596-4617	4450bp product used to produce pTrcHis/pmpD-1218
DpmpD-5'	TGG TAT ATG TAG GCC CTC AAG CGG		167-190	
DpmpG-3'	TTC CTG CAC TCA AAC CAT AAC CTC G	pmpG	3028-3052	2925bp product used to produce pTrcHis/pmpG-1410
DpmpG-5'	TCA AGG AAT TTA CGA TGG GGA GAC G		126-150	
DpmpG2-5'	CAA GGA ATT TAC GAT GGG GAG A	pmpG	127-148	2924 bp product used to produce pBAD/pmpG
DpmpG2-3'	TCC TGC ACT CAA ACC ATA ACC		3031-3051	

Table 2-1: Primer sequences used for PCR

2.1.2.1 Clontech Advantage® PCR

PCR amplification was undertaken using the Advantage PCR Kit (Clontech, Basingstoke, UK) according to the manufacturers instructions. The amplification mix consisted of 1x manufacturer's reaction buffer (40mM Tris-HCl, 15mM potassium acetate (KOAc)), 1x Advantage Genomic PCR polymerase mix (0.1 units/µl *Tth* DNA polymerase, 0.01µg/ml TthStart antibody, 1% glycerol, 0.2mM Tris-HCl, 4.6mM KCl, 1.5µM EDTA, 15µM dithiothreitol, 7.3µg/ml bovine serum albumin), 0.2mM each deoxynucleotide triphosphate (dNTP), 1.1mM magnesium acetate ($Mg(OAc)_2$), 1µM each primer and 40ng *Chlamydia trachomatis* serovar A DNA in a 100µl reaction volume. The mixture was overlaid with 30µl of mineral oil (Sigma). Cycling consisted of denaturation at 94^{O}C for 1 minute then 35 cycles of 30 seconds denaturation at 94^{O}C and 3 minutes annealing/extension at 68^{O}C, then 3 minutes final extension at 68^{O}C, and was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA).

PCR product of the predicted size was visualised on an ethidium-stained agarose gel (1% agarose (Sigma), 0.004% ethidium bromide (Sigma)) by comparison with a λ/*Pst*1 marker on exposure to UV light at 254nm

2.1.2.2 Hi-fidelity Pfu amplification

Pfu DNA polymerase (Promega) is a high-fidelity DNA polymerase claimed to have an error rate of only 1 x 10^{-6} per base.[233] Since the PCR products generated ranged from 3-5 kb we would thus anticipate 0.003 – 0.005 errors to occur within the PCR product. The reaction mixture comprised 1X Pfu buffer (Promega), 0.2mM each dNTP (Boehringer, Ingelheim, Germany), 1μM each primer, 20ng *Chlamydia trachomatis* serovar A DNA and 2.5 units Pfu DNA polymerase (Promega) in a 50μl reaction volume. The mixture was overlaid with a drop of mineral oil (Sigma). Cycling consisted of denaturation at 95^{O}C for 2 minutes then 35 cycles of 45 seconds denaturation at 95^{O}C, 30 seconds annealing at 55^{O}C and 5 minutes extension at 72^{O}C, then 5 minutes final extension at 72^{O}C, and was performed in a DNA thermal cycler (Perkin Elmer Cetus). In order to add overhanging single adenine nucleotides to each strand of the blunt PCR product, following completion of amplification, 2.5 units of Taq DNA polymerase was added and a further 10 minutes incubation undertaken at 72^{O}C. PCR product of the predicted size was visualised on an ethidium bromide-stained agarose gel by comparison with a λ/*Pst*1 marker by exposure to UV light at 254nm.

2.1.3 Purification of PCR products

2.1.3.1 Ethidium bromide gel electrophoresis

2μl 10X agarose gel loading buffer was added to the entire aqueous phase of the PCR reaction and mixed thoroughly. The mixture was then electrophoresed in a 0.8-1% low melting point agarose (Promega) gel containing 0.5μg/ml ethidium bromide using TAE buffer (0.04M Tris.acetate, 0.001M EDTA) at 96 volts for 90 minutes with a λ /*Pst*1 marker and the products visualised under 254 nm UV light. Exposure to UV light was kept to a minimum to reduce radiation damage to the double-stranded product. Amplimers of the appropriate size were excised using a clean scalpel blade and transferred to a polyethylene microfuge tube and the DNA extracted using either Qiacx II DNA extraction kit® (Qiagen, Dorking, UK) or Invitrogen Snap columns®.

2.1.3.2 Crystal violet gel electrophoresis

In the later stages of the project, crystal violet gels were used for the visualisation of PCR products, restriction fragments and vectors and for isolation prior to cloning. Crystal violet gels allow direct visualisation of the DNA bands and obviates the requirement for ethidium bromide and UV light both of which may damage DNA[234].

5µl 10X crystal violet loading buffer (2% Ficoll 400, 0.002% xylene cyanol) was added to the entire aqueous phase of the PCR reaction mix and this was then loaded into a 1% low melting point agarose (Promega) gel containing 10µg/ml crystal violet (BDH, Poole, UK). A λ/*Pst*1 marker was used to determine the molecular weights. This was then electrophoresed for 1 hour at 97v in TAE buffer and the bands visualised directly. Amplimers of the appropriate size were excised using a clean scalpel blade and transferred to a polyethylene microfuge tube and the DNA extracted using either Qiaex II DNA extraction kit® or Invitrogen Snap columns® using the manufacturers recommended protocols as below.

2.1.3.3 Qiaex II DNA extraction kit

300µl QX1 solubilization buffer (proprietary formulations not available) was added to the gel fragment and incubated at 50^{O}C until the gel had melted. The Qiaex II® beads were re-suspended by vortexing for 30 seconds then 10µl of the suspension added to the melted agarose solution. The mixture was incubated at 50^{O}C for 10 minutes with gentle vortexing every 2 minutes to maintain the suspension. The beads were then pelleted by centrifugation at 16,000g for 2 minutes and the supernatant removed and discarded. The pellet was washed by re-suspending in 500µl QX1 buffer and then centrifugation at 16,000g; the supernatant was discarded and the pellet washed twice, in the same way, with 500µl PE wash buffer. The pellet was allowed to air-dry for 15 minutes. The DNA was then eluted from the beads by re-suspension in 20µl 10mM TE buffer (pH 8.5) followed by centrifugation for 2 minutes at 16,000g with collection of the supernatant. The process was then repeated with a further 10µl 10mM TE buffer (pH 8.5) to increase the total yield of DNA. The two supernatants were then combined and stored at –20^{O}C.

2.1.3.4 Invitrogen Snap Columns®

Two hundred and fifty microlitres of 6.6 molar (M) sodium iodide (Invitrogen, Groningen, The Netherlands) was added to the excised gel fragment and mixed by

gentle inversion. The mixture was then incubated for two minutes at 50^{O}C until the gel had melted. Proprietary binding buffer (525µl) was then added and mixed thoroughly. The solution was then loaded into a Snap® column and centrifuged at 2000g. The flow-through was poured back into the top of the column and the process repeated twice. The column was then washed twice by the addition of 400µl 1X proprietary final wash buffer and centrifugation at 2000g. After the second wash, the column was dried by centrifugation at 16,000g for one minute. The column was placed in a fresh microfuge tube and 40µl TE buffer (pH 8.5) added. The column was spun for 1 minute at 16,000g and the eluted DNA stored at –20^{O}C.

2.1.4 Preparation of vectors and inserts for ligation

2.1.4.1 pGEM-T®

The cloning vector pGEM-T (Promega) is designed for easy cloning of PCR products through the presence of single 3'-thymydine overhangs. The vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-complementation peptide coding region of β-galactosidase, which enables blue/white colony selection of recombinant clones. As pGEM-T is designed for direct cloning of PCR products, no vector preparation was required.

2.1.4.2 pTrcHis®

The expression vector pTrcHis (Invitrogen) is designed for the expression of non-toxic gene products in *E. coli*. Expression is under the control of the *trc* promoter (a hybrid promoter derived from the *trp* and *lac* promoters). Expressed products have an N-terminal polyhistidine (6xHis) tag to facilitate purification and an N-terminal Xpress epitope for easy detection with the manufacturer's anti-Xpress antibody. pTrcHis is supplied in circular form, it was therefore digested with the appropriate restriction enzyme(s) and purified either by ethidium bromide gel electrophoresis and Qiaex II® extraction of vector DNA from the gel or by the phenol/chloroform method. Where a single enzyme was used to cut the vector, the 5' termini were de-phosphorylated as described in section 2.1.4.8.

2.1.4.3 pBluescript® II phagemid (SKII)

pBluescript II (Stratagene, Amsterdam, The Netherlands) is a high copy number, ColE1-based phagemid with a large polylinker, an f1 origin available in either (+) or (-) orientation and T3 and T7 promoters for *in vitro* RNA transcription. Recombinant selection is facilitated by ampicillin resistance and blue/white colony colour selection. Bluescript II is supplied in circular form; it was therefore digested with the appropriate restriction enzyme(s) and purified by the phenol/chloroform method (section 3.1.1.1). Where a single enzyme was used to cut the vector, the 5' termini were de-phosphorylated as described below.

2.1.4.4 pBAD-TOPO TA®

The expression vector pBAD-TOPO TA (Invitrogen) is designed for direct cloning of PCR products through the presence of single 3'-T overhangs. Ligation is achieved rapidly using topoisomerase, which is bound to the vector. Expression in *E. coli* is driven by the *ara*BAD promoter. Low levels of transcription occur in the absence of arabinose and the level of expression is arabinose dose-dependent allowing accurate titration of the level of expression. pBAD is designed for direct cloning of PCR products, therefore no vector preparation was required.

2.1.4.5 pCR-II-TOPO®

The cloning vector pCR-II-TOPO (Invitrogen) is designed for direct cloning of PCR products through the presence of single 3'-T overhangs. Ligation is achieved rapidly using topoisomerase, which is bound to the vector. It incorporates kanamycin and ampicillin resistance, blue/white colony selection through disruption of the *lacZα* gene, M13 forward and reverse priming sites for insert sequencing and T7 promoter/primer for *in vitro* RNA transcription/translation. pCR-II is designed for direct cloning of PCR products and therefore no vector preparation was required.

2.1.4.6 PET-30®

The expression vector pPET (Novagen, Nottingham, UK) ensures that target genes are cloned under the control of strong bacteriophage T7 transcription/translation signals and thus expression is induced by the T7 RNA polymerase of the expression host. Cloning in hosts that do not contain T7 RNA polymerase renders the cloned gene transcriptionally silent thus eliminating plasmid instability due to the expression of

proteins potentially toxic to the host cell. During the expression phase, T7 RNA polymerase is highly selective and active such that almost all of the cell's resources are converted to target gene expression; the target protein may compose more than 50% of the total cell protein after a few hours of induction. pPET-30 is supplied in circular form; it was therefore digested with the appropriate restriction enzyme(s) and purified by the phenol/chloroform method. Where a single enzyme was used to cut the vector, the 5' termini were de-phosphorylated as described below.

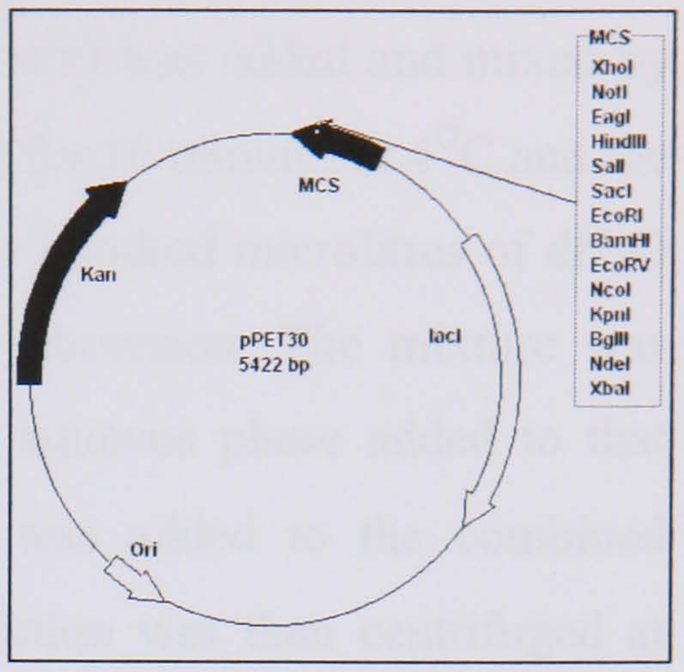


Figure 2-1: PET-30a expression vector

2.1.4.7 Isolation of restriction fragments for use as inserts

1.25µg of plasmid, containing the restriction fragment of interest, was digested with 10-20 units restriction enzyme, 1.5µl manufacturer's restriction enzyme buffer and dH_2O in a final volume of 15µl for one hour at $37^{O}C$. Following digestion the restriction fragments were separated using either ethidium bromide or crystal violet gel electrophoresis as described above. DNA bands of the appropriate size were excised using a clean scalpel blade and transferred to a polyethylene microfuge tube and the DNA extracted using the Qiaex II DNA extraction kit®. An assumption was made that 80% of the original DNA would be extracted using this method.

2.1.4.8 De-phosphorylation of vector or insert termini prior to cloning

De-phosphorylation of the 5'-termini of vectors or inserts was performed to prevent re-linking of compatible vector termini or the formation of concatemers by the linkage of several inserts together. De-phosphorylated termini can only be joined by the action of DNA ligase.

DNA (1.25µg) was added to 100µl 1X calf intestinal alkaline phosphatase buffer (10mM NaCl, 5mM Tris-HCl, 1mM $MgCl_2$, 0.1mM dithiothreitol - New England Biolabs, Hitchin, Herts, UK.) and 1µl calf intestinal alkaline phosphatase (New England Biolabs). The mixture was incubated at $37^{O}C$ for 30 minutes. 1µl shrimp alkaline phosphatase was then added and incubated for a further 30 minutes to ensure that de-phosphorylation was complete. The mixture was then heated to $65^{O}C$ for 15 minutes to

inactivate the enzymes. 100µl phenol/chloroform (50:50 v/v) was added and mixed by gentle inversion. The solution was centrifuged at 16,000g for 10 minutes at 4^{O}C and the aqueous phase transferred to a fresh microfuge tube. One hundred microlitres of dH_2O was added to the organic phase and mixed by gentle inversion. The mixture was centrifuged at 16,000g for 10 minutes at 4^{O}C and the aqueous phase added to that obtained with the first extraction. 200µl isopropanol was added to the combined aqueous phase and mixed by gentle inversion. The solution was then centrifuged at 16,000g for 15 minutes. The supernatant was removed and discarded and the DNA pellet washed with 500µl of 70% ethanol (Sigma) then centrifuged at 16,000g for 5 minutes. The supernatant was discarded and the pellet spun at 16,000g for a further 1 minute. The remaining supernatant was removed and discarded and the pellet allowed to air dry. The DNA pellet was re-suspended in 20µl 10mM TE buffer pH 8.5. An arbitrary assumption was made that 80% of the DNA would be recovered. Thus, the concentration of the DNA solution was taken to be 50ng/µl.

2.1.4.9 Filling in 5' single stranded extensions with Klenow fragment of DNA polymerase

Klenow fragment of DNA polymerase was used to fill in 5' single stranded extensions of double-stranded DNA to provide blunt ends for cloning.

After restriction enzyme digestion the restriction enzyme was de-activated either by heating to 65^{O}C for 10 minutes or by phenol/chloroform extraction of the DNA. 1µl dNTPs, 0.2µl bovine serum albumin (BSA - Promega) and 2.5 units Klenow fragment (Promega) was added and the mixture incubated for 10 minutes at room temperature. If further digestion of the DNA was required, the Klenow fragment was heat-inactivated at 75^{O}C for 10 minutes.

2.1.5 Ligation

2.1.5.1 Calculation of vector/insert ratio

For ligation the following formula was used to calculate the amount of insert required for a given quantity of vector. In most cases a vector: insert ratio of 1:3 was used.

$$\left(\frac{ng\ of\ vector \times size\ of\ insert\ (kb)}{size\ of\ vector\ (kb)}\right) \times insert : vector\ ratio = ng\ of\ insert\ required$$

2.1.5.2 pGEM-T®

One microlitre of PCR product was mixed with 1µl T4 DNA ligase buffer (30mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 10mM DTT, 1mM ATP - Promega), 1µl (10 units) T4 DNA ligase (Promega), 25ng pGEM-T vector and 6.5µl dH_2O. The mixture was incubated overnight at 4^OC.

2.1.5.3 pTrcHis®

The vector: insert ratio was calculated as above. The appropriate quantity of insert was added to 10ng appropriately digested pTrcHis vector in a 10µl solution containing 1µl T4 ligase buffer and 10 units T4 DNA ligase. The mixture was incubated overnight at 4^OC.

2.1.5.4 pBluescript II®

The vector: insert ratio was calculated as above. The appropriate quantity of insert was added to 10ng appropriately digested pTrcHis vector in a 10µl solution containing 1µl T4 ligase buffer and 10 units T4 DNA ligase. The mixture was incubated overnight at 4^OC.

2.1.5.5 pBAD-TOPO®

Two microlitres of PCR product was mixed with 0.5µl (5ng) pBAD-TOPO and 1.5µl dH_2O. The mixture was incubated at room temperature for 5 minutes then transferred to ice and used for immediate transformation.

2.1.5.6 pCR-II-TOPO®

Four and a half microlitres of PCR product was mixed with 0.5µl (5ng) pCR-II-TOPO. The mixture was incubated at room temperature for 5 minutes then transferred to ice and used for transformation as soon as possible.

2.1.5.7 pPET®

The vector: insert ratio was calculated as above. The appropriate quantity of insert was added to 10ng appropriately digested pPET vector in a 10µl solution containing 1µl T4 ligase buffer and 10 units T4 DNA ligase. The mixture was incubated overnight at 4^OC.

2.1.5.8 Preparation of competent cells

Laboratory stocks of the appropriate strain, stored at –70^{O}C, were streaked out onto Luria-Bertani (LB) agar (1% w/v enzymatic casein digest, 0.5% w/v yeast extract, 0.5% sodium chloride (NaCl), 1.5% agar - Difco/BD, Oxford, UK) plates and incubated overnight at 37^{O}C. A single colony was then selected and used to inoculate 5ml sterile SOB medium (2% w/v bacto-tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 2.5mM potassium chloride (KCl), 10mM magnesium chloride ($MgCl_2$)) which was incubated on a shaking water bath overnight at 37^{O}C. One millilitre of this culture was then used to inoculate 100ml SOB medium and incubated on a shaking water bath at 37^{O}C with hourly monitoring of the OD_{550}. When the culture reached an OD_{550} of 0.55 – 0.65 the solution was transferred to centrifuge bottles and incubated on ice for 30 minutes before being centrifuged at 6174g. The supernatant was discarded and the bacterial pellet re-suspended in 28ml ice-cold simple frozen storage (SFS) buffer (100mM KCl, 50mM calcium chloride ($CaCl_2$), 10mM KOAc, 10% w/v glycerol). The suspension was incubated on ice for 15 minutes then centrifuged at 6174g for 15 minutes. The supernatant was discarded and the bacterial pellet re-suspended in 6.4ml ice-cold SFS buffer. The suspension was then transferred to sterile cryopreservation tubes in 200µl aliquots and frozen in liquid nitrogen. The aliquots were stored at –70^{O}C.

In order to test the efficiency of transformation of the competent cells, an aliquot was transformed with 1ng of the super-coiled plasmid pT7-7 according to the standard protocol described below. The whole transformation volume was plated onto an LB agar plate containing 100µg/ml ampicillin (Sigma) and incubated overnight at 37^{O}C. The number of colonies was counted and the transformation efficiency calculated using the formula:

$$transformation\ efficiency = \left(\frac{volume\ plated}{total\ volume} \right) \times number\ of\ colonies \times 1000$$

2.1.6 Transformation into competent cells

2.1.6.1 Competent cells used

XL1-Blue: A recombination-deficient strain that will support the growth of vectors carrying some amber mutations, but not those with the Sam100 mutation. Transfected

DNA is modified but not restricted. The F' in this strain allows blue/white colony screening[235] and the strain is also tetracycline resistant.

Invitrogen Top10 One-Shot®: A high-efficiency cloning strain of *E. coli* capable of cloning efficiencies as high as 1 x 10^9.[233]

BL21: A strain employed for high-level expression of genes cloned into expression vectors containing the bacteriophage T7 promoter. Bacteriophage T7 RNA polymerase is carried on the bacteriophage λ DE3, which is integrated into the chromosome of BL21[236].

2.1.6.2 Transformation into XL1-Blue

Aliquots of XL1-Blue competent cells were thawed on ice. 10µl of ligation mix was diluted with 200µl ice-cold SFS buffer in a glass tube. 200µl suspension of competent cells was added to the diluted ligation mix and incubated on ice for 10 minutes. The mixture was then subjected to a heat-shock by immersion in a water bath at 42^{O}C for 60 seconds then transferred immediately to ice and left for 5 minutes. 1ml SOC medium (2% w/v bacto-tryptone, 0.5% w/v yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM magnesium sulphate ($MgSO_4$), 20mM glucose) at 37^{O}C was then added and the mixture incubated in a rotating incubator at 37^{O}C for 1 hour. After incubation, the mixture was transferred to a 1.5ml polyethylene microfuge tube and centrifuged at 10,000g for 30 seconds. 1200µl of the supernatant was removed and discarded and the bacteria re-suspended in the remaining 200µl supernatant. The suspension was then plated onto agar plates containing appropriate antibiotics together with the indicator sugar derivative 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma).

2.1.6.3 Transformation into Top10 One-Shot® competent cells

A vial of Top 10 One-Shot® cells was removed from storage at –70^{O}C and thawed on ice. 2µl 0.5M β-mercaptoethanol (Invitrogen) was gently stirred into the competent cells. 2µl ligation mix was added and incubated on ice for 30 minutes. The cells were then heated, in a water bath, to 42^{O}C for 30 seconds and returned to ice for two minutes. 250µl SOC medium was added and the cells incubated in a rotating incubator at 37^{O}C for 30 minutes after which the suspension was mixed by gentle vortexing then plated out, in aliquots of 20, 40 and 200µl, onto agar plates containing appropriate antibiotics.

2.1.6.4 Transformation into BL21

Aliquots of BL21 competent cells were thawed on ice. 10ng of purified expression construct DNA was diluted with 200µl ice-cold SFS buffer in a glass tube. 200µl suspension of competent cells was added to the diluted ligation mix and incubated on ice for 10 minutes. The mixture was then subjected to a heat-shock by immersion in a water bath at 42^{O}C for 60 seconds then transferred immediately to ice and left for 5 minutes. 1ml SOC medium at 37^{O}C was then added and the mixture incubated on a rotating incubator at 37^{O}C for 1 hour. After incubation, the mixture was transferred to a 1.5ml polyethylene microfuge tube and centrifuged at 10,000g for 30 seconds. 1200µl of the supernatant was removed and discarded and the bacteria re-suspended in the remaining 200µl supernatant. The suspension was then plated onto agar plates containing appropriate antibiotics.

2.1.7 Screening of transformants

2.1.7.1 Screening by antibiotic selection

Screening of bacterial colonies containing recombinants was facilitated through the use of antibiotic resistance encoded within the vector as detailed in the table. LB agar containing the appropriate antibiotic was used for the initial plating of transformants and for subsequent maintenance of the plasmid-bearing strains.

Vector	Antibiotic resistance
pBAD	Ampicillin
pBluescript	Ampicillin
pCRII	Ampicillin + kanamycin
pGEM-T	Ampicillin
pPET	Kanamycin
pTrcHis	Ampicillin

Table 2-2: Plasmid antibiotic resistance

2.1.7.2 Blue/white screening for lacZ

Following transformation of plasmids bearing the *lacZ* gene the transformation mix was plated out onto agar containing the appropriate antibiotic(s) together with X-gal and incubated overnight at 37^{O}C. Bacterial colonies not containing an insert were identified by their blue colouration due to their ability to metabolise the X-gal, through α-complementation to a blue coloured pigment. Conversely, those bacteria containing an

insert, which disrupts the *lacZ* gene, were unable to metabolise X-gal and therefore remained white. White colonies were transferred to a fresh agar plate, containing the appropriate antibiotic(s) together with X-gal, in a gridded pattern to allow identification. These plates were then incubated overnight to confirm the colour selection.

Plasmids allowing blue/white selection were: pGEM-T, pBluescript II and pCR-II-TOPO.

2.1.7.3 Quick plasmid preparation

This method was used as a rapid method of isolating DNA from overnight cultures of plasmid-bearing bacteria. The method was used to obtain DNA for restriction enzyme analysis but not for cloning or other procedures.

Individual colonies were selected from the gridded colony plates and inoculated into glass tubes containing 2ml of LB broth with the appropriate antibiotic(s) – see Table 2.2. The cultures were incubated overnight at 37^{O}C in a shaking incubator. 1.5ml of the culture was transferred to 1.5ml polyethylene microfuge tubes and centrifuged at 16,000g for 30 seconds. The supernatant was discarded and the bacterial pellet re-suspended in 110µl STET buffer (0.1M NaCl, 10mM Tris.Cl (pH 8), 1mM EDTA (pH 8), w/v 5% Triton X-100) containing 500µg/ml lysozyme (Sigma). The mixture was incubated for 10 minutes at room temperature then boiled for 2 minutes in a water bath. After boiling the mixture was cooled on ice, then centrifuged at 16,000g for 15 minutes. The pellet, containing cell debris and denatured protein, was then removed with a toothpick. 110µl 5M lithium chloride (LiCl - Sigma) was then added, mixed by gentle inversion and incubated on ice for 15 minutes to precipitate the RNA. The solution was then centrifuged at 16,000g for 15 minutes and the supernatant transferred to a clean microfuge tube. 550µl of absolute ethanol (Sigma) was then added and mixed by gentle inversion. The solution was then centrifuged at 16,000g for 15 minutes and the supernatant discarded. The DNA pellet was solubilized in 35µl 10mM TE buffer pH 8.5 and the solution stored at –20^{O}C.

2.1.7.4 Restriction enzyme analysis of plasmid DNA

Screening of white colonies for the presence of the insert was performed on plasmid DNA extracted by the 'quick plasmid preparation' protocol described above (section 2.1.7.3). Restriction enzyme analysis was performed on these plasmid preparations by

digesting 3µl DNA with 10 units of appropriate restriction enzyme, 1.5µl manufacturer's restriction enzyme buffer and dH_2O in a total volume of 15µl in a sealed 96 well micro-titre plate. Digestions were allowed to progress at 37^{O}C for 2 hours. 1.5µl 10X gel loading buffer was then added to each restriction mix and the entire volume loaded into the well of a 10 x 10cm 1% w/v agarose (Promega) gel, which was then electrophoresed at 97 volts (V) with TAE buffer for 1 to 1.5 hours until adequate separation of the fragments had been achieved. A *λ/Pst*1 marker was used to allow estimation of the size of fragments which were visualised under 254 nm UV light and recorded with a Polaroid® Gel Documentation camera.

2.1.8 Purification and archiving of plasmid constructs and plasmid-bearing bacterial strains

2.1.8.1 Plasmid purification – QIAwell 8® kit

The QIAwell 8® kit (Qiagen) is a proprietary anion-exchange column designed for the vacuum-driven isolation of ultra-pure plasmid DNA from minipreps. Five millilitres of LB broth was inoculated with a single colony of the plasmid-bearing strain and incubated overnight in a shaking incubator at 37^{O}C. The broth was transferred to a microfuge tube and centrifuged at 16,000g for 1 minute. The supernatant was discarded and the pellet re-suspended in 300µl P1 re-suspension buffer (proprietary formulations not available). 300µl P2 lysis buffer was then added, mixed by inversion and incubated for 5 minutes at room temperature. 300µl chilled P3 neutralisation buffer was then added and mixed by gentle inversion. The solution was incubated on ice for 10 minutes, re-mixed, and then centrifuged at 16,000g for 15 minutes. The supernatant was then applied to a QIAwell 8 column in the manufacturer's manifold and suction applied. The columns were washed twice by the addition of 1ml QW wash buffer under suction. A clean 1.2ml collection tube was then placed under the column and the DNA eluted by the addition of 500µl QF elution buffer and the application of suction. The eluate was transferred to a fresh 1.5ml microfuge tube and 350µl 100% isopropanol added and mixed thoroughly. The mixture was then centrifuged at 16,000g for 30 minutes. The supernatant was removed and discarded and the DNA pellet washed with 1ml 70% ethanol (Sigma) then centrifuged at 16,000g for 5 minutes. The supernatant was discarded and the pellet spun at 16,000g for a further 1 minute. The remaining supernatant was removed and discarded and the pellet allowed to air dry. The DNA

pellet was re-suspended in 50µl 10mM TE buffer pH 8.5. DNA concentration, yield and purity were measured by spectrophotometry as described (section 2.1.1.2) and the DNA frozen in 25µl aliquots at –20^{O}C.

2.1.8.2 Plasmid purification – Plasmid Midi Kit®

The Plasmid Midi Kit® (Qiagen) is a proprietary anion-exchange column designed for the gravity-driven isolation of ultra pure plasmid DNA from 25-100ml cultures. Twenty five millilitres of LB broth was inoculated from a single colony of the plasmid-bearing strain and incubated overnight in a shaking incubator at 37^{O}C. The broth was transferred to a 40ml centrifuge tube and centrifuged at 3951g for 5 minutes at 4^{O}C. The supernatant was discarded and the pellet re-suspended in 4ml P1 re-suspension buffer (proprietary formulations not available). 4ml P2 lysis buffer was then added, mixed by inversion and incubated for 5 minutes at room temperature. 4ml chilled P3 neutralisation buffer was then added and mixed by gentle inversion. The solution was incubated on ice for 15 minutes, re-mixed, and then centrifuged at 17,640g for 30 minutes. Meanwhile a Qiagen-tip 100 was equilibrated with 4ml QBT equilibration buffer. The supernatant was then applied to the Qiagen-tip 100 and allowed to drain through by gravity. The columns were washed twice by the addition of 10ml QC wash buffer. A clean 14ml centrifuge tube was then placed under the column and the DNA eluted by the addition of 5ml QF elution buffer. 3.5ml cold isopropanol was added to the eluate and mixed thoroughly. The mixture was then centrifuged at 17,640g at 4^{O}C for 30 minutes. The supernatant was removed and discarded and the DNA pellet washed with 5ml 70% ethanol then centrifuged at 11,290g for 10 minutes. The supernatant was discarded and the pellet spun at 16,000g for a further 1 minute. The remaining supernatant was removed and discarded and the pellet allowed to air dry. The DNA pellet was re-suspended in 100µl 10mM TE buffer pH 8.5. DNA concentration, yield and purity were measured by spectrophotometry as described (section 2.1.1.2) and the DNA frozen in 50µl aliquots at -20^{O}C.

2.1.8.3 Freezing of plasmid-bearing bacterial strains

Two microlitres of LB broth was inoculated from a single colony of the plasmid-bearing strain and incubated overnight in a shaking incubator at 37^{O}C. The following morning 800µl LB broth/glycerol (50:50 v/w) was added and mixed by gentle vortexing. The mixture was then divided into three aliquots and frozen in liquid nitrogen, dry ice or a

commercial freezing box (Nalgene, Rochester, NY, USA) at –70^{O}C. The frozen strains were stored at –70^{O}C.

2.1.9 Individual cloning strategies

2.1.9.1 Cloning of pTrcHis/pmpG-1410

pmpG DNA was amplified using the Clontech Advantage® PCR system as above. Restriction enzyme analyses with *Sac* I, *Bam* H1, *Hind* III, *Bgl* II were used to confirm the correct product had been amplified. The PCR product was ligated into pGEM-T® as described and the ligation mix transformed into XL1-Blue competent cells. DNA was extracted from 10 clones by the quick plasmid preparation method and restriction enzyme analyses with *Sac* II, *Bam* H1, *Hind* III, *Bgl* II and *Sac* I (NEB) was performed and used to confirm correct insertion and orientation. A high purity stock of pGEM-T/pmpG was generated using the QIAwell 8 method and XL1-Blue bacteria containing the plasmid were frozen. A 1410 base pair (bp) *Hind* III fragment from the pGEM-T was isolated by restriction enzyme digestion and electrophoresis followed by purification using the Qiaex II® method. The purified fragment was then ligated into pTrcHisC® (previously digested with *Hind* III, de-phosphorylated and extracted by the phenol/chloroform method), in frame to allow expression, and transformed into XL1-Blue. Transformants were screened by purification of plasmid DNA, by the quick plasmid preparation method, followed by restriction enzyme analysis with *Hind* III and *Bam* H1. A high purity stock of pTrcHis/pmpG-1410 was purified using the QIAwell 8 method and XL1-Blue bacteria containing the plasmid were stored as frozen glycerol stocks. The high purity DNA preparation was again screened by restriction enzyme analysis with *Hind* III and *Bam* H1.

2.1.9.2 Cloning of pBluescript/pmpD-1218

pmpD DNA was amplified using the Clontech Advantage® PCR system as above. Restriction enzyme analyses with *Sac* I, *Bam* H1, *Hind* III and *Bgl* II was used to confirm the correct product had been amplified. A 1218 base pair *Bam* H1 fragment from the PCR product was isolated by restriction digestion and electrophoresis followed by purification using the Qiaex II® method. The purified fragment was then ligated into pBluescript SKII+ (previously digested with *Bam* H1, de-phosphorylated and purified by the phenol/chloroform method) and transformed into XL1-Blue. Transformants were

initially identified by blue/white selection and ampicillin resistance and 10 clones screened by purification of plasmid DNA by the quick plasmid preparation method, followed by restriction enzyme analysis with *Hind* III, *Sac* I and *Bam* H1. A high purity stock of pSKII/pmpD-1218 was purified using the QIAwell 8 method and XL1-Blue bacteria containing the plasmid were stored as frozen glycerol stocks. The high purity DNA preparation was again screened by restriction enzyme analysis with *Hind* III and *Bam* H1.

2.1.9.3 Cloning of pBAD/pmpG

pmpG DNA was amplified using Pfu high-fidelity DNA polymerase as described. The PCR product was incubated with Taq DNA polymerase in order to add overhanging adenine nucleotides, visualised by ethidium bromide gel electrophoresis and the correct sized band (2924bp) purified by the Qiaex II® kit. The purified PCR product was ligated into pBAD-TOPO® and transformed into One-Shot® competent cells. Transformants were initially identified by ampicillin resistance and 10 clones screened by purification of plasmid DNA by the quick plasmid preparation method, followed by restriction enzyme analysis with *Bam* H1, *Eco* RV and *Nco* I. A high purity stock of pBAD/pmpG was purified using the QIAwell 8 method and One-Shot® bacteria containing the plasmid were stored as frozen glycerol stocks.

2.1.9.4 Cloning of pPET/pmpGc

An aliquot of the high-purity pBAD/pmpG DNA was digested with *Xho* I and *Sac* I and a 1761 bp fragment of *pmpG* demonstrated by ethidium bromide gel electrophoresis. The remainder of the reaction volume was then electrophoresed on a 1% crystal violet agarose gel and the fragment purified using the Qiaex II® kit. pPET-30a vector was similarly digested with *Xho* I and *Sac* I and purified in the same manner. The fragment was ligated into the digested pPET-30a vector and transformed into commercial TOP10 competent cells (Invitrogen). Transformants were initially identified by kanamycin resistance and 9 clones screened by purification of plasmid DNA by the quick plasmid preparation method, followed by restriction enzyme analysis with *Bgl* II. A high purity stock of pPET/pmpGc was purified using the QIAwell 8 method and TOP10 bacteria containing the plasmid were stored as frozen glycerol stocks. In order to increase the yield of recombinant protein, pPET/pmpGc was transformed in BL21 for expression. BL21- pPET/pmpGc strains were frozen for storage as frozen glycerol stocks.

2.1.9.5 Cloning of pCR-II/pmpG

pmpG DNA was amplified using Pfu high-fidelity DNA polymerase as described. The PCR product was incubated with Taq DNA polymerase in order to add overhanging adenine nucleotides. The crude PCR product was visualised by ethidium bromide gel electrophoresis and the correct sized band (2924 bp) purified by the Qiaex II® kit. The purified PCR product was ligated into pCR-II-TOPO® and transformed into commercial TOP10 competent cells (Invitrogen). Transformants were initially identified by ampicillin resistance and 9 clones screened by purification of plasmid DNA by the quick plasmid preparation method, followed by restriction enzyme analysis with *Eco* RV. A high purity stock of pCR-II/pmpG was purified using the QIAwell 8 method and TOP10 bacteria containing the plasmid were stored as frozen glycerol stocks. The high purity DNA preparation was again screened by restriction enzyme analysis with *Eco* RI + *Eco* RV.

2.1.9.6 Cloning of pPET/pmpGa

An aliquot of the high-purity pCR-II/pmpG DNA was digested with *Eco* RI and *Eco* RV and a 1504 bp fragment of *pmpG* demonstrated by ethidium bromide gel electrophoresis. *Eco* RV was used to cut the vector fragment in order to facilitate separation of the fragment by gel electrophoresis for later cloning. The remainder of the reaction volume was then electrophoresed on a 1% w/v crystal violet agarose gel and the fragment purified using the Qiaex II® kit. pPET 30a vector was digested with *Eco* RI, de-phosphorylated and purified in the same manner. The fragment was ligated into the digested pPET30a vector and transformed into commercial TOP10 competent cells. Transformants were initially identified by kanamycin resistance and 10 clones screened by purification of plasmid DNA by the quick plasmid preparation method, followed by restriction enzyme analysis with *Bam* HI. A high purity stock of pPET/pmpGa was purified using the QIAwell 8 method and TOP10 bacteria containing the plasmid were stored as frozen glycerol stocks. In order to increase the yield of recombinant protein, pPET/pmpGa was transformed in BL21 for expression. BL21- pPET/pmpGa strains were stored as frozen glycerol stocks.

2.2 Expression

2.2.1 Basic techniques

2.2.1.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The BioRad Mini-Protean II (BioRad, Hemel Hempstead, UK) was used for SDS-PAGE electrophoresis[237,238]. The glass plates were assembled according to the manufacturer's instructions.

10ml of 10% separating gel was made up by mixing monomer solution (40% acrylamide solution (Sigma), 0.008% N,N'-methylenebisacrylamide) with 4X separating gel buffer (375mM Tris-HCl, pH 6.8), 10% v/v SDS, dH_2O, 10% ammonium persulphate (Sigma) and N,N,N',N'-tetramethylethylenediamine (TEMED - Sigma) in the quantities given in the table as appropriate for the size of proteins to be differentiated. The gel mix was then poured between the glass plates and overlaid with isopropanol. Once the gel had set, the isopropanol was poured off and the gel surface washed with dH_2O and dried with blotting paper. The 15-well comb was then inserted between the plates. 4ml of stacking gel was then made by mixing 532µl monomer solution with 1ml 4X stacking gel buffer (125mM Tris-HCl, pH 6.8), 40µl 10% SDS, 2.4ml dH_2O, 20µl 10% ammonium persulphate and 2µl TEMED. The stacking gel mix was then poured between the glass plates and allowed to set. Once the stacking gel had set, the comb was removed and the wells washed with dH_2O. The gels were then fastened to the electrode assembly and placed in the buffer tank. Tris-glycine electrophoresis buffer (25mM tris(hydroxymethyl)aminomethane, 250mM glycine (Sigma), 0.1% SDS was added to the top and bottom reservoirs.

Protein molecular weight	Acrylamide conc^n (%)	Monomer sol^n	4X separating gel buffer	10% SDS	dH_2O	Ammonium phosphate	TEMED
40 – 200	7.5	2.5ml	2.5ml	100µl	4.9ml	50µl	3.3µl
21 – 100	10	3.3ml	2.5ml	100µl	4ml	50µl	3.3µl
10 - 40	12.5	4.2ml	2.5ml	100µl	3.2ml	50µl	3.3µl

Table 2-3: SDS-PAGE gel composition

The protein sample to be electrophoresed was mixed with an equal volume of 2X SDS-PAGE loading buffer (125mM Tris-HCl, 5% β-mercaptoethanol (Sigma), 2% SDS, 0.6% bromophenol blue (Sigma), 20% glycerol (Sigma)) and heated to 100^{O}C in a boiling water-bath for 3 minutes to denature the proteins. The protein/buffer mix was

then loaded into the wells of the stacking gel and 200v applied vertically across the gel until the bromophenol blue dye-line reached the bottom of the gel. The gels were then removed from the assembly and either stained with Coomassie brilliant blue or used for Western blotting.

SDS-PAGE gels were stained with Coomassie Brilliant Blue R250 to demonstrate separated protein bands. The gels were immersed in staining solution (0.25% Coomassie Brilliant Blue R250 (BDH), 45% methanol (BDH), 45% dH_2O, 10% glacial acetic acid (BDH)) for 1 to 2 hours on a rocking platform. The gel was then transferred to de-staining solution (45% methanol (BDH), 45% dH_2O, 10% glacial acetic acid (BDH)) and left on the rocking platform overnight. After adequate de-staining gels were dried between plastic sheets for long-term archiving.

2.2.1.2 Western blotting & immuno-detection

A standard Western blotting protocol was used[239]. Hybond P® nylon membrane (Amersham, Chalfont, UK) was used for immunoblotting. A piece of membrane was cut to the size of the gel, wetted briefly in methanol (Sigma), then soaked in Towbin buffer (39mM glycine, 48mM Tris, 0.037% SDS, 20% methanol) for at least 10 minutes. The components of the Western blot were then assembled in the BioRad MiniProtean II blotting cassettes (BioRad), under Towbin buffer, in the order: porous pad, Whatman 3MM blotting paper, gel, Hybond P® membrane, Whatman 3MM paper, porous pad. The cassette was then closed and inserted in the blotting electrode assembly in the Towbin buffer filled tank, such that the Hybond P® membrane was closest to the anode. Current was applied across the cassettes at 200mA for one hour with an ice pack to prevent heating.

After 1 hour, the cassette was removed and opened, and the membrane transferred to blocking buffer (Tris-buffered saline (TBS – 150mM NaCl, 100mM Tris-HCl, pH 7.5), 0.0005% v/v Tween-20, 3% w/v dried skimmed milk powder (Tesco, Cheshunt)) for one hour. After blocking, the membrane was transferred to an individual plastic bag, in which it was sealed with primary antibody, diluted in rinse buffer (TBS, 0.0005% v/v Tween-20, 1% w/v dried skimmed milk powder) at the appropriate dilution. The membrane was incubated with the antibody solution for 1 hour on a rotating platform.

The membranes were then removed from the bags and washed, individually, three times in rinse buffer. They were then sealed into plastic bags with the secondary antibody, diluted appropriately in rinse buffer and incubated on a rotating platform for one hour.

The membranes were then washed twice in rinse buffer and once in TBS, each for 8 minutes. Finally, the membranes were equilibrated in detection buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5) then incubated with 0.33mg/ml nitro blue tetrazolium (NBT - Sigma) and 0.165mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in detection buffer for 5 minutes to allow the colour to develop, then washed and allowed to dry.

2.2.2 pTrcHis constructs

A single colony of XL1-Blue containing the expression construct of interest was inoculated into 2ml of LB broth containing 50µg/ml ampicillin and 15µg/ml tetracycline and incubated overnight at 37°C in a shaking incubator. 1ml of this culture was then transferred to 40ml pre-warmed LB broth, also containing ampicillin and tetracycline, and incubated in a shaking incubator with hourly monitoring of the OD_{600}. When the OD_{600} reached ~ 0.6, five millilitres of culture were aliquoted into each of six tubes labelled A to F. Each tube received either 6.25µl, 0.625µl or no O.8M isopropyl β-D-thiogalactopyranoside (IPTG - Sigma) to give final concentrations of 1mM, 0.1mM or 0mM IPTG, and was then transferred to a shaking incubator at either 25°C or 37°C as detailed in . After 4 hours, 2ml of the culture was removed and centrifuged at 16,000g for 30 seconds. The pellet was stored at –20°C pending analysis. The remaining culture was returned to the incubator and a further 2ml aliquot taken and centrifuged after 20 hours.

Table 2-4: IPTG concentrations used to induce expression in pTrcHis constructs

Tube	IPTG Concentration (mM)	Temperature (°C)
A	0	37
B	0.1	37
C	1	37
D	0	25
E	0.1	25
F	1	25

The bacterial pellets were re-suspended in 200µl phosphate buffered saline (PBS: 138mM NaCl, 2.7mM KCl, pH 7.4) and subjected to four freeze/thaw cycles alternating between solid carbon dioxide (CO_2) and a 37°C water-bath. 10µl of each suspension was then mixed with 10µl 2X SDS-PAGE loading buffer and electrophoresed in duplicate 10% SDS-PAGE gels; each lane therefore containing the equivalent of 50µl original culture. One gel was stained with Coomassie blue stain and the other was used for Western blotting. Anti-Xpress® antibody (Invitrogen), diluted 1:5000, was used as the primary antibody and goat anti-mouse-alkaline phosphatase conjugate (Promega), diluted 1:7500, was used as the secondary antibody. Nitro blue

tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as the chromogenic substrates.

2.2.3 pBAD constructs

A single colony of One-Shot® bacteria containing the expression construct of interest was inoculated into 3ml of LB broth containing 20µg/ml ampicillin and incubated overnight at 37°C in a shaking incubator. 250µl of this culture was then used to inoculate each of 10 labelled tubes, each containing 10ml LB broth (+ ampicillin) and incubated in a shaking incubator with hourly monitoring of the OD_{600}. When the OD_{600} reached ~ 0.5 a 1ml aliquot was removed from each tube and centrifuged at 16,000g for 30 seconds. The pellet was stored at –20°C pending analysis. Each tube then received 90µl of the appropriate arabinose stock solution (Invitrogen) to give final concentrations as shown in Table 2-5. The tubes were then transferred to a shaking incubator at either 25°C or 37°C as detailed in Table 2-5. After 4 hours, 1ml of the culture was removed and centrifuged at 16,000g for 30 seconds. The pellet was stored at –20°C pending analysis. The remaining culture was returned to the incubators and a further 1ml aliquot taken and centrifuged after 20 hours.

Tube	Arabinose stock solution	Final arabinose concentration	Temperature (°C)
1	2%	0.02%	37
2	0.2%	0.002%	37
3	0.02%	0.0002%	37
4	0.002%	0.00002%	37
5	0.0002%	0.000002%	37
6	-	0%	37
7	2%	0.02%	25
8	0.2%	0.002%	25
9	0.02%	0.0002%	25
10	0.002%	0.00002%	25
11	0.0002%	0.000002%	25
12	-	0%	25

Table 2-5: Arabinose concentrations used to induce expression in pBAD constructs

The bacterial pellets were re-suspended in 100µl phosphate-buffered saline and subjected to four freeze/thaw cycles using solid CO_2 and a 37°C water bath. 10µl of each suspension was then mixed with 10µl 2X SDS-PAGE loading buffer and electrophoresed in duplicate 10% SDS-PAGE gels. One gel was stained with Coomassie blue stain and the other was used for Western blotting. Anti-V5® monoclonal antibody (Invitrogen), diluted 1:5000, was used as the primary antibody. Anti-V5® monoclonal antibody recognises the paramyxovirus P and V protein sequence –Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr- which is encoded in the V5 epitope of the pBAD vectors. Goat anti-mouse-alkaline phosphatase conjugate

(Promega), diluted 1:7500, was used as the secondary antibody. NBT/BCIP was used as the chromogenic substrate.

2.2.4 pPET constructs

A single colony of BL21 containing the expression construct of interest was inoculated into 2ml of LB broth containing 50µg/ml ampicillin and 15µg/ml tetracycline and incubated overnight at 37^{O}C in a shaking incubator. 1ml of this culture was then transferred to 40ml pre-warmed LB broth, also containing ampicillin and tetracycline, and incubated in a shaking incubator with hourly monitoring of the OD_{600}. When the OD_{600} reached ~ 0.6 5ml of culture was aliquoted into each of six tubes labelled A to F. Each tube received 6.25µl, 0.625µl or no O.8M IPTG to give final concentrations of 1mM, 0.1mM or 0mM IPTG, and was then transferred to a shaking incubator at either 25^{O}C or 37^{O}C as detailed in Table 2-6. After 4 hours, 2ml of the culture was removed and centrifuged at 16,000g for 30 seconds. The pellet was stored at –20^{O}C pending analysis. The remaining culture was returned to the incubators and a further 2ml aliquot taken and centrifuged after 20 hours.

Table 2-6: IPTG concentrations used to induce expression in pPET constructs

Tube	IPTG Concentration (mM)	Temperature (°C)
A	0	37
B	0.1	37
C	1	37
D	0	25
E	0.1	25
F	1	25

The bacterial pellets were re-suspended in 200µl PBS and subjected to four freeze/thaw cycles using solid CO_2 and a 37^{O}C water-bath. 10µl of each suspension was then mixed with 10µl 2X SDS-PAGE loading buffer and electrophoresed in duplicate 10% SDS-PAGE gels, each lane therefore containing the equivalent of 50µl original culture. One gel was stained with Coomassie blue stain and the other was used for Western blotting. Anti-S protein monoclonal antibody-alkaline phosphatase conjugate (Novagen), diluted 1:5000, was used as the sole antibody. NBT/BCIP was used as the chromogenic substrate.

2.2.5 rMOMP construct

The construct used to prepare *Chlamydia trachomatis* serovar A rMOMP was kindly provided by Lyn Appleby and Michael Ward (Southampton Medical School, UK). A

single colony of the expression host containing the expression plasmid was inoculated into 40ml LB and incubated overnight at 37^{O}C in a shaking incubator. The following morning the culture was added to 1000ml pre-warmed LB. The culture was then grown in a shaking incubator at 37^{O}C to an OD_{600} of 0.6. IPTG was then added to a final concentration of 0.5mM and the cultures induced at 37^{O}C in a shaking incubator for 5 hours. The broth was then divided into four 250ml aliquots and centrifuged at 10,976g for 15 minutes at 4^{O}C. The pellets were stored for purification.

2.3 Recombinant Protein Purification

2.3.1 General methods

2.3.1.1 Determination of the solubility of recombinant proteins in PBS

Each of the recombinant proteins produced was assessed to determine its solubility in PBS. As described above, an aliquot of bacterial culture for each time point and inducer concentration was centrifuged, then re-suspended in 1/10 the original volume of PBS and subjected to four freeze/thaw cycles using dry ice and a 37^{O}C water-bath. 50µl of this was then taken and centrifuged. The supernatant was transferred to a fresh microfuge tube and the pellet re-suspended in 50µl PBS. 5µl of each was then analysed by SDS-PAGE as described above.

2.3.1.2 Purification of inclusion bodies

All the recombinant proteins used for the field studies were mostly present in PBS insoluble form in the bacterial cells after expression. Purification of inclusion bodies was therefore performed first and the inclusion bodies then used for further affinity purification.

A pellet from 500ml of culture was re-suspended in 20ml BugBuster® Protein Extraction Reagent (Novagen – proprietary formulation not available) to which 25µl protease inhibitor cocktail (Sigma - contains 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), bestatin, pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), and N-(alpha-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp (phosphoramidon)) and 20µl Benzonase® Nuclease mix (Novagen) had been added. The suspension was

mixed on a rocking platform for 20 minutes at room temperature until no longer viscous. The suspension was then centrifuged at 20,070g for 20 minutes at 4^{O}C. The supernatant was removed and stored for SDS-PAGE analysis.

The pellet was re-suspended in 5ml BugBuster® reagent and 50µl lysozyme 20ml/ml added. The mixture was incubated at room temperature for 5 minutes then 30ml 0.1X BugBuster® reagent was added and the suspension vortexed for 1 minute. The suspension was then centrifuged at 20,070g for 15 minutes at 4^{O}C. The supernatant was removed, an aliquot stored for SDS-PAGE analysis, and the pellet re-suspended in 30ml 0.1X BugBuster® reagent. The suspension was vortexed for 1 minute then centrifuged at 20,070g for 15 minutes at 4^{O}C. The supernatant was removed and an aliquot stored for SDS-PAGE analysis. The wash process was repeated once more. The pellet, containing the purified inclusion bodies was frozen.

2.3.2 Purification of rMOMP

Two pellets of inclusion bodies, each from 250ml of culture were used. Each pellet was re-suspended in 12.5ml 2% sarkosyl (Sigma) + 40mM dithiothreitol (Sigma) and mixed on a rotating platform at room temperature for 1 hour. The suspension was then centrifuged at 15,366g for 20 minutes and the supernatant removed and stored. The pellet was again re-suspended in the sarkosyl/dithiothreitol solution and the process repeated. The pellet was re-suspended in 5ml TBS and an aliquot stored for SDS-PAGE analysis. The two supernatants were combined and placed into a 10kDa exclusion size Slide-A-Lyzer® dialysis cassette (Pierce, Tattenhall, Cheshire, UK) and dialysed against a saturated solution of $(NH_4)_2SO_4$ (Fluka, Poole, UK) overnight at 4^{O}C. The suspension, containing the precipitated rMOMP, was then removed from the cassette, transferred to a clean centrifuge tube and centrifuged at 15,366g for 15 minutes at 4^{O}C. The pellet was then re-suspended in 9ml of 2% octylglucoside (Sigma) + 40mM dithiothreitol and mixed overnight at 4^{O}C. The rMOMP remains insoluble in the octylglucoside. The suspension was centrifuged at 15,366g for 15 minutes at 4^{O}C and the rMOMP re-suspended in 1ml PBS.

The aliquots collected during the purification process were then analysed by SDS-PAGE.

2.3.3 Ni-NTA Protein Affinity Purification

Ni-NTA Spin Columns® (Qiagen) were used for the purification of recombinant proteins carrying a 6X histidine tag. The Ni-NTA Spin Columns® contain silica beads to which nickel-chelating resin is bound. The histidine-tag containing recombinant proteins bind strongly to the nickel tetradentate complexes allowing stringent washing. As the recombinant proteins were all insoluble in PBS, they were purified under denaturing conditions.

250ml of culture was centrifuged and the resultant pellet re-suspended in 2.5ml PBS. 100µl of this suspension (equivalent to 10ml original culture) was added to 900µl lysis buffer B (8M urea, 0.1M NaH_2PO_4, 10mM Tris-HCl pH 8.0) and mixed on a rotary shaker for 1 hour at $4^{O}C$. Once completely lysed the suspension was centrifuged at 16,000g for 20 minutes at $4^{O}C$. 20µl supernatant (equivalent to 200µl original culture) was stored for SDS-PAGE analysis. Meanwhile a Ni-NTA Spin Column® was equilibrated with 600µl lysis buffer B then centrifuged for 2 minutes at 920g. 600µl of the cleared lysate was then added and spun for 2 minutes at 920g. 20µl flow-through (equivalent to 200µl original culture) was stored for SDS-PAGE analysis. The column was then washed with 600µl wash buffer C (8M urea, 0.1M NaH_2PO_4, 10mM Tris-HCl pH 5.9) and centrifuged for 2 minutes at 920g. 20µl flow-through (equivalent to 200µl original culture) was stored for SDS-PAGE analysis. This wash process was then repeated. The recombinant protein was then eluted from the column by adding 200µl elution buffer E (8M urea, 0.1M NaH_2PO_4, 10mM Tris-HCl pH 4.5) and then centrifuging for 2 minutes at 920g. The flow-through contained the recombinant protein. Five microlitres of the flow-through (equivalent to 250µl original culture) was set aside for SDS-PAGE analysis. The elution process was then repeated.

The aliquots collected during the purification process were then analysed by SDS-PAGE.

2.3.4 ProBond® Column Resin Affinity Purification

ProBond® resin contains divalent nickel cations immobilised onto ProBond resin and is used for affinity purification of recombinant proteins with a 6X His tag. As the recombinant proteins were all insoluble in PBS, they were purified under denaturing conditions. This method was used for the purification of recombinant proteins from the

expression of pPET-30a, pPET/pmpGa and pPET/pmpGc used in the field studies in The Gambia.

The ProBond® column (Invitrogen) was prepared by first re-suspending the resin, and then centrifuging at 920g for 2 minutes. The supernatant was then aspirated and discarded. 7ml sterile dH_2O was then added and the resin re-suspended. The column was then centrifuged at 920g for 2 minutes and the supernatant again removed and discarded. This wash process was then repeated with a further 7ml sterile dH_2O. 7ml of denaturing binding buffer (8M urea, 20mM sodium dihydrogen orthophosphate (NaH_2PO_4), 500mM NaCl, pH 7.8) was then added and the resin re-suspended. The column was then centrifuged at 900g for 2 minutes and the supernatant discarded. This process was then repeated a further two times.

Pelleted bacterial cells or purified inclusion bodies equivalent to 250ml of culture were used for each purification. 20ml guanidinium lysis buffer (6M guanidinium hydrochloride (GuHCl), 20mM sodium phosphate ($NaPO_4$), 500mM NaCl, pH 7.8) was warmed to 37°C. Each pellet of either bacteria or inclusion bodies was re-suspended in 10ml guanidinium lysis buffer and mixed on a rocking platform at room temperature for 10 minutes. The bacterial suspensions were then sonicated, on ice, at high intensity at 20 kHz for 3 bursts of 5 seconds each. The suspensions were then centrifuged at 1200g for 15 minutes and the supernatant transferred to a clean tube and the pH adjusted to 7.8. The pellet was then re-suspended in 10ml PBS and an aliquot stored for later SDS-PAGE analysis.

Five millilitres of the cleared lysate was added to the column and the resin re-suspended. The suspension was mixed on a rocking platform for 10 minutes. The column was then centrifuged for 2 minutes at 920g and the supernatant removed and stored at –20°C. The second 5ml of the cleared lysate was then added to the column and the process repeated for all four 5ml aliquots. The column was then washed by adding 4ml denaturing binding buffer, mixing on the rocking platform for 5 minutes, centrifuging for 2 minutes at 920g and aspirating and storing the supernatant. This process was repeated one further time. The washing process was then repeated twice each with denaturing wash buffer pH 6.0 (8M urea, 20mM $NaPO_4$, 500mM NaCl, pH 6.0) and denaturing wash buffer pH 5.3 (8M urea, 20mM $NaPO_4$, 500mM NaCl, pH 5.3). 1ml denaturing elution buffer (8M urea 20mM $NaPO_4$, 500mM NaCl, pH 4.0) was then added and the resin re-suspended. The suspension was mixed on the rocking platform for 5 minutes, and then centrifuged for 2 minutes at 920g. The supernatant was

aspirated and stored. The elution process was repeated four more times with further 1 ml aliquots of denaturing elution buffer.

The aliquots collected during the purification process were then analysed by SDS-PAGE.

2.3.5 TALON® Metal Affinity Resin Column

TALON Metal Affinity Resin (Clontech) is used for the purification of 6XHis tagged proteins. The resin contains sepharose beads to which cobalt-chelating resin is bound. The histidine-tag containing recombinant proteins bind strongly to the cobalt tetradentate complexes. As the recombinant proteins were all insoluble in PBS, they were purified under denaturing conditions. This method was used for the purification of recombinant proteins from the expression of the pPET/pmpGa and pPET/pmpGc.

Bacterial cells from 500ml of induced culture were centrifuged in two 250ml aliquots at 10,976g for 15 minutes and the supernatants discarded. The pellets were each re-suspended in 10ml lysis buffer (6M GuHCl, 50mM NaH_2PO_4, 10mM Tris-HCl, 100mM NaCl, pH 8.0), and then mixed on a rocking platform at room temperature for 30 minutes until completely lysed. The suspension was passed through an 18 gauge needle several times to shear the DNA and reduce viscosity. The suspension was then centrifuged at 11,290g for 20 minutes at $4^{O}C$. The supernatant was transferred to a clean tube and used for further purification, a small aliquot being stored for SDS-PAGE analysis. The pellet was re-suspended in 10ml PBS and an aliquot stored for SDS-PAGE analysis.

1ml of a 50% suspension of TALON® metal affinity resin was transferred to each of two 40ml centrifuge tubes and centrifuged at 22,658g for 10 minutes. The supernatant was removed and discarded. 2.5ml lysis buffer was added, mixed on a rocking platform for 5 minutes then the suspension was centrifuged at 22,658g for 10 minutes and the supernatant discarded. The 10ml cleared bacterial lysate was then added to each tube, the pellet re-suspended and the suspension mixed on a rocking platform at room temperature for 20 minutes. The tubes were then centrifuged at 22,658g for 10 minutes and the supernatants removed and stored. The pellets were then each re-suspended in 5ml lysis buffer and mixed on a rocking platform for 5 minutes. The tubes were again centrifuged at 22,658g for 10 minutes and the supernatant removed, an aliquot being saved for SDS-PAGE analysis. Two further wash processes were carried out. Finally 500µl elution buffer (8M urea, 50mM NaH_2PO_4, 100mM NaCl, 20mM piperazine-N,N'-

bis(2-ethanesulfonic acid) pH 6.2 - (PIPES, Sigma) was added, the pellet re-suspended and the suspension mixed on a rocking platform for 5 minutes. The tubes were then centrifuged at 22,658g for 10 minutes and the supernatant removed and stored. This elution procedure was then repeated three further times each with 500µl elution buffer.

The aliquots collected during the purification process were then analysed by SDS-PAGE.

2.4 Whole Blood Assay

2.4.1 Preparation of antigens

2.4.1.1 Phytohaemagglutinin

Phytohaemagglutinin (PHA – Sigma) was purchased as a 1mg/ml solution. This solution was diluted as required to a final concentration of 20µg/ml with supplemented Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco/Life Technologies/Invitrogen, Paisley, Scotland) when making up the antigen plates for the whole blood assay.

2.4.1.2 Purified protein derivative

Purified protein derivative (PPD – Statens Seruminstitute, Copenhagen, Denmark) was purchased as a 1mg/ml solution. This solution was diluted as required to a final concentration of 20µg/ml with supplemented RPMI 1640 medium when making up the antigen plates for the whole blood assay.

2.4.1.3 *Candida albicans*

Candida albicans was purchased as a freeze-dried powder (Alk Laboratories, Wallingford CT, USA). The powder was suspended in PBS/10% glycerol to a final concentration of 1mg/ml. This solution was diluted as required to a final concentration of 20µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.1.4 Induced pPET without insert

As a control for the specificity of the recombinant proteins in subsequent immunological studies, the pPET vector without an insert was induced with IPTG and the bacterial cells subjected to the same recombinant protein extraction procedure and affinity purification of putative protein in the exactly the same way as the pPET *pmpG* constructs. No recombinant protein bands were visible on SDS-PAGE analysis, and so the resultant extract was diluted by the mean of the dilution factors for pPET/pmpGa and pPET/pmpGc as required to obtain a 1mg/ml suspension. This solution was diluted

as required as for the pPET/pmpG recombinants with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.1.5 *Chlamydia trachomatis* elementary bodies

Chlamydia trachomatis serovar A elementary bodies (EB's), grown from an isolate from an individual with trachoma in Jali, The Gambia, were kindly provided by Professor Michael Ward (Southampton Medical School, UK) as a 1.35 mg/ml suspension. This was diluted with sterile PBS to give a stock solution of 0.5mg/ml. This solution was diluted as required to a final concentration of 10µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.1.6 MOMP

Recombinant MOMP was prepared as described above. The final concentration after purification was 5mg/ml. This was diluted with sterile PBS to give a stock solution of 1mg/ml. This solution was diluted as required to a final concentration of 20µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.1.7 OMP2

Recombinant *Chlamydia trachomatis* serovar AOMP2 was kindly provided by Professor Hill Gaston (Cambridge University School of Medicine) as a 1mg/ml solution. This solution was diluted as required to a final concentration of 20µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay. The recombinant OMP2 was produced by cloning the appropriate PCR product into a pQE60 expression vector (Qiagen) which was then transformed into *E. coli*. The induced proteins could then be purified using their C-terminal histidine tag. Bacterial lysates containing the antigen were sonicated and centrifuged six times in buffer and solubilized in 6 M urea. The recombinant was then purified by elution from nickel containing His-Trap columns (Pharmacia) using 300 mM imidazole.

2.4.1.8 HSP60

Recombinant *Chlamydia trachomatis* serovar A HSP60 was kindly provided by Professor Hill Gaston (Cambridge University School of Medicine) as a 0.7mg/ml solution. The recombinant was produced as described above. This solution was diluted

as required to a final concentration of 20µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.1.9 PMPGc

Recombinant PMPGc was prepared as described above. The concentration was estimated by comparison against known standards using SDS-PAGE electrophoresis and staining with Coomassie Blue. The recombinant protein was then diluted to give a stock solution of 1mg/ml. This solution was diluted as required to a final concentration of 20µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.1.10 PMPGa

Recombinant PMPGa was prepared as described above. The concentration was estimated by comparison against known standards using SDS-PAGE electrophoresis and staining with Coomassie Blue. The recombinant protein was then diluted to give a stock solution of 1mg/ml. This solution was diluted as required to a final concentration of 20µg/ml with supplemented RPMI when making up the antigen plates for the whole blood assay.

2.4.2 Preparation of antigen plates

Assay plates containing antigens and controls were prepared in advance and stored frozen at –20^{O}C. All preparation was undertaken with full aseptic technique using a Class II microbiology safety cabinet (British Standard 5726: 1992). The cabinet was regularly serviced, and cleaned daily with 70% ethanol before and after use, and sterile disposable equipment was used within the hood space.

Antigen plates were prepared in batches of forty. RPMI (20.5ml), supplemented with 100 units/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (all Gibco/Life Technologies/Invitrogen), was placed in each of eleven 50ml sterile plastic, screw-topped, labelled tubes. Sufficient stock solution of each antigen was added to the appropriate tube to give a final concentration of 20µg/ml (except for EB's which were used at 10µg/ml).

Forty 96-well, lidded, round-bottom cell culture plates (Nalge Nunc International) were then opened in the cabinet and 200µl sterile PBS pipetted into the outside wells as shown in Figure 2-2. 100µl of each antigen was then pipetted, in turn, into the appropriate wells. Once all the wells had been filled the plates were frozen at –20^{O}C until required.

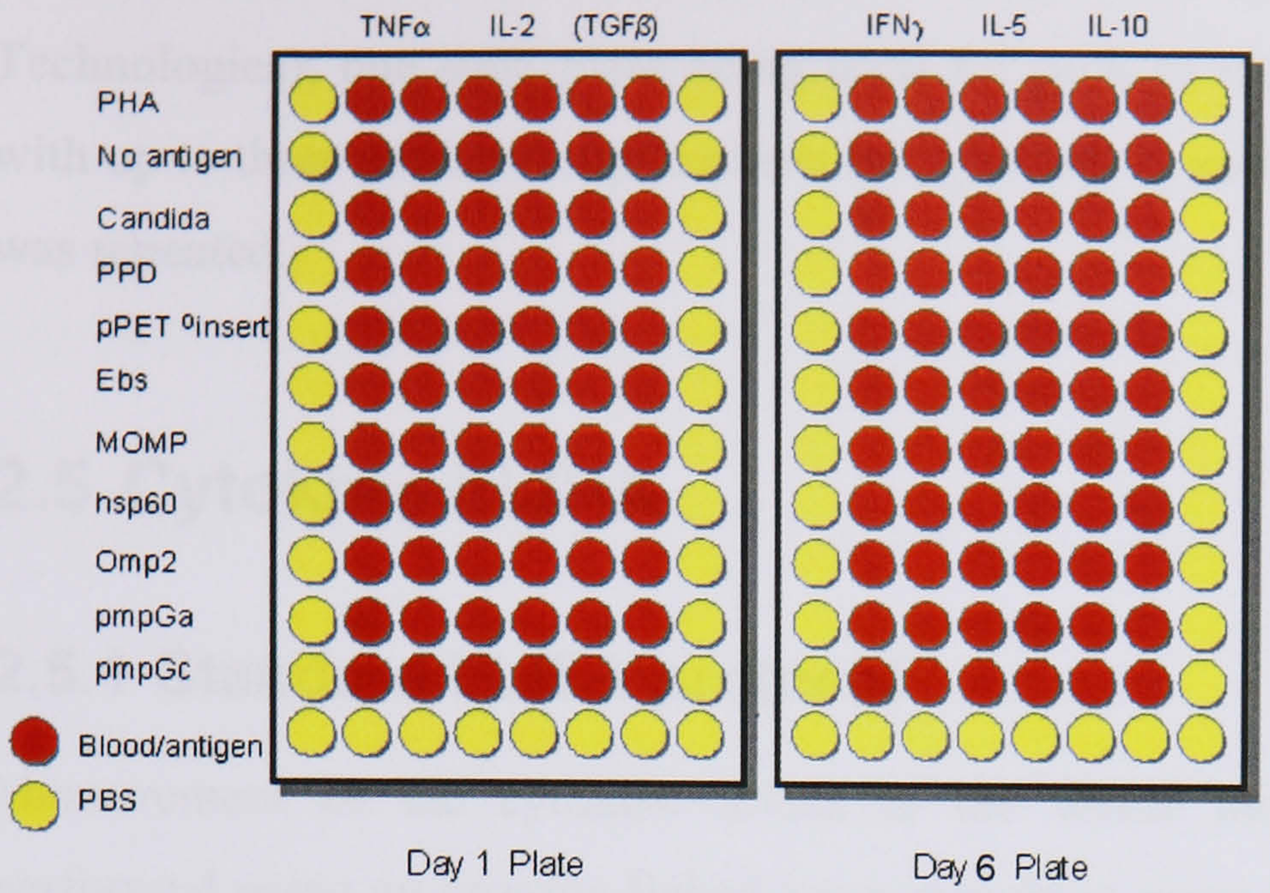


Figure 2-2: Whole blood assay plate layout

2.4.3 Setting up whole blood assays

Whole blood assays were performed using methods established by Elliot[320] and Dockrell.[319] Approximately 10ml of blood was taken from each patient into a Vacutainer® tube (BD, Oxford, UK) containing 143 units/ml sodium heparin for the whole blood assay. These samples were used to set up the whole blood assays within 1 hour of venesection. Meanwhile the requisite number of antigen plates were allowed to thaw in the class II microbiology safety cabinet. All manipulations of the blood were conducted within the safety cabinet and all contaminated disposables were soaked in 1% Virkon® (Antec International, Sudbury, UK) then incinerated. For each patient two plates were used, the first to be harvested on day 1 for the assays of TNF-α, IL-2 and transforming growth factor β (TGF-β) and the second plate to be harvested on day 6 for IFNγ, IL-5 and IL-10.

Two volumes of blood/RPMI were used, the larger where TGFβ was to be assayed in addition to the other five cytokines. For six cytokines 6ml of blood was diluted with 9ml of supplemented RPMI, for five cytokines 5ml of blood was diluted with 7.5ml supplemented RPMI. The blood/RPMI was mixed thoroughly by pipetting up and down. The blood/RPMI mix was then transferred into a sterile reservoir and then 100µl pipetted into each of the antigen/control wells in the antigen plate. The lid was then replaced, fastened with porous dressing tape and the plates incubated at 37^{O}C in a humidified incubator with 5% CO_2.

After 24 hours incubation the day 1 plates were removed from the incubator and transferred to the safety cabinet. Using a multi-channel pipette, the supernatants were

removed from the wells and transferred to labelled 96-well microtitre plates (Life Technologies); one such plate being used for each of the supernatants to be assayed, with up to three patients' supernatants being stored in each plate. On day 6, the process was repeated.

2.5 Cytokine ELISA

2.5.1 Standard ELISA protocol

Measurement of the cytokine levels in the whole blood assay supernatants was performed using an enzyme-linked immunosorbent assay (ELISA) technique (OptEIA, BD PharMingen, San Diego, USA). Each well from the whole blood assay duplicates was assayed individually, rather than being pooled, as it was considered that the whole blood assay was a more likely source of variability than the ELISA measurements. The supplied, lyophilised cytokine standards were reconstituted with sterile water to give a stock solution of 100ng/ml. This stock solution was then aliquoted and stored at $-20^{O}C$. For the assays the cytokine standards were prepared by doubling dilutions prepared in tubes to ensure thorough mixing, then pipetted onto the ELISA plate. Details of dilutions used are given below.

The ELISA plates were first coated overnight with capture antibody. The supplied anti-human cytokine antibody was diluted 1:250 with coating buffer (0.1M $NaHCO_3$, 33mM Na_2CO_3, pH 9.5) and used, at 100µl per well, to coat the wells of the microtitre plate (Dynex Technologies, Middlesex, UK). The plates were covered and allowed to incubate overnight at $4^{O}C$. The following morning the plates were washed in an automated microtitre plate-washer (ELP-40, Bio-Tek Instruments, Winooski, Vermont, USA). The wells were aspirated then washed three times with 300µl/well of wash buffer (PBS, 0.05% Tween-20). After the last wash, the plates were inverted onto a blotting pad to remove any last traces of wash buffer. The plates were then blocked with 200µl/well of assay diluent (PBS, 10% foetal calf serum, Sigma) for 1 hour at room temperature, and then washed three times in the same manner as before. Meanwhile, doubling dilutions of the appropriate cytokine standard were prepared to the appropriate dilutions (see below).

100µl of each supernatant from the whole blood assay, the control supernatants and the cytokine controls were then transferred to the appropriate wells of the microtitre plate.

Any necessary dilutions (see below) being first carried out in the storage plate. The plates were covered and allowed to incubate for 2 hours at room temperature. The wells were then aspirated and washed as before, but with a total of 5 washes. Meanwhile the detection solution was mixed. The biotinylated anti-human-cytokine detection antibody and avidin-horseradish peroxidase conjugate were diluted together 1:250 in assay diluent. 100µl detection solution was then pipetted into each well and allowed to incubate at room temperature for 1 hour. The wells were then washed again as before, but with a total of 7 washes.

ABTS substrate (Kirkegaard & Perry Laboratories, Guildford, UK) was then prepared by adding equal volumes of 0.01% H_2O_2 and 0.3g/l 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate) in glycine/citric acid buffer. 100µl of the ABTS substrate mix was then added to each well and allowed to develop in the dark for ~30 minutes. The reaction was then stopped by the addition of 100µl 1% SDS. The absorbance was then read at 410nm using an automated ELISA plate reader (MRX ELISA plate reader, Dynex Technologies), a standard curve generated from the cytokine standard results and the concentration calculated from each sample and control well. Concentrations were calculated automatically as the mean of the replicates although the plates and the individual results were checked manually for any obvious contamination.

2.5.2 Variations on the standard method for individual cytokine ELISA

2.5.2.1 TNFα

Cytokine standards: 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 0 pg/ml

PHA, Candida and EB whole blood assay supernatants diluted X 5

2.5.2.2 IFNγ

Cytokine standards: 1200, 600, 300, 150, 75, 37.5, 18.75, 9.75, 4.69, 0 pg/ml

Control supernatant diluted X 500

PHA whole blood assay supernatants diluted X 40

2.5.2.3 TGFβ

Cytokine standards: 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/ml

Control supernatant diluted X 10

2.5.2.4 IL-2

Cytokine standards: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 0 pg/ml

Control supernatant diluted X 10

2.5.2.5 IL-5

Cytokine standards: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 0 pg/ml

PHA supernatant diluted X 10

2.5.2.6 IL-10

Cytokine standards: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 0 pg/ml

Control supernatant diluted X 10

PHA supernatant diluted X 10

2.5.3 Preparation of whole blood assay control supernatants

In order to compare plate-to-plate variation, a control supernatant was produced to be used on every ELISA plate. 40ml of blood was taken from two members of the UK laboratory staff and used to set up 10 whole blood assay plates each. The blood was diluted 1:1.5 with supplemented RPMI and pipetted out into 10 x 96-well microtitre plates where each well contained 20μg/ml PHA in RPMI. The plates were incubated for either one or six days and the supernatants from each day's harvesting pooled, mixed thoroughly then frozen in aliquots at –20^{O}C. These control supernatants were then used on each ELISA plate, diluted if necessary as above. Due to restricted time and reagents undiluted control was used for the first few plates and in some instances this gave results outside the most accurate part of the standard curve; hence results for the cytokine levels in the control wells for these plates may well have been less accurate than in subsequent plates where the control sera were diluted.

2.6 Immunoblotting

SDS-PAGE electrophoresis, Western blotting and immuno-detection of antibodies was performed with the sera of all individuals to demonstrate, in a semi-quantitative manner, the presence of antibodies to a number of the chlamydial antigens (MOMP, OMP2, HSP60, PMPGa and PMPGc) together with the negative control 'PET-no antigen'.

A 10% SDS-PAGE gel with 4% stacking buffer was made as described in 2.2.1.1. Each gel was loaded with 5µl Precision Pre-Stained Protein Marker (Bio-Rad), then 2µg each of PET-no antigen, MOMP, OMP2, HSP60, PMPGa and PMPGc. Using a 14-well comb allowed 2 sets of antigens to be electrophoresed on each gel. Two gels were electrophoresed in parallel. After electrophoresis, Western blotting was performed as described in 2.2.1.2. After the blocking stage the membranes were cut in half such that a full set of antigens, markers and control were on each half. These membranes were then transferred to individual plastic bags, in which they were sealed with individual patient sera, diluted 1:100 in 5ml rinse buffer (TBS, 0.0005% Tween-20, 1% dried milk powder). The membranes were incubated with the sera overnight on a rotating platform.

The membranes were then removed from the bags and washed individually, three times in rinse buffer. They were then sealed, in pairs, into plastic bags with affinity-purified, goat anti-human IgG (H+L) alkaline phosphatase-conjugated antibody (Invitrogen), diluted 1:3000 in 5ml rinse buffer and incubated on a rotating platform for one hour. The membranes were then washed twice in rinse buffer and once in TBS, each for 8 minutes. Finally, the membranes were equilibrated in detection buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5) then incubated with 0.33mg/ml nitro blue tetrazolium (NBT) and 0.165mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in detection buffer for 5 minutes to allow the colour to develop, then washed and allowed to dry.

The intensity of each band was assessed by three observers independently and graded from 0 to 3, according to the criteria: 0 – not visible, 1 – just visible, 2 – clearly visible, 3 – strongly positive. This grading scheme worked well with little variation between the observers. The mean of the three values was used for analysis.

2.7 Full blood count

3ml blood was taken into EDTA-containing Vacutainers®. An aliquot was used, on the day of venesection, for automated full blood count analysis, including differential, using a Medonic Cellanayzer CA530 (Medonic, Bromma, Sweden). The remaining blood was stored at –80^{O}C until extraction of DNA.

2.8 Extraction of DNA from whole blood

DNA extraction was performed using the commercial Geneclean® kit (Qbiogene, Harefield, UK). The 5ml EDTA blood sample used for the full blood count was removed from the freezer and thawed rapidly in a 37^{O}C water bath. As soon as the blood had thawed it was transferred to ice. For each blood sample, 9ml of RBC Lysis solution (proprietary formulations not available) was placed in a labelled 15ml tube. 3ml of the blood was added and, after mixing, left to incubate for 10 minutes at room temperature. The mixture was then centrifuged at 3000g for 10 minutes and all but ~100µl of the supernatant was removed and discarded. The pellet was re-suspended by vortexing, and then 3ml Cell Lysis solution added and mixed. If the presence of any cell clumps was noted the mixture was incubated at 37^{O}C until they had dispersed. 15µl RNAse A was then added and the mixture incubated at 37^{O}C for 30 minutes. The solution was cooled to room temperature, then 1ml Protein Precipitation solution added and mixed. The sample was centrifuged at 3000g for 10 minutes. The supernatant was then transferred to a separate tube containing 3ml isopropanol and mixed. The solution was centrifuged at 3000g for 5 minutes and the supernatant carefully poured off and discarded. The pellet was washed with 3ml 70% ethanol, then centrifuged at 3000g for 5 minutes. The pellet was allowed to air dry, then solubilized in 250µl DNA Hydration solution. The DNA solution was stored in two separate aliquots of 125µl at –80^{O}C.

2.9 Chlamydia PCR

2.9.1 Extraction of DNA from eye swabs

After collection eye swabs were stored in 500µl TE buffer at –20^{O}C. After thawing, as much solution as possible was expressed from the swabs into the TE buffer. The tubes were then centrifuged at 9500g for 30 minutes to pellet cellular material. After removal

of supernatants, the pellets were re-suspended in 20µl of proteinase solution (0.5% Tween 20 (BDH), 0.5% Nonidet P-40 (BDH) and 100µg/ml proteinase K (Boehringer Mannheim, Lewes, UK)). The suspension was incubated for one hour at 60^{O}C after which the proteinase K was inactivated by boiling for 10 minutes. The crude DNA preparation was stored at –20^{O}C until required.

2.9.2 Amplicor CT® PCR for *Chlamydia*

The Amplicor CT® test for *Chlamydia trachomatis* (Roche Diagnostics, Branchburg, NJ, USA) is a commercial test in kit form which a 207 bp target sequence from the *Chlamydia trachomatis* cryptic plasmid is amplified by PCR using biotinylated primers. Following amplification, the amplimers are denatured to form single-stranded DNA and added to proprietary microtitre plates coated with oligonucleotide probes specific for the *Chlamydia trachomatis* target sequence. After removal of excess amplimers, avidin-horseradish peroxidase conjugate is added to the wells and binds to the biotin-labelled amplimers. After further washing a 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide substrate is added to the wells and the substrate is oxidised to a coloured complex in the presence of horseradish peroxidase. The absorbance of the complex is measured at 450nm using an automated microtitre plate reader.

2.9.3 Stool microscopy

A modified Stoll's method was used for counting helminth eggs in faecal samples. 4ml of 10% formalin in water was placed in a 7ml bijou. 1g of stool was added to the formal water with an orange stick and mixed thoroughly by vortexing. Samples so prepared could be stored for examination at a later date.

A 50µl aliquot of the stool suspension was placed on a microscope slide and covered with a coverslip. Any helminth eggs seen were identified using the x40 objective and the numbers under the entire coverslip were counted using the x10 objective. The number of eggs per gram of faeces was calculated by multiplying the number of eggs seen in the 50µl aliquot by 100.

2.10 Recruitment

The study protocol was approved by the ethics committee of the London School of Hygiene & Tropical Medicine and by the MRC/Gambian Government Ethical

Committee. All individuals gave informed, written consent either by signature or fingerprint. For children under the age of 16 consent was obtained from their parent or guardian. All cases of active trachoma were treated with tetracycline eye ointment and any other illnesses identified were either treated immediately or an appropriate referral for assessment and treatment was made. Cases and controls were sex matched in all cases. Age matching was to within three years for those under 20 and within 10 years for those aged 20 and above though in most cases age matching was exact. Cases and controls were matched by ethnic group as much as possible.

2.10.1 Sample size calculation

Sample size calculations were carried out for a two-sample test of equality of means using the formula derived by Rosner.[240] For each group the sample size was:

- Active trachoma
    - 50 cases and 25 controls – providing 80% power to detect a 35% difference in response at a level of $p < 0.05$
- Scarred trachoma
    - 25 cases and 25 controls – providing 80% power to detect a 40% difference in response at a level of $p < 0.05$
- Infertile women
    - 20 cases and 20 controls – providing 80% power to detect a 50% difference in response at a level of $p < 0.05$

2.10.2 WHO classification of trachoma

All cases of active and scarred trachoma were classified, using the criteria proposed by Thylefors et al,[241] as normal, follicular trachoma (TF), intense trachoma (TI), scarred (TS) or trichiasis (TT). Examples of each are illustrated below.

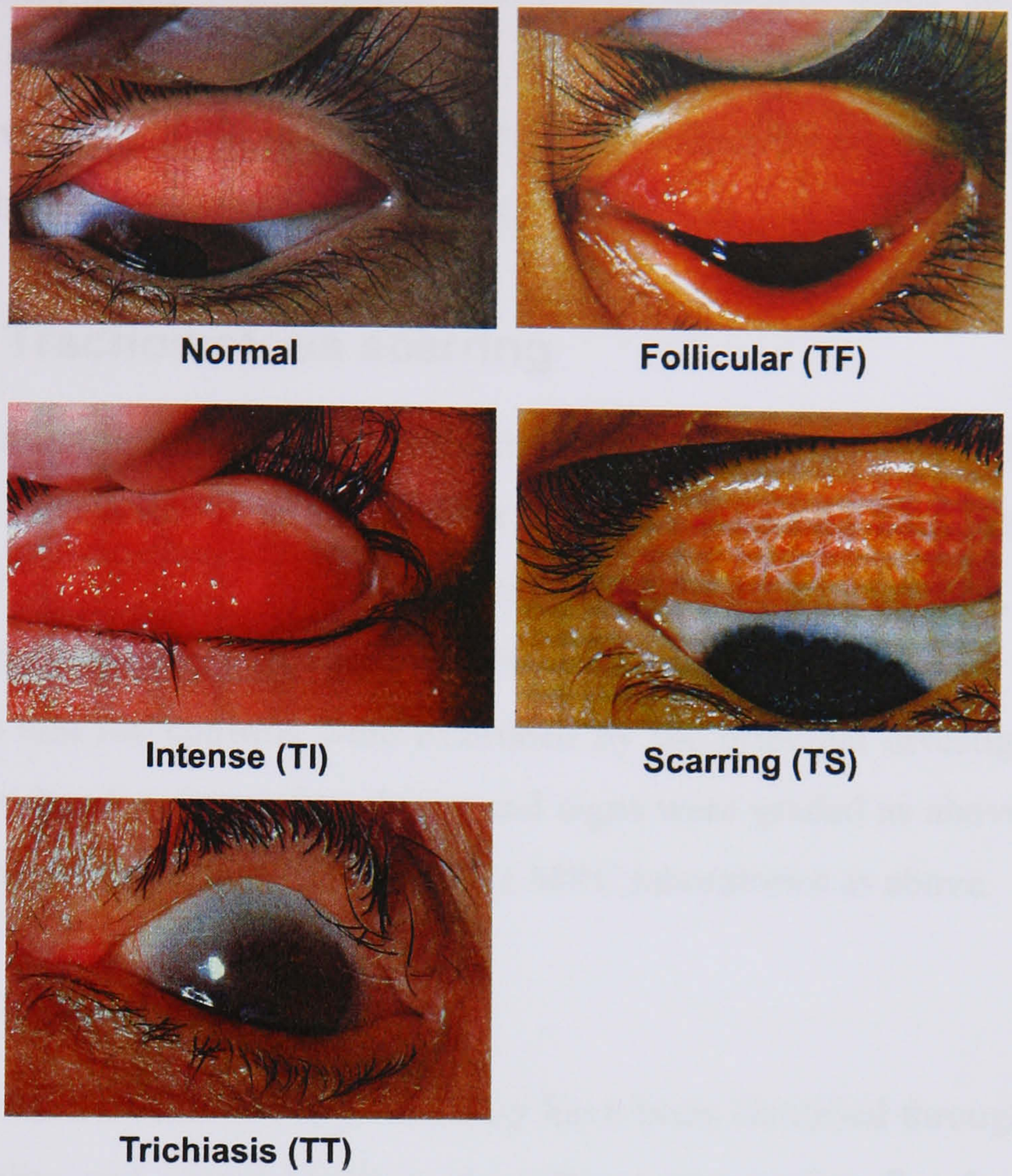


Figure 2-3: WHO classification of trachoma (from Dawson et al)[6]

The criteria for classification, as described by Thylefors are:

Follicular trachoma: "The presence of five or more follicles (at least 0.5mm) in the upper tarsal conjunctiva"

Intense trachoma: "Pronounced inflammatory thickening of the upper tarsal conjunctiva that obscures more than half of the normal deep tarsal vessels"

Trachomatous scarring: "The presence of scarring in the tarsal conjunctiva"

Trachomatous trichiasis: "At least one eyelash rubs on the eyeball"

2.10.3 Active trachoma

Individuals with active trachoma were identified by a trained field-worker as part of ongoing national screening. As trachoma control has been very effective in the study villages of Jali and Berending, a number of other villages and schools were visited. Individuals with active trachoma were identified and their disease graded according to World Health Organisation criteria. One age, sex, ethnicity and locality-matched control

was identified for every two children with active trachoma. Since the whole blood assays needed to be set up within one hour, and as the villages were located more than one hour's drive from the laboratory, the study participants were brought to the MRC laboratories where venesection was performed.

2.10.4 Trachomatous scarring

Individuals resident in the villages of Jali and Berending in The Gambia have been co-operating in a number of studies for over 14 years. Through these studies a large number of scarred individuals have been identified together with age, sex, ethnicity and locality-matched controls. These individuals were invited to participate in this study. Both the cases and the controls were examined by the principal investigator and/or a trained field-worker for signs of trachoma and signs were graded as above. Individuals agreeing to participate in the study visited the MRC laboratories as above.

2.10.5 Infertile women

A cohort of women with secondary infertility have been identified through an ongoing study of infertility and sexual health in the villages surrounding Farafenni in Central Gambia, and age, ethnicity and locality-matched controls identified. All the cases have undergone hysterosalpingography to identify any structural lesions and only women with tubal factor infertility were included. Individuals were approached in their villages and invited to participate in the study. For this group, venesection was performed in the villages and the blood brought to the laboratory for analysis.

2.11 Summary of recruitment, investigations and data sets generated

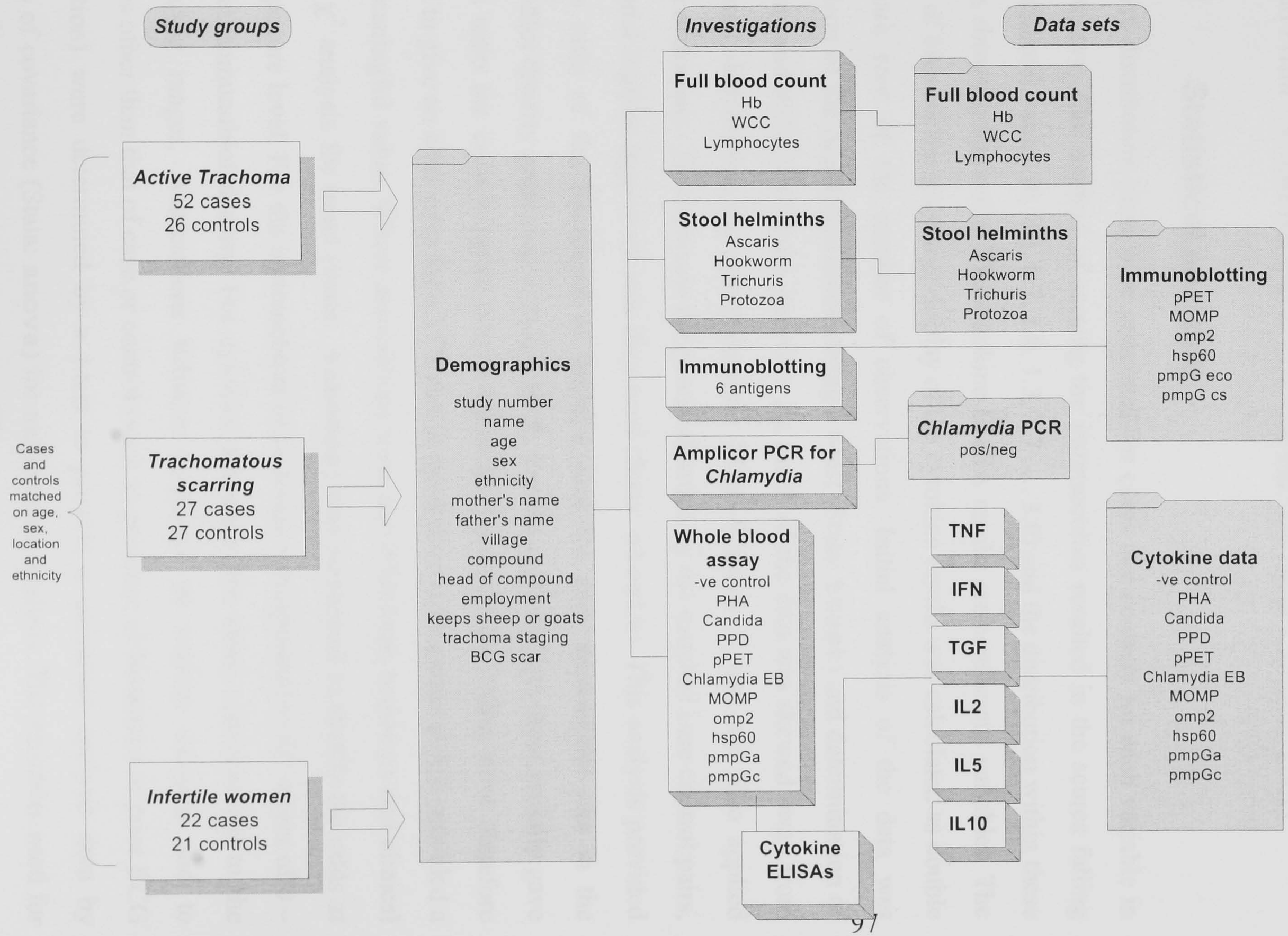


Figure 2-4: Summary of recruitment, investigations and data sets generated

2.12 Statistical analysis

2.12.1 General data management

Data were entered into a Microsoft Access® database, access to which was restricted to protect the confidentiality of the study participants. Statistical analysis was undertaken using the Stata® statistical package v.6 (Stata Corporation, College Station, Texas, USA). Most graphs were constructed using Stata although a few were generated using GraphPad Prism® v. 3.02 (GraphPad Software Inc, San Diego California USA).

2.12.2 Statistical analysis

Frequency distribution plots were generated for cases and controls for each variable in each category. The method of scoring the immunoblots resulted in the scores falling into 10 discrete values (0, 0.3, 0.7, 1.0, 1.3, 1.7 …. 3.0) and the distribution within these scores is shown graphically. The cytokine ELISA results are continuous variables. The number of bins for the graphical display of the cytokine results was calculated as double the square root of the number of observations. Initial analysis of the data was comparison of the population means using a t-test (Stata: **`ttest`**) and determination of the means and 95% confidence intervals. As much of the data was skewed away from the normal distribution the non-parametric Mann-Whitney U test was also applied (Stata: **`ranksum`**). To maximise the power afforded by the matched case-control pairs, conditional logistic regression was then used (Stata: **`clogit`**). This analysis provided an odds ratio of the likelihood of being a case for each incremental rise in the immunoblot density score (e.g. 1 to 2, 2 to 3). For the cytokine data these initially gave an odds ratio for each 1 pg/ml rise in cytokine concentration, these were therefore recoded to give an odds ratio for a 25% rise in cytokine concentration as this provided a more meaningful value. Where associations were noted between responses and clinical state, a χ^2 analysis for trend (Stata: **`tabodds`**) was performed to stratify the odds at each exposure level. For the immunoblots these levels corresponded to the scores of 0 – 3 for the immunoblot density. For cytokine responses the strata corresponded to the interquartile ranges. Associations between responses to various antigens and to variables other than that of case or control (such as presence of helminths or prior BCG vaccination) were determined by a t-test to provide a univariate analysis then by analysis of covariance (Stata: **`anova`**) for multivariate analysis. The variables used for

this were: age, sex, prior BCG vaccination, presence of intestinal helminths, presence of intestinal protozoa and ethnicity.

Thus, in the results chapter these results are presented in the following format:

- Frequency distribution plot broken down into cases and controls
- Table showing mean values and 95% confidence intervals for the cases and controls for each antigen, together with the results of the t-test and Mann-Whitney U-test
- Graph illustrating the mean and 95% confidence intervals for the cases and controls
- Table showing the results of logistic regression and the F figure and p value for the multivariate analysis
- Tables showing the results of a χ^2 analysis for trend for any significant associations

A general graphical summary of all results is shown in section 4.6

Chapter Three

Results 1 – Generation of plasmid constructs and expression of recombinant protein

3 Results 1 – Generation of plasmid constructs and expression of recombinant protein

3.1 Cloning

3.1.1 Purification of *Chlamydia trachomatis* serovar A DNA

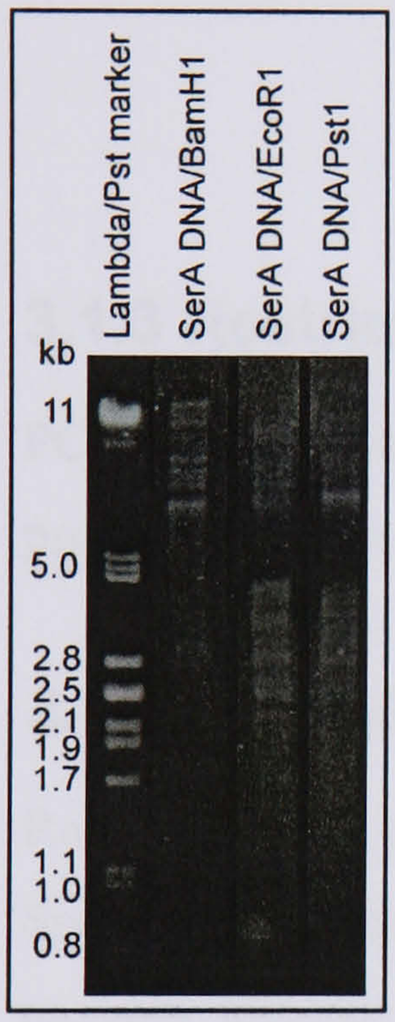


Figure 3-1: Restriction enzyme analysis of *Chlamydia trachomatis* genomic DNA

Chlamydia trachomatis serovar A DNA was purified as described in 2.1.1.1. Spectrophotometry of the DNA, solubilized in TE buffer demonstrated a concentration of 463ng/µl, giving a total yield of 58.8µg. The A_{260}/A_{280} ratio however was 1.03, indicating organic solvent and/or protein contamination. After ether extraction of the phenolic phase the A_{260}/A_{280} ratio was 1.17 suggesting continuing protein contamination. After re-extraction the final DNA concentration had reduced to 200ng/µl, and as further purification would probably result in the loss of more DNA, it was decided to continue with cloning and undertake further purification only if required.

The purified DNA was incubated with *Bam*H1, *Eco*R1 and *Pst*1 and the resulting products electrophoresed as described. The resultant electrophoretic pattern is seen in Figure 3-1.

3.1.2 PCR Amplification

Chlamydia trachomatis serovar *pmp* gene A DNA was amplified by PCR using either the Clontech Advantage® PCR kit or using Pfu to ensure the fidelity of amplification. The resultant product was analysed by agarose gel electrophoresis to ensure amplicons of the anticipated size had been generated. The PCR products are shown in Figure 3-2 and Figure 3-3. (Predicted PCR product sizes are shown in Table 2-1, page 56).

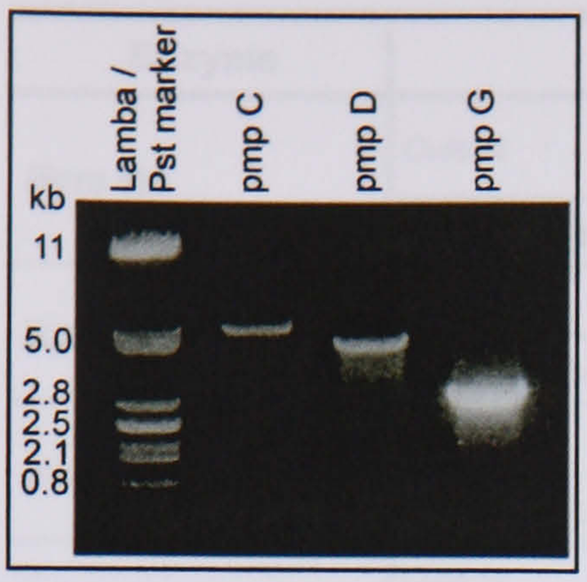


Figure 3-2: *pmpC, pmpD* and *pmpG* PCR products amplified using the Clontech Advantage® PCR kit and visualised on an ethidium bromide-stained agarose gel under 254nm UV illumination

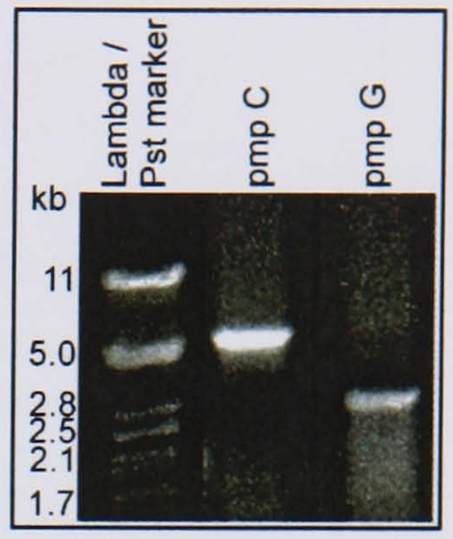


Figure 3-3: *pmpC* and *pmpG* PCR products amplified using Pfu DNA polymerase and visualised on an ethidium bromide-stained agarose gel under 254nm UV illumination

3.1.3 Restriction enzyme analysis of PCR products

PCR products were examined by restriction enzyme analysis to confirm that the correct product had been generated.

3.1.3.1 pmpG

Restriction sites predicted using serovar D sequence data for *pmpG* from the Chlamydia Sequencing Project are shown in Figure 3-4 and fragment sizes predicted on restriction enzyme analysis of *pmpG* PCR product with *Sac* I, *Bam*H1, *Hind*III and *Bgl*II, individually and in combination are shown in Table 3-1 together with the actual fragment sizes seen on restriction enzyme analysis of the PCR product obtained by PCR (Figure 3-5).

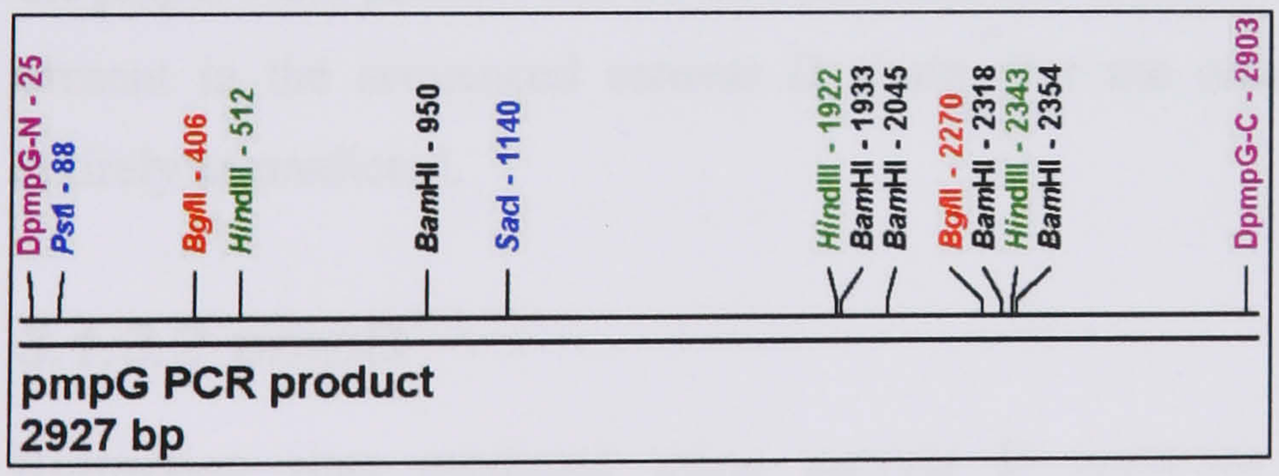


Figure 3-4: Predicted *pmpG* PCR product restriction map
Purple = primers, other colours = restriction sites

Figure 3-5: Fragments obtained on restriction enzyme analysis of *pmpG* PCR product and visualised under 254nm UV illumination after agarose gel electrophoresis

Enzyme		Predicted	Actual
***Bam* H1**	*Cuts at*	950, 1933, 2045, 2318, 2354	950, 1933, 2045, 2318, 2354
	Fragment sizes	950, 983, 112, 273, 36, 573	950, 983, 112, 273, 36, 573
***Bgl* II**	*Cuts at*	406, 2270	406, 2270
	Fragment sizes	406, 1864, 657	406, 1864, 657
***Hind* III**	*Cuts at*	512, 1922, 2343	512, 1922
	Fragment sizes	512, 1410, 421, 584	512, 1410, 1005
***Pst* I**	*Cuts at*	88	?88, either ~1300 or ~1700
	Fragment sizes	88, 2839	~1600, ~1200, ?88
***Pvu* I**	*Cuts at*	Not cut	Not cut
	Fragment sizes	2927	2927
***Msp* I**	*Cuts at*	Not cut	Not cut
	Fragment sizes	2927	2927
***Sac* I**	*Cuts at*	1140	1140
	Fragment sizes	1140, 1787	1140, 1787
***Bam* H1 + *Hind* III**	*Cuts at*	512, 950, 1922, 1933, 2045, 2318, 2343, 2354	512, 950, 1922, 1933, 2045, 2318, 2354
	Fragment sizes	512, 438, 972, 11, 112, 273, 25, 11, 573	512, 438, 972, 11, 112, 273, 36, 573
***Bgl* II + *Sac* I**	*Cuts at*	406, 1140, 2270	406, 1140, 2270
	Fragment sizes	406, 734, 1130, 657	406, 734, 1130, 657

Table 3-1: Predicted and observed fragments of the *pmpG* PCR product after restriction enzyme analysis

As the table shows there is a discrepancy between the predicted and observed digestion patterns with *Hind*III. This is most likely explained by a change in the enzyme recognition sequence in the DNA substrate leading to a loss of the *Hind*III site. Without further investigation it is not possible conclude that this represents a difference in sequence between our serovar A strain and the reference serovar D strain. Additionally, the *pmpG* PCR product had a *Pst*I restriction site at position either ~1300 or ~1700, not present in the sequenced serovar D strain. For the other enzymes the results were entirely as predicted.

3.1.3.2 pmpD

Restriction sites predicted using serovar D sequence data from the Chlamydia Sequencing Project are shown in fragment sizes predicted on restriction enzyme analysis of *pmpD* PCR product with *Bam*H1, *Bgl*II, *Hind*III and *Sac*I, individually and in combination are shown in Table 3-2 together with the actual fragment sizes seen on restriction enzyme analysis of the PCR product obtained by PCR (Figure 3-7).

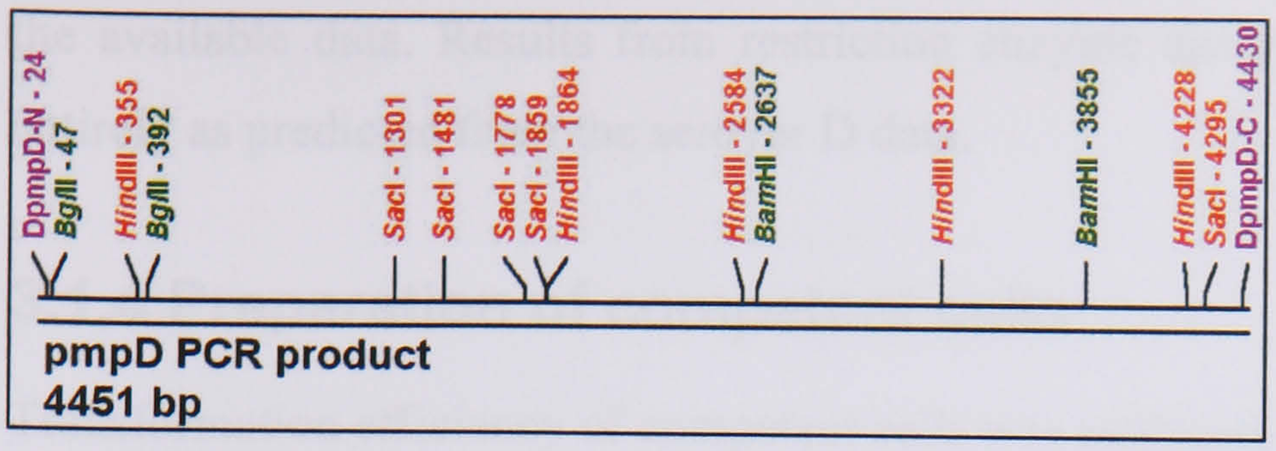


Figure 3-6: *pmpD* PCR product. Predicted restriction map

Figure 3-7: pmpD PCR product. Results of restriction enzyme analysis (*Bam*H1 + *Bgl*II digestion not shown)

Enzyme		Predicted	Actual
***Bam* H1**	*Cuts at*	2637, 3855	2637, 3855
	Fragment sizes	2637, 1218, 596	2637, 1218, 596
***Bgl* II**	*Cuts at*	47, 392	47, 392
	Fragment sizes	47, 345, 4059	47, 345, 4059
***Hind* III**	*Cuts at*	355, 1864, 2584, 3322, 4228	355, 1864, 2584, 3322, 4228
	Fragment sizes	355, 1509, 720, 738, 906, 223	355, 1509, 720, 738, 906, 223
***Sac* I**	*Cuts at*	1301, 1481, 1778, 1859, 4295	1301, 1481, 1859, either ~3000 or ~3200, 4295
	Fragment sizes	1301, 180, 297, 81, 2436, 156	1301, 180, 378, ~1300, ~1000, 156
***Bam* H1 + *Bgl* II**	*Cuts at*	47, 392, 2637, 3855	47, 392, 2637, 3855
	Fragment sizes	47, 345, 2245, 1218, 596	47, 392, 2637, 3855, *2637 - due to incomplete BglII digestion*
***Bam* H1 + *Hind* III**	*Cuts at*	355, 1864, 2584, 2637, 3322, 3855, 4228	355, 1864, 2584, 2637, 3322, 3855, 4228
	Fragment sizes	355, 1509, 720, 53, 685, 533, 373, 223	355, 1509, 720, 53, 685, 533, 373, 223
***Bgl* II + *Sac* I**	*Cuts at*	47, 392, 1301, 1481, 1778, 1859, 4295	47, 392, 1301, 1481, 1859, ~3000, 4295
	Fragment sizes	47, 345, 909, 180, 297, 81, 2436, 156	47, 345, 909, 180, 378, 1141, 1295, 156

Table 3-2: Predicted and observed fragments of the pmpD PCR product after restriction enzyme analysis

These results demonstrate a discrepancy between the results predicted from the sequence of serovar D and those seen with serovar A for restriction with *Sac*I. It appears that that our serovar A DNA has lost the *Sac*I site at 1778 but gained an additional *Sac*I site at either ~ 3000 or ~ 3200. It is not possible to determine this more accurately with

the available data. Results from restriction enzyme analysis with other enzymes was entirely as predicted from the serovar D data.

3.1.4 Preparation of competent cells

Transformation efficiency of competent cells was estimated as shown below.

Strain	Date	Transformation efficiency
XL1 Blue	Dec 97	2.0×10^6
XL1 Blue	May 98	1.4×10^6
TOP 10	July 98	2.1×10^5
BL21	July 99	5.1×10^5

Table 3-3: Transformation efficiencies of competent cells

3.1.5 Individual cloning strategies

3.1.5.1 Cloning of pTrcHis/pmpG-1410

PmpG DNA obtained by PCR was first ligated into pGEM-T® and the vector transformed in XL-1 Blue as described in section 2.1.9.1. The quick plasmid preparation method was used to extract plasmid DNA from the successfully-transformed cells and this was subjected to restriction enzyme analysis with *Sac*I, *Bam*H1, *Hind*III, *Bgl*II *Pst*I and *Hpa*II (*Msp*I), both individually and in combination. The predicted structure of this recombinant plasmid is shown in Figure 3-8. The results of electrophoretic analysis of the restriction products is shown in Figure 3-9, and the results of predicted and observed fragment sizes shown in Table 3-4.

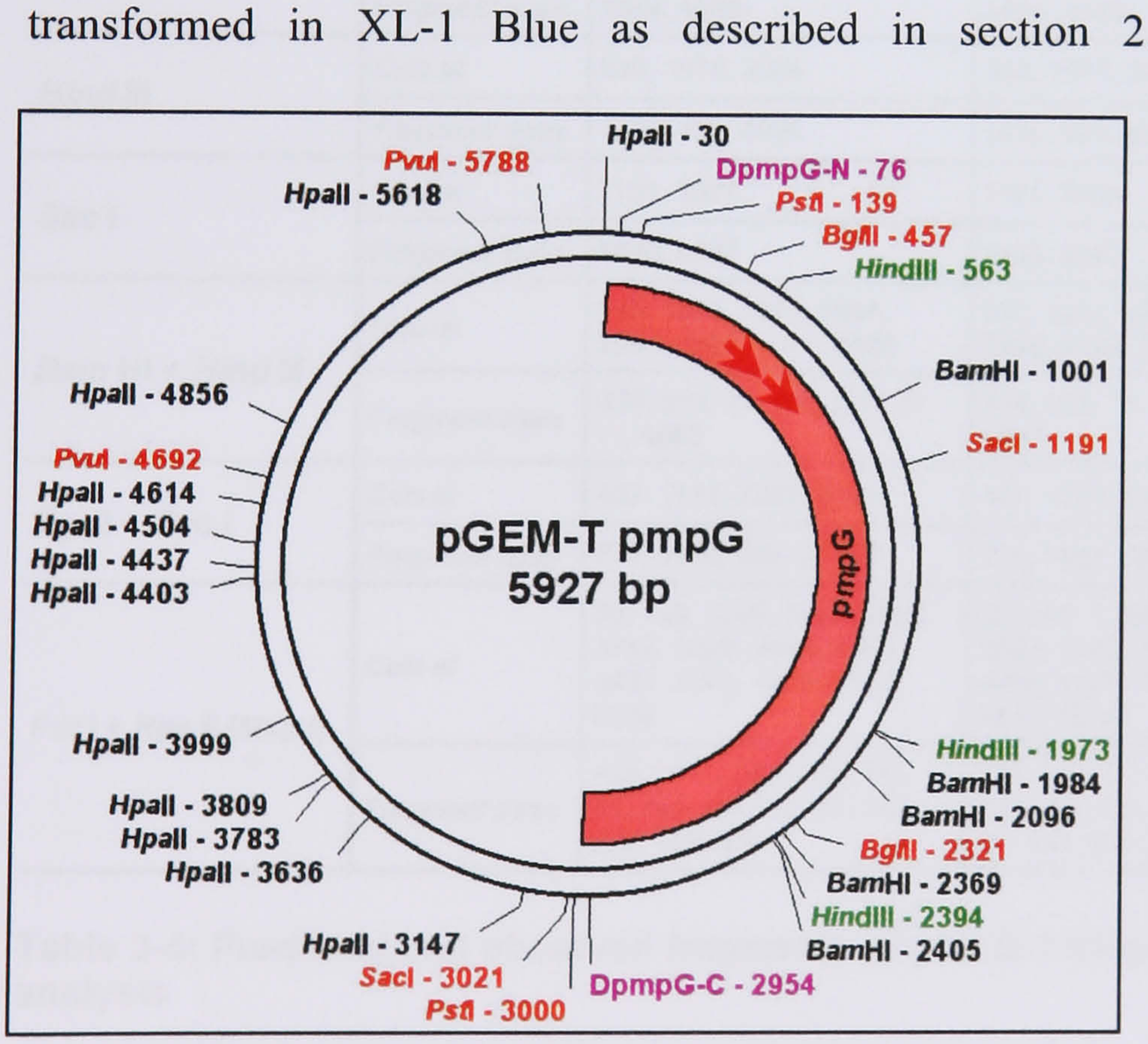


Figure 3-8: Predicted restriction map of pGEM-T/pmpG

Note: In this and subsequent figures the numbering of restriction sites begins at position 1 on the host vector, hence numbering on the insert will be different from that seen in the PCR maps.

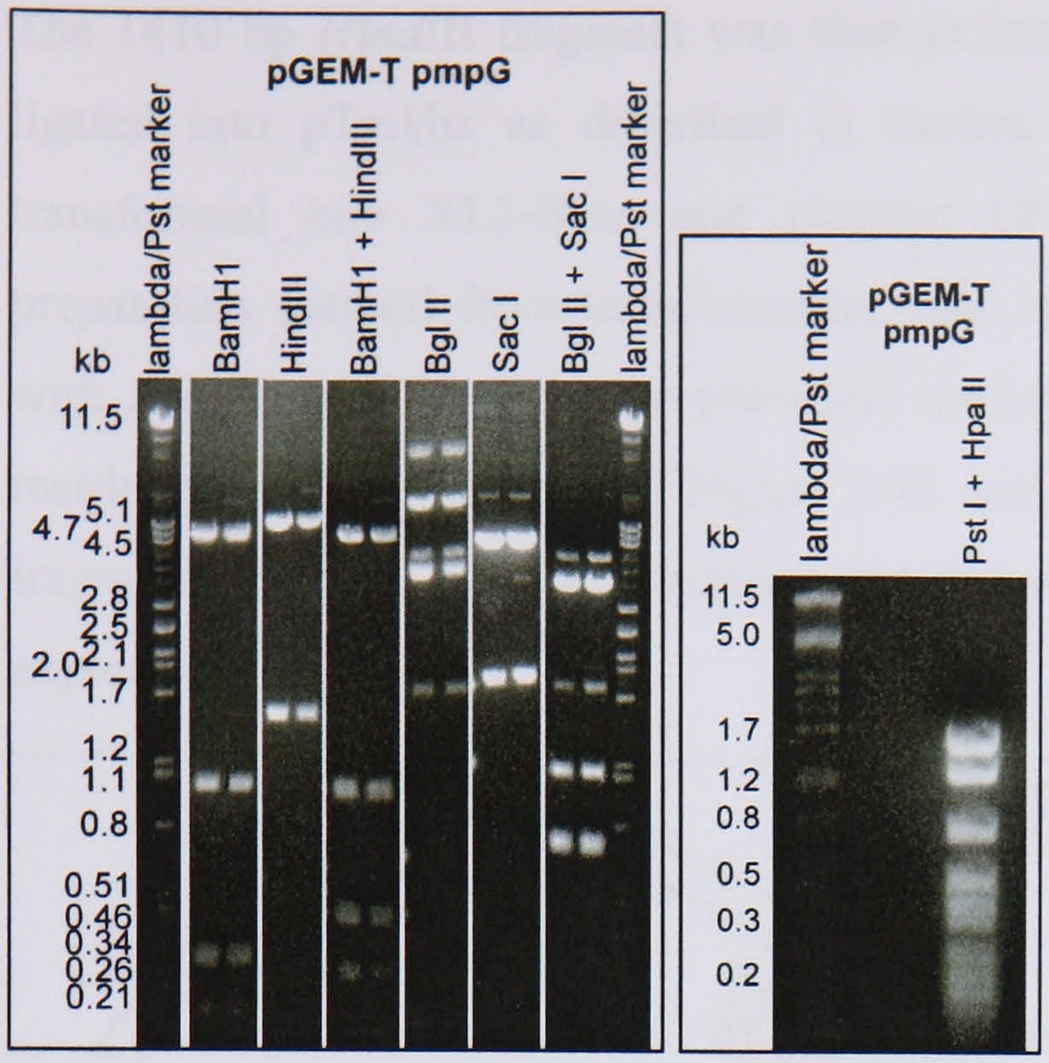


Figure 3-9: Restriction enzyme analysis of pGEM-T/pmpG

Enzyme		Predicted	Observed
***Bam* HI**	*Cuts at*	1001, 1984, 2096, 2369, 2405	1001, 1984, 2096, 2369, 2405
	Fragment sizes	983, 112, 273, 36, 4523	983, 112, 273, 36, 4523
***Bgl* II**	*Cuts at*	457, 2321	457, 2321
	Fragment sizes	1864, 4063	1864, 4063
***Hind* III**	*Cuts at*	563, 1973, 2394	563, 1973, 2394
	Fragment sizes	1410, 421, 4096	1410, 421, 4096
***Sac* I**	*Cuts at*	1191, 3021	1191, 3021
	Fragment sizes	1830, 4097	1830, 4097
***Bam* HI + *Hind* III**	*Cuts at*	563, 1001, 1973, 1984, 2096, 2369, 2394, 2405	563, 1001, 1973, 1984, 2096, 2369, 2405
	Fragment sizes	438, 972, 11, 112, 273, 25, 11, 4085	438, 972, 11, 112, 273, 36, 4085
***Bgl* II + *Sac* I**	*Cuts at*	457, 1191, 2321, 3021	457, 1191, 2321, 3021
	Fragment sizes	734, 1130, 700, 3363	734, 1130, 700, 3363
***Pst* I + *Hpa* II (*Msp* I)**	*Cuts at*	30, 139, 3000, 3147, 3636, 3783, 3809, 3999, 4403, 4437, 4504, 4614, 4856, 5618	30, 139, ?, 3000, 3147, 3636, 3783, 3809, 3999, 4403, 4437, 4504, 4614, 4856, 5618
	Fragment sizes	109, 2861, 147, 489, 147, 26, 190, 404, 34, 67, 110, 242, 762, 339	109, ~1600, ~1200, 147, 489, 147, 26, 190, 404, 34, 67, 110, 242, 762, 339

Table 3-4: Predicted and observed fragments of pGEM-T/pmpG after restriction enzyme analysis

These results are in agreement with those seen on restriction enzyme analysis of the *pmpG* PCR product and appear to indicate a loss of the *Hind*III site at position 2394. The results also confirm the presence of an additional *Pst*I site within the *pmpG* PCR fragment. In all other respects the results are as predicted, confirming the expected insert.

The 1410 bp *Hind*III fragment was then isolated from high-purity plasmid DNA and ligated into pTrcHis as described in section 2.1.9.1. The resultant construct was transformed into XL1-Blue and plasmid DNA, extracted by the quick plasmid preparation method from transformants, was screened by restriction enzyme analysis with *Hind*III and *Bam*HI. The predicted restriction map is shown in Figure 3-10, the restriction enzyme analysis in Figure 3-11 and the predicted and observed restriction fragments in Table 3-5. These confirm the correct fragment in the correct orientation for expression.

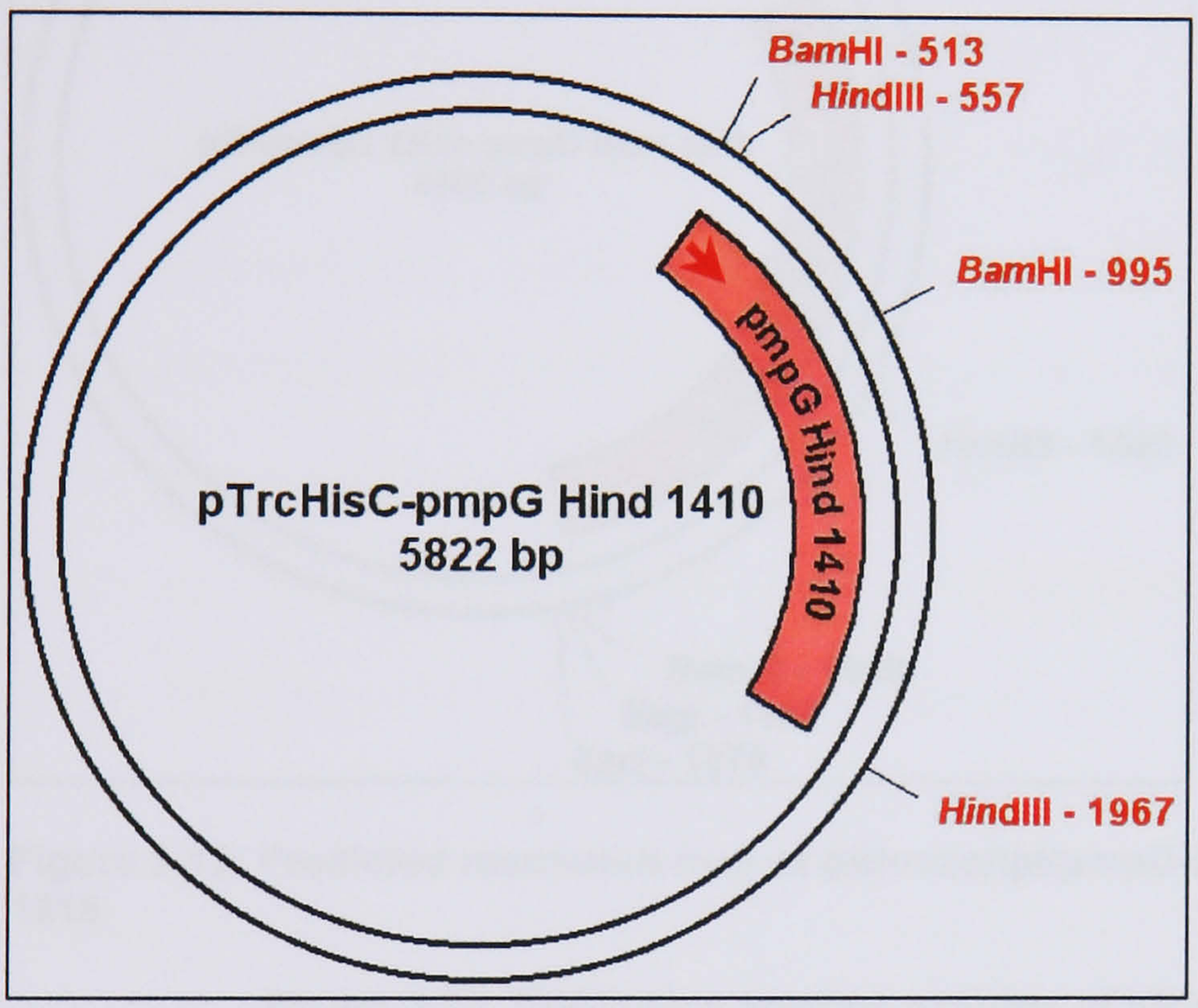


Figure 3-10: Predicted restriction map of pTrcHis/pmpG-1410

Figure 3-11: Restriction enzyme analysis of pTrcHis/pmpG-1410

Enzyme		Predicted	Observed
***Bam* HI**	*Cuts at*	513, 995	513, 995
	Fragment sizes	482, 5340	5340, 482
***Hind* III**	*Cuts at*	557, 1967	557, 1967
	Fragment sizes	1410, 4412	1410, 4412

Table 3-5: Predicted and observed restriction fragments of pTrcHis/pmpG-1410

3.1.5.2 Cloning of pBluescript/pmpD-1218

PmpD DNA was obtained by PCR as shown (Section 3.1.2). A 1218bp *Bam*HI restriction fragment (Figure 3-7) was purified and ligated into pBluescript SKII+ (Figure 3-12) as described in section 2.1.9.2.

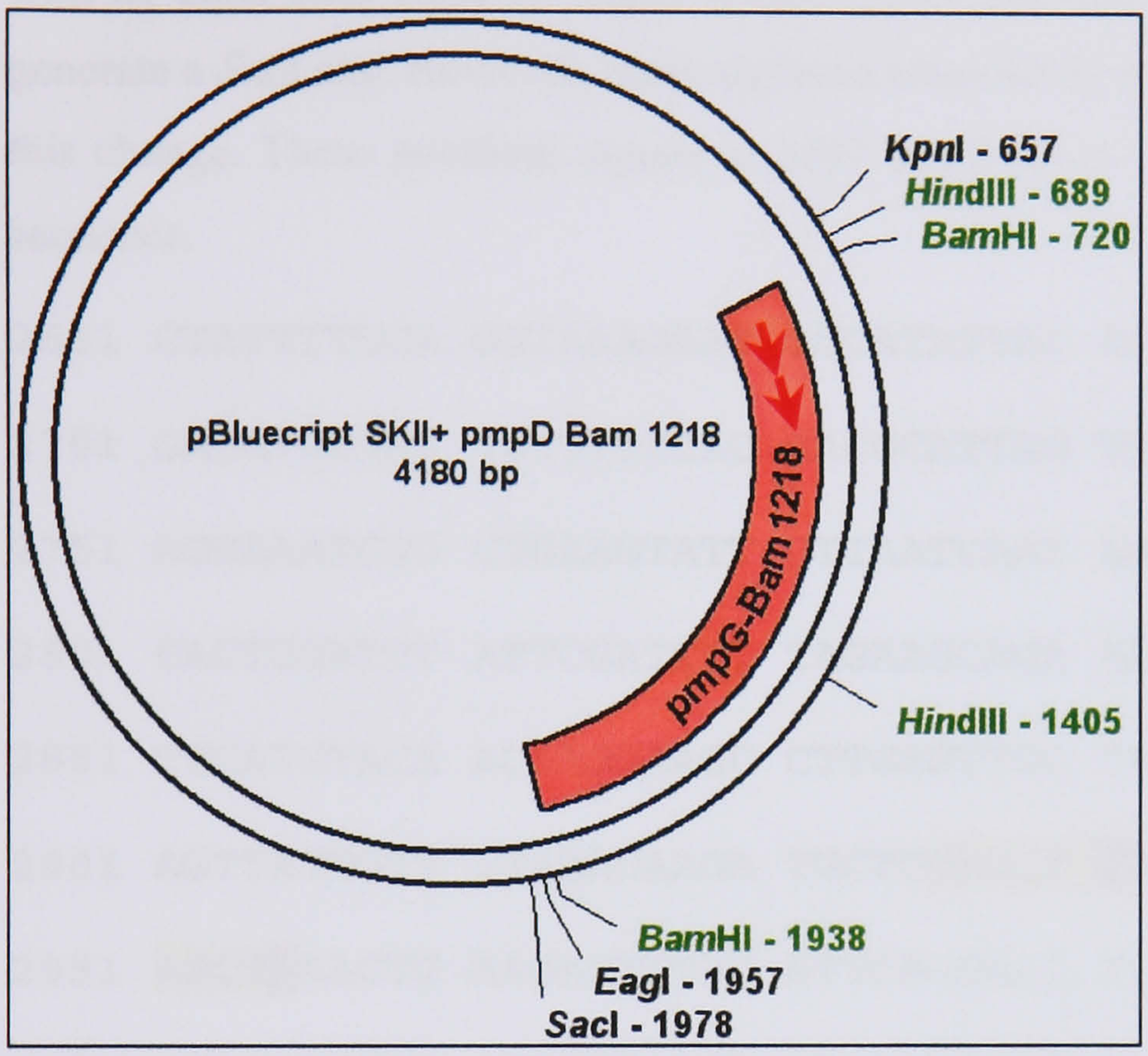


Figure 3-12: Predicted restriction map of pBluescript/pmpD-Bam-1218

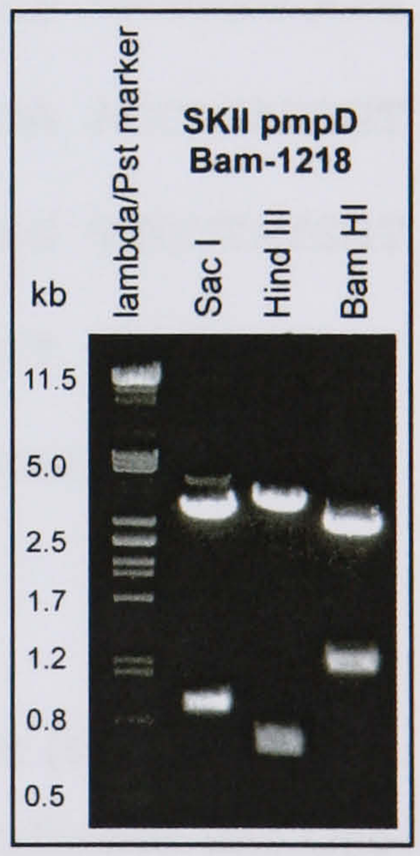


Figure 3-13: Restriction enzyme analysis of pBluecript/ pmpD-Bam-1218 (*Kpn*I + *Eag*I digestion not shown)

Enzyme		Predicted	Observed
***Bam* H1**	*Cuts at*	720, 1938	720, 1938
	Fragment sizes	1218, 2962	1218, 2962
***Hind* III**	*Cuts at*	689, 1405	689, 1405
	Fragment sizes	716, 3464	716, 3464
***Sac* I**	*Cuts at*	1978	1978, ~2950
	Fragment sizes	4180	3230, 950
***Kpn* I + *Eag* I**	*Cuts at*	657, 1957	657, 1957
	Fragment sizes	1300, 2880	1300, 2880

Table 3-6: Predicted and observed fragments of pBluescript/pmpD-Bam-1218 after restriction enzyme analysis

Confirmation of the correct fragment was obtained by restriction enzyme analysis with *Bam*HI, *Hind*III, *Kpn*I and *Eag*I as shown in Figure 3-13 and Table 3-6.

The additional *Sac*I site within the cloned *pmpD* DNA was again detected and can now be placed more accurately at around position 2950. On examining the sequence there are two sites at which single point mutations from adenine to cytosine might lead to the formation of a novel *Sac*I site (GAGCTC) at positions 2931 and 2955 (Figure 3-14), with no other sites close by where another point mutation, insertion or deletion would generate a *Sac*I site. However, more rigorous sequencing would be necessary to confirm this change. These positions equate to 3097 and 3121 in the original serovar D *pmpD* sequence.

```
2651 CTATTTTGCA GGTAAAGAAT CGCATATTAC AGCCCTGAAT GCTACGGAAG

2701 GACATGCTAT TGTTTTCCAC GACGCATTAG TTTTTGAAAA TCTAGAAGAA

2751 AGGAAATCTG CTGAAGTATT GTTAATCAAT AGTCGAGAAA ATCCAGGTTA

2801 CACTGGATCT ATTCGATTTT TAGAAGCAGA AAGTAAAGTT CCTCAATGTA

2851 TTCATGTACA ACAAGGAAGC CTTGAGTTGC TAAATGGAGC CACATTATGT

2901 AGTTATGGTT TTAAACAAGA TGCTGGAGCT AAGTTGGTAT TGGCTGCTGG

2951 AGCTAAACTG AAGATTTTAG ATTCAGGAAC TCCTGTACAA CAAGGGCATG

3001 CTATCAGTAA ACCTGAAGCA GAAATCGAGT CATCTTCTGA ACCAGAGGGT

3051 GCACATTCTC TTTGGATTGC GAAGAATGCT CAAACAACAG TTCCTATGGT

3101 TGATATCCAT ACTATTTCTG TAGATTTAGC CTCCTTCTCT TCTAGTCAAC
```

Figure 3-14: Potential point mutations leading to a novel *Sac*I site in *pmpD*

3.1.5.3 Cloning of pBAD/pmpG

PmpG DNA was obtained by high-fidelity PCR as described above (section 2.1.2.2). The purified PCR product was ligated into pBAD-TOPO® and transformed into One-Shot® competent cells. DNA, extracted from the clones by the quick plasmid preparation method was subjected to restriction enzyme analysis with *Eco*RV and *Nco*I, individually and in combination. The predicted map of this recombinant plasmid is shown in Figure 3-15. The results of electrophoretic analysis of the restriction products is shown in Figure 3-16, and the results of predicted and observed fragment sizes shown in Table 3-7.

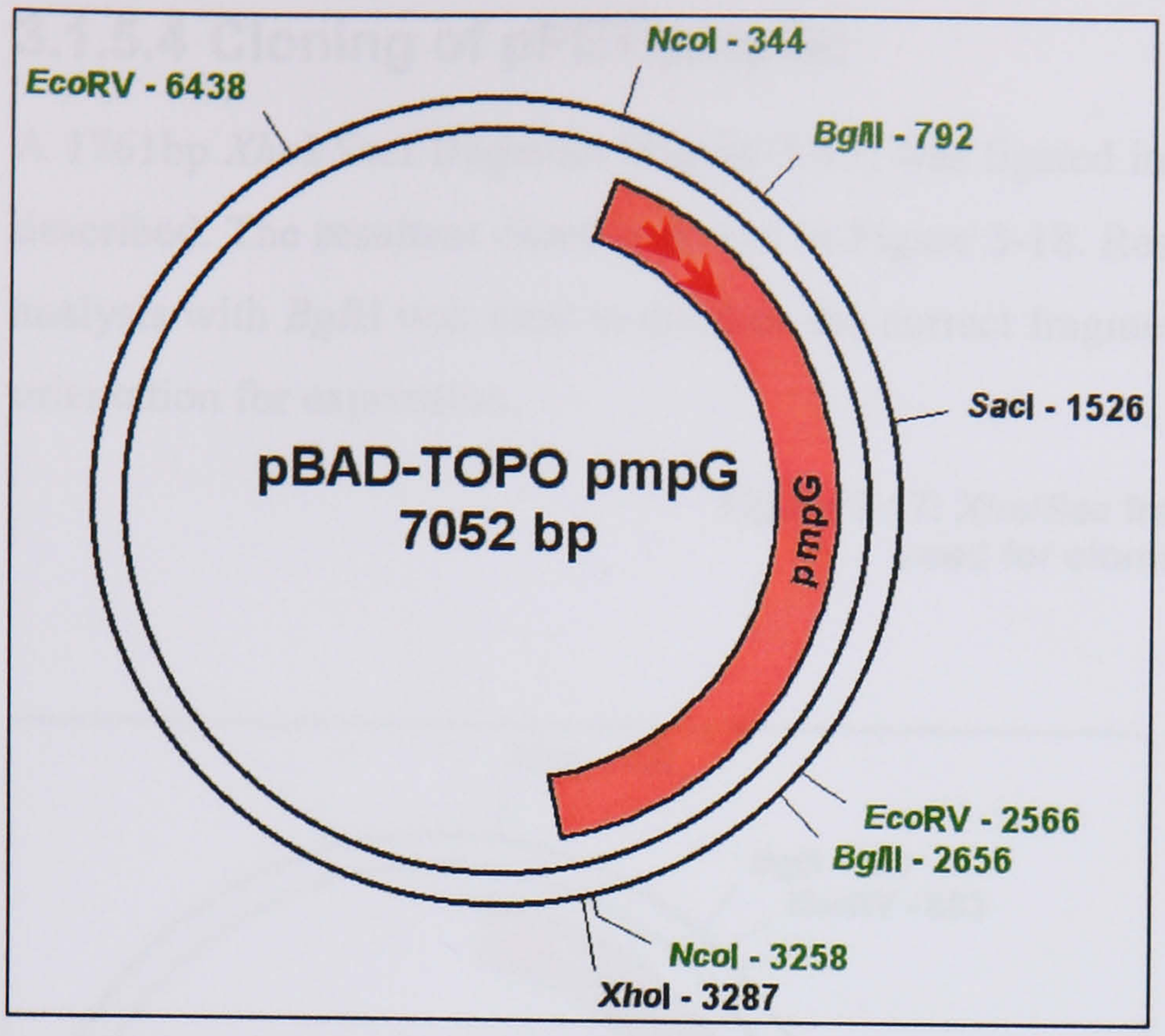
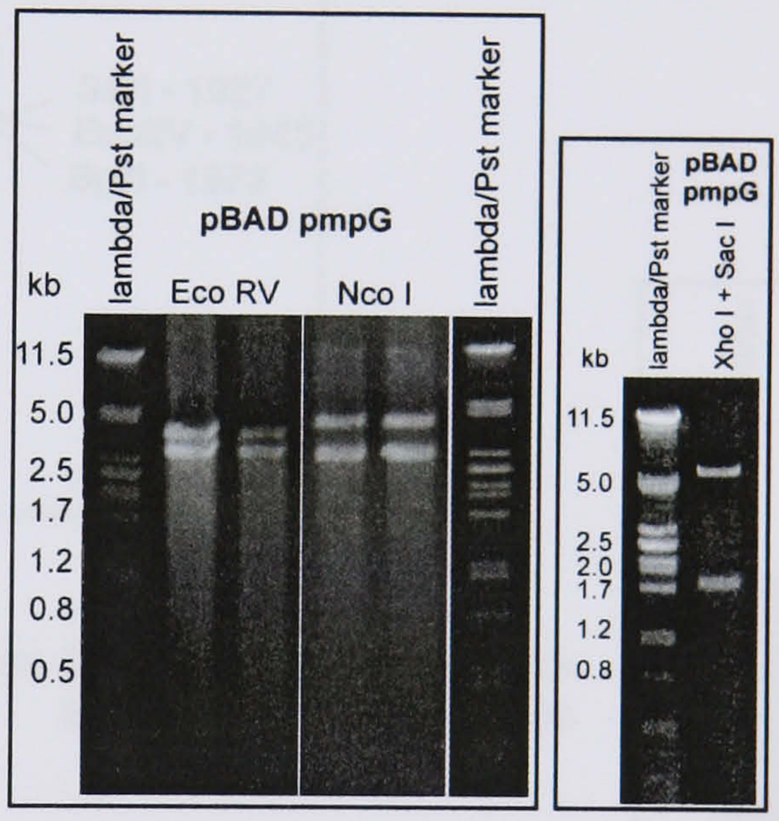


Figure 3-15: Predicted restriction map of pBAD/pmpG

Figure 3-16: Restriction enzyme analysis of pBAD/pmpG



Enzyme		Predicted	Observed
***Eco* RV**	*Cuts at*	2566, 6438	2566, 6438
	Fragment sizes	3180, 3872	3180, 3872
***Nco* I**	*Cuts at*	344, 3258	344, 3258
	Fragment sizes	2914, 4138	2914, 4138
***Xho* I + *Sac* I**	*Cuts at*	1526, 3287	1526, 3287
	Fragment sizes	1761, 5291	1761, 5291

Table 3-7: Predicted and observed fragments of pBAD/pmpG after restriction enzyme analysis

The restriction enzyme analysis demonstrates that the vector contains the expected insert in the correct orientation for expression.

3.1.5.4 Cloning of pPET/pmpGc

A 1761bp *Xho*I/*Sac*I fragment (Figure 3-17) was ligated into pPET30a® as described. The resultant clone is shown in Figure 3-18. Restriction enzyme analysis with *Bgl*II was used to confirm the correct fragment in the correct orientation for expression.

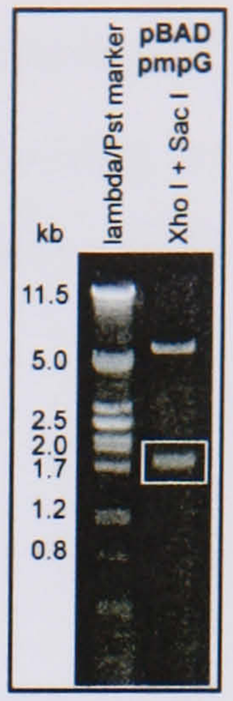


Figure 3-17: *Xho*/*Sac* fragment of pmpG used for cloning pPET/pmpGc

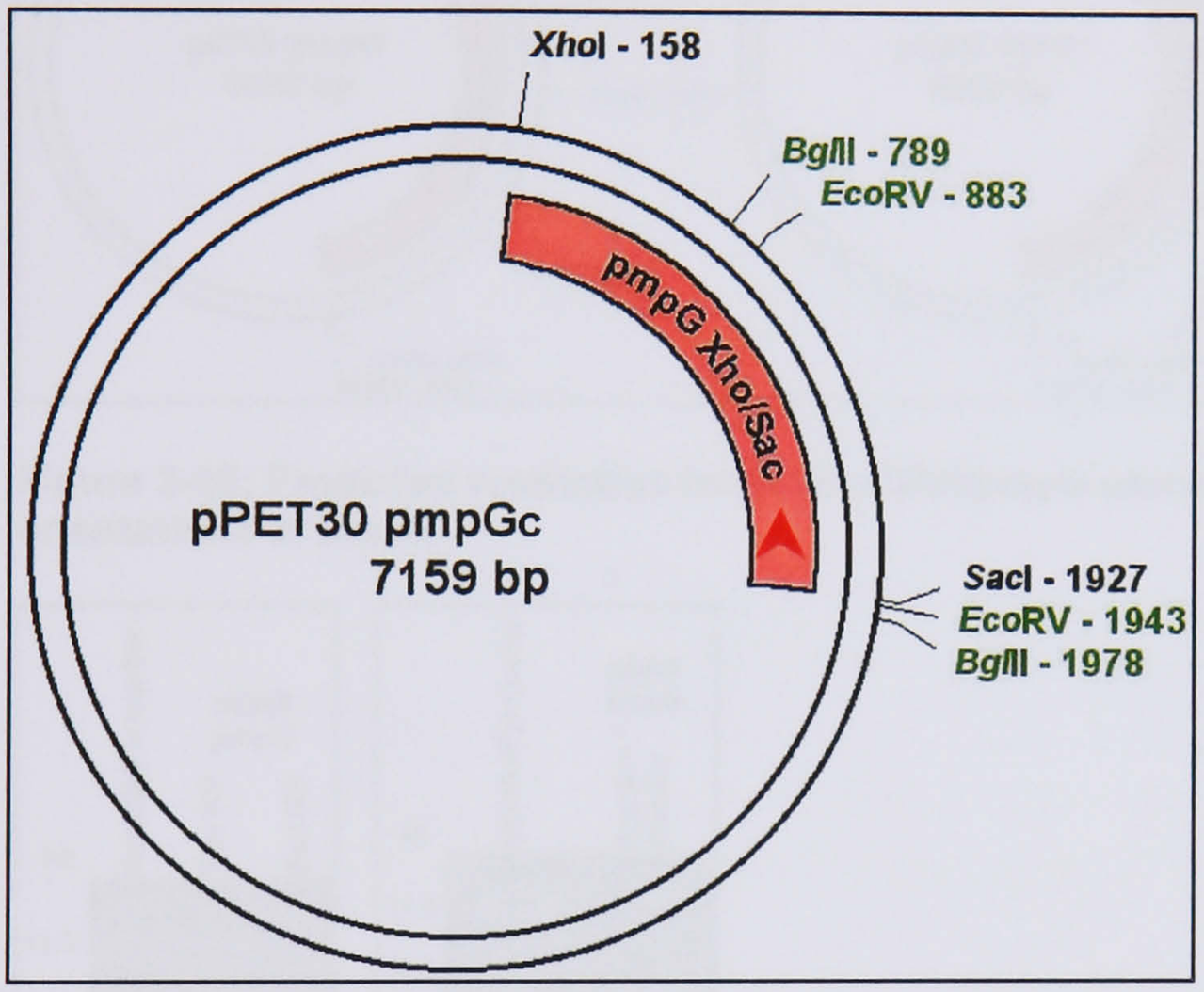


Figure 3-18: Predicted restriction map of pPET/pmpGc

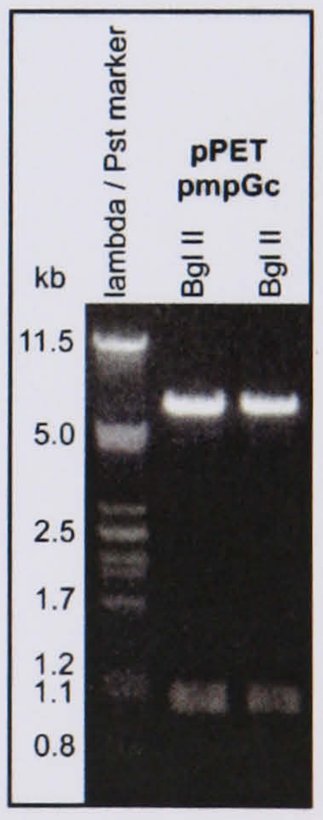


Figure 3-19: Restriction enzyme analysis of pPET/pmpGc

Enzyme		Predicted	Observed
***Bgl* II**	*Cuts at*	789, 1978	789, 1978
	Fragment sizes	1189, 5970	1189, 5970

Table 3-8: Predicted and observed fragments of pPET/pmpGc after restriction enzyme analysis

The restriction enzyme analysis confirms that the vector contains the fragment in the correct orientation.

3.1.5.5 Cloning of pCR-II/pmpG

pmpG DNA obtained through Pfu PCR amplification was ligated into pCR-II-TOPO® as described (section 2.1.9.5). The possible orientations of this insert are shown in Figure 3-20. Restriction enzyme analysis with *Eco*RI and *Eco*RV (Figure 3-21) indicated that the *pmpG* DNA had been inserted in reverse orientation.

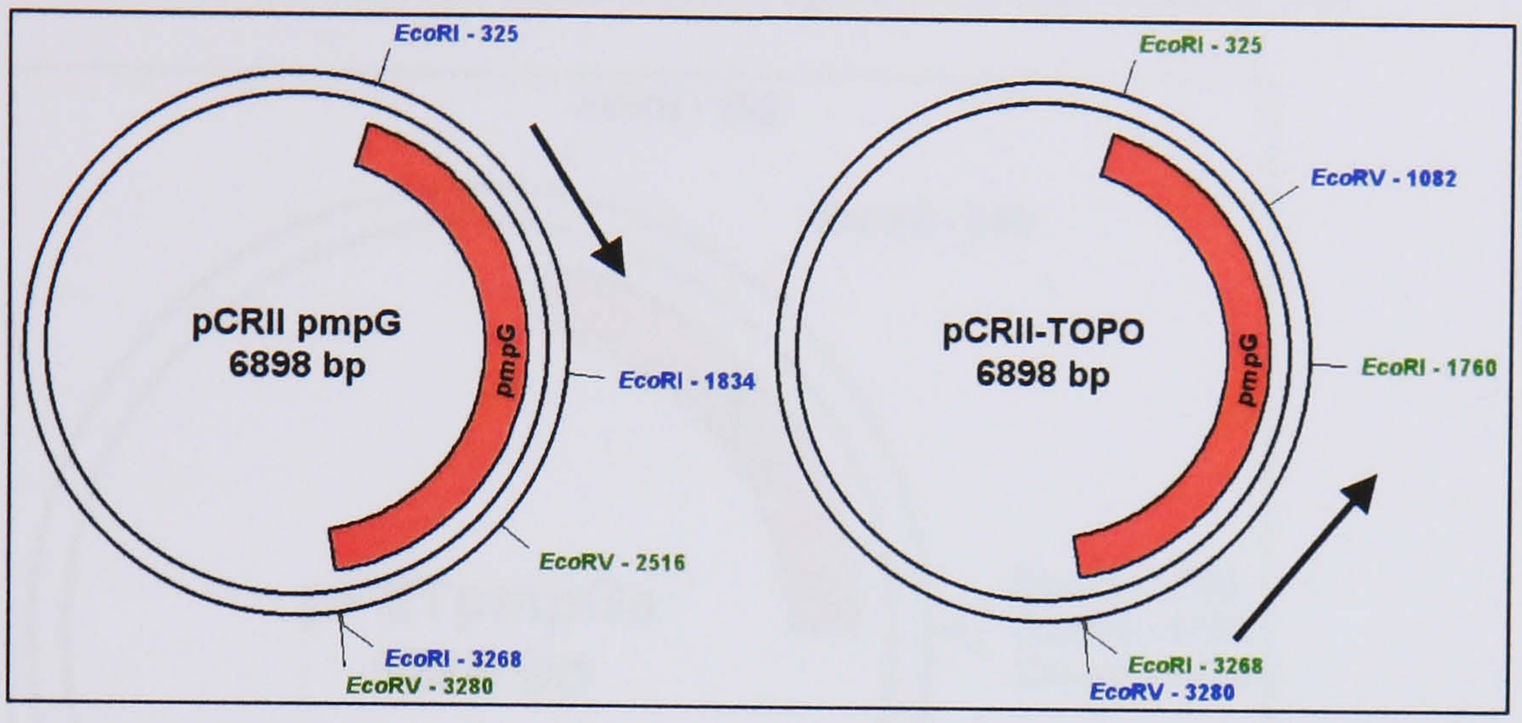


Figure 3-20: Predicted restriction maps of pCRII/pmpG showing both forward and reverse orientations of insert

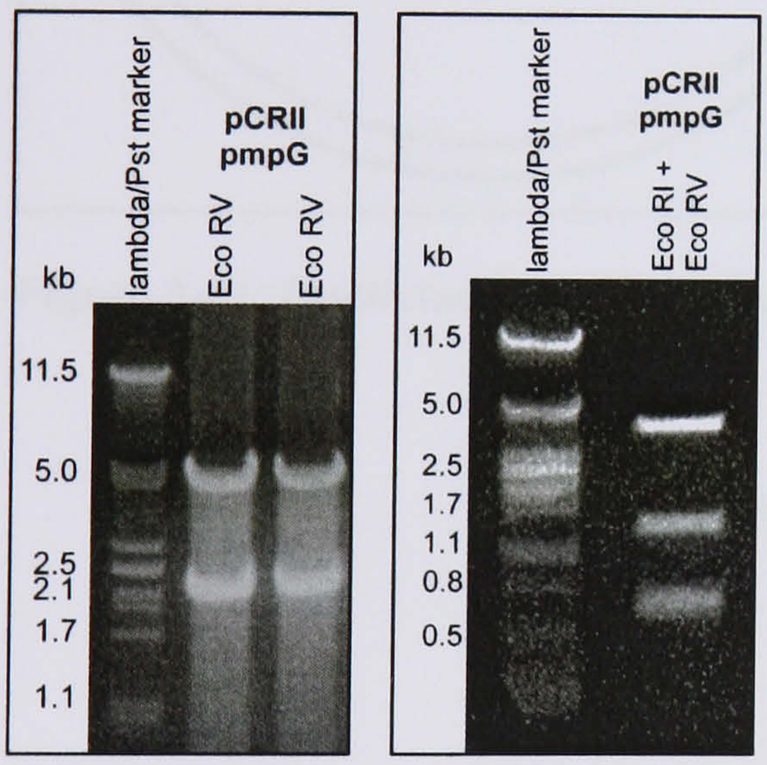


Figure 3-21: Restriction enzyme analysis of pCRII/pmpG

Enzyme		Predicted	Predicted (reversed)	Observed
***Eco* RV**	*Cuts at*	2516, 3280	1082, 3280	1082, 3280
	Fragment sizes	764, 6134	2198, 4700	4700, 2198
***Eco* RV + *Eco* RI**	*Cuts at*	325, 1834, 2516, 3268, 3280	325, 1082, 1760, 3268, 3280	325, 1082, 1760, 3268, 3280
	Fragment sizes	1508, 682, 752, 12, 3943	757, 678, 1508, 12, 3943	757, 682, 1503, 12, 3943

Table 3-9: Predicted and observed fragments of pCRII/pmpG after restriction enzyme analysis

3.1.5.6 Cloning of pPET/pmpGa

A 1509bp *Eco*R1 fragment was released from pCR-II/pmpG and ligated into *Eco*R1 digested pPET30a® described. The predicted clone is shown in Figure 3-22. Restriction enzyme analysis with *Bam*H1 confirmed all recombinants contained the fragment in the correct orientation for expression (Figure 3-23 and Table 3-10)

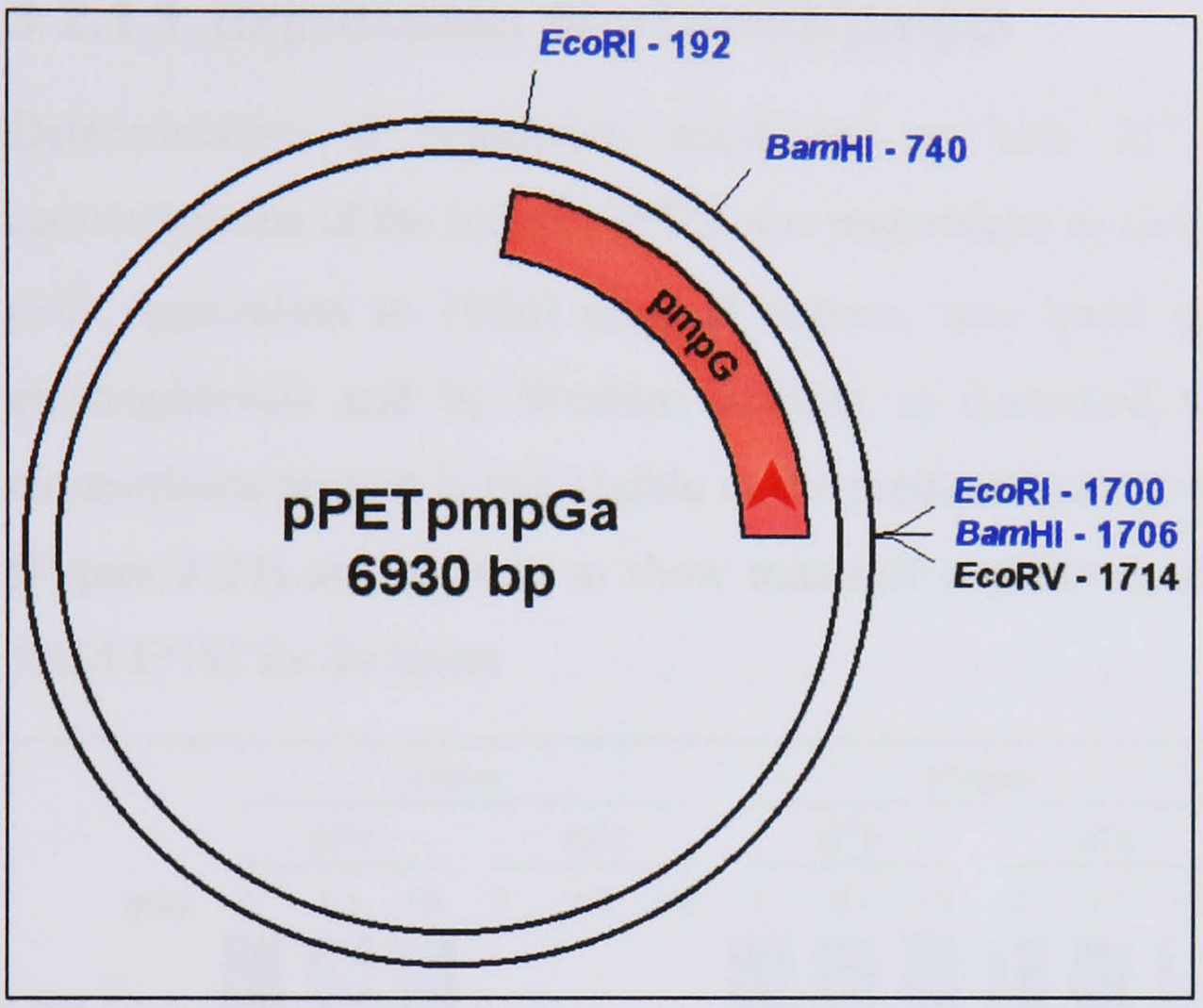


Figure 3-22: Predicted restriction map of pPET/pmpGa

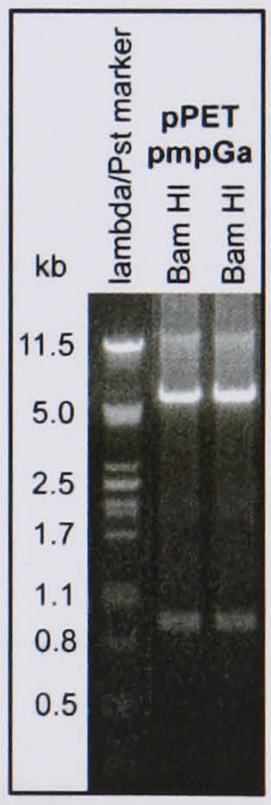


Figure 3-23: Restriction enzyme analysis of pPET/pmpGa

Enzyme		Predicted	Observed
***Bam* HI**	*Cuts at*	740, 1706	740, 1706
	Fragment sizes	966, 5964	966, 5964

Table 3-10: Predicted and observed fragments of pPET/pmpGa after restriction enzyme analysis

3.2 Expression and purification of recombinant proteins

3.2.1 pTrcHis/pmpG

3.2.1.1 Expression of pTrcHis/pmpG

Determination of expression conditions at both 25^{O} and 37^{O}C under varying concentrations of the inducer IPTG was undertaken as described. An aliquot of pelleted cells, equivalent to 100µl original culture, was lysed and analysed by SDS-PAGE electrophoresis and by Western blotting as described in section 2.2.1. A band of recombinant protein is just visible at the predicted size (54kDa) on the SDS-PAGE gel (Figure 3-24) and appears to show maximal expression after incubation at 37^{O}C with 1mM IPTG for 20 hours.

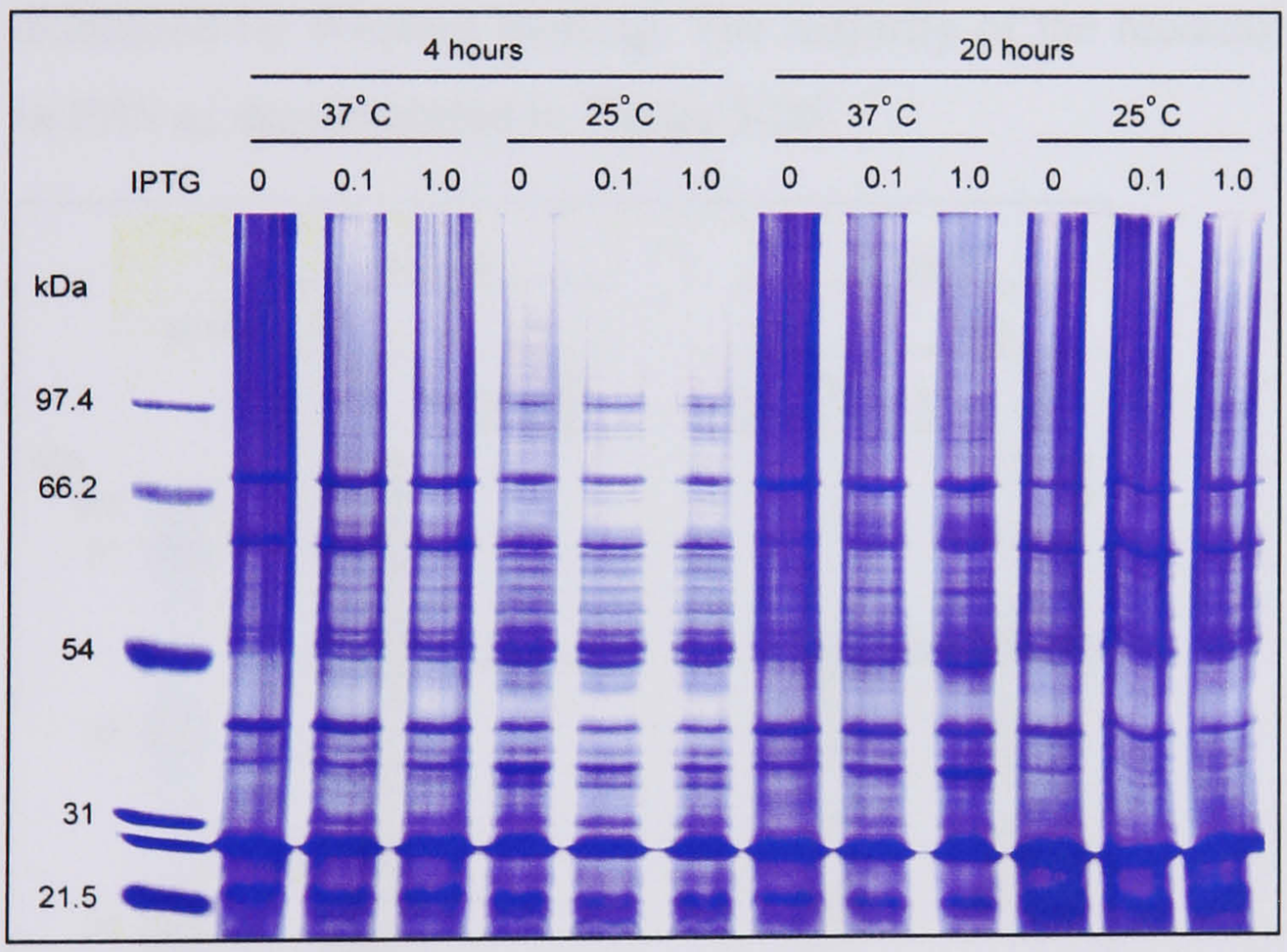
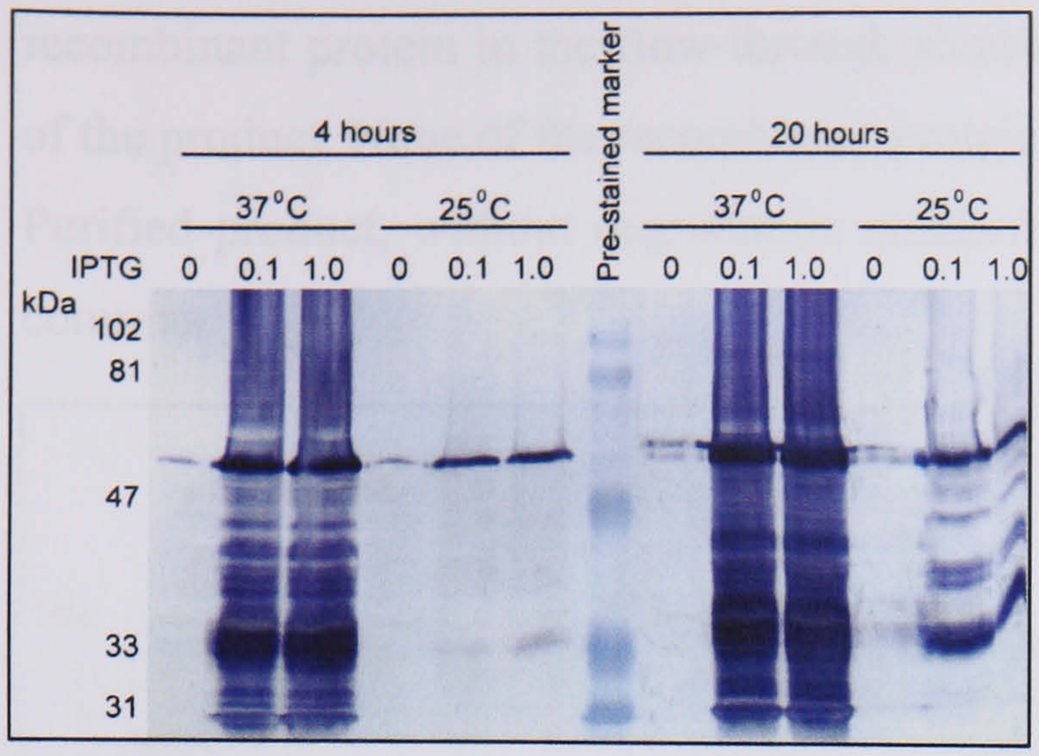


Figure 3-24: Expression of recombinant protein from pTrcHis/pmpG - SDS-PAGE

Inspection of the Western blot detected with anti-Xpress® antibody (Figure 3-24) revealed that observable quantities of recombinant proteins were produced at 0.1mM at 25^{O}C after 4 hours, with fewer degradation products than were apparent at 37^{O}C or after 20 hours. These incubation conditions were therefore chosen as the optimal expression conditions for larger-scale production of recombinant protein.

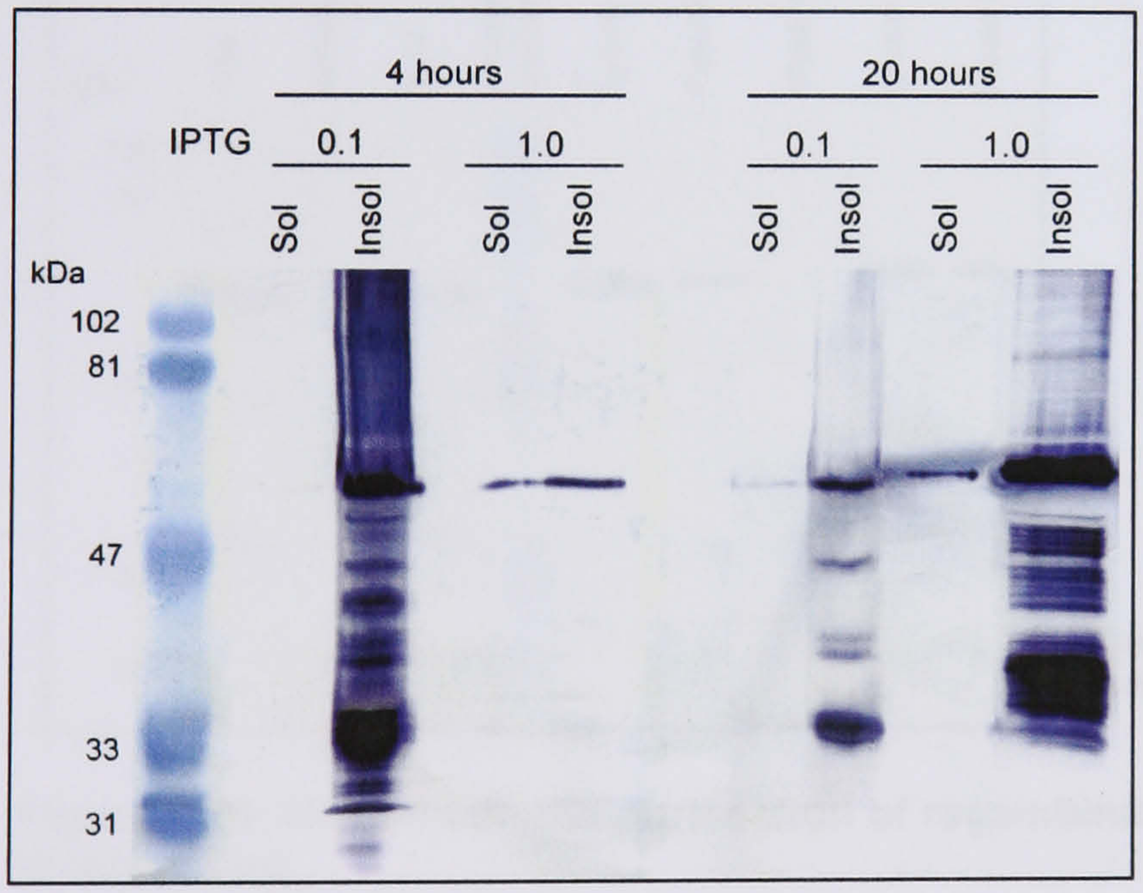


Western blot probed with Anti-Xpress® antibody (Invitrogen)

Figure 3-25: Expression of recombinant protein from pTrcHis/pmpG - Western blot

3.2.1.2 Determination of solubility of recombinant proteins from pTrcHis/pmpG

The solubility of the recombinant proteins in PBS was determined as described in section 2.3.1.1. Five microlitre aliquots, equivalent to 100µl original culture were examined by Western blotting. The majority of the recombinant product was insoluble in PBS as demonstrated in Figure 3-26.



Western blot probed with Anti-Xpress® antibody (Invitrogen)

Figure 3-26: Solubility of recombinant protein from pTrcHis/pmpG - Western blot

3.2.1.3 Purification of recombinant proteins from pTrcHis/pmpG

Purification of an induced bulk culture of pTrcHis/pmpG was undertaken using Ni-NTP columns under denaturing conditions as described in section 2.3.3. The recombinant protein was eluted first with 200µl of elution buffer and then again with 100µl. The recombinant protein band was not visible on SDS-PAGE analysis (Figure 3-27) but was clearly seen on Western blotting (Figure 3-28). The presence of

recombinant protein in the flow-through showed that the column was not able bind all of the product. None of the recombinant protein was removed during the washing phase. Purified product, without degradation contaminants, was successfully eluted from the columns.

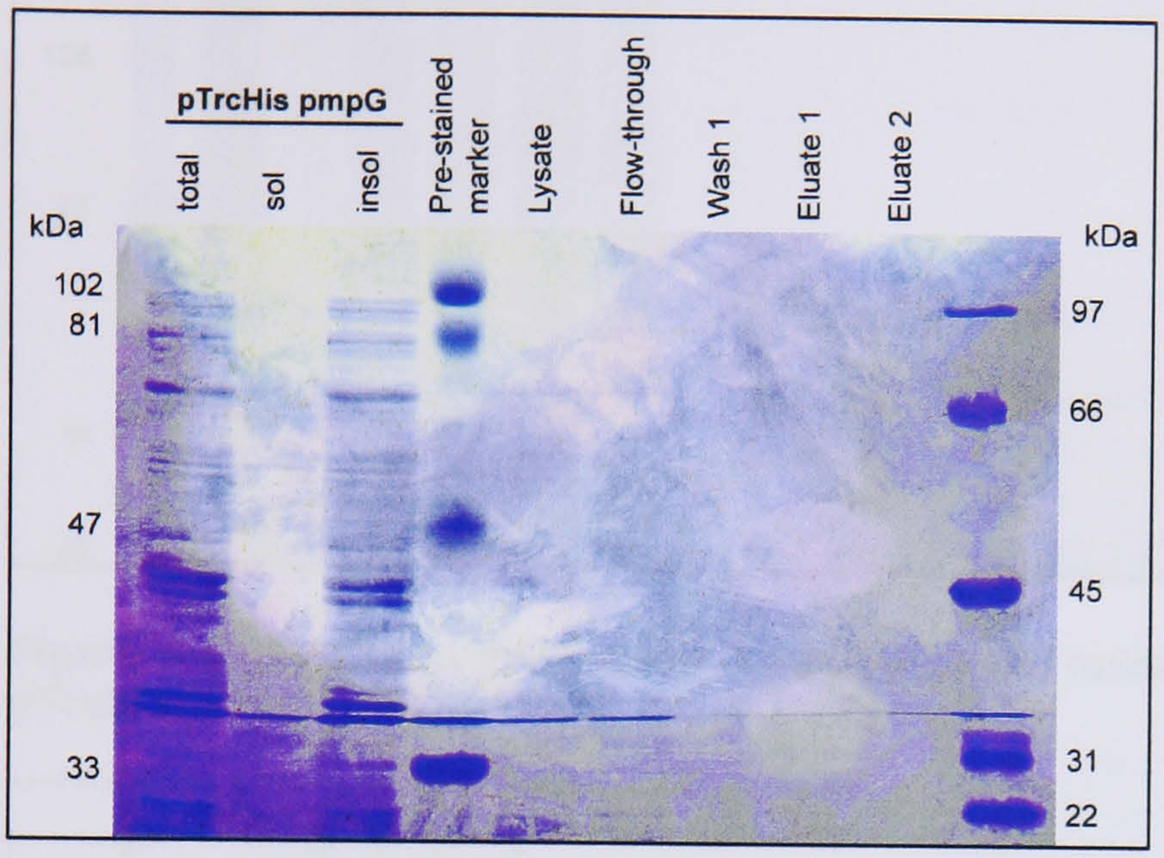
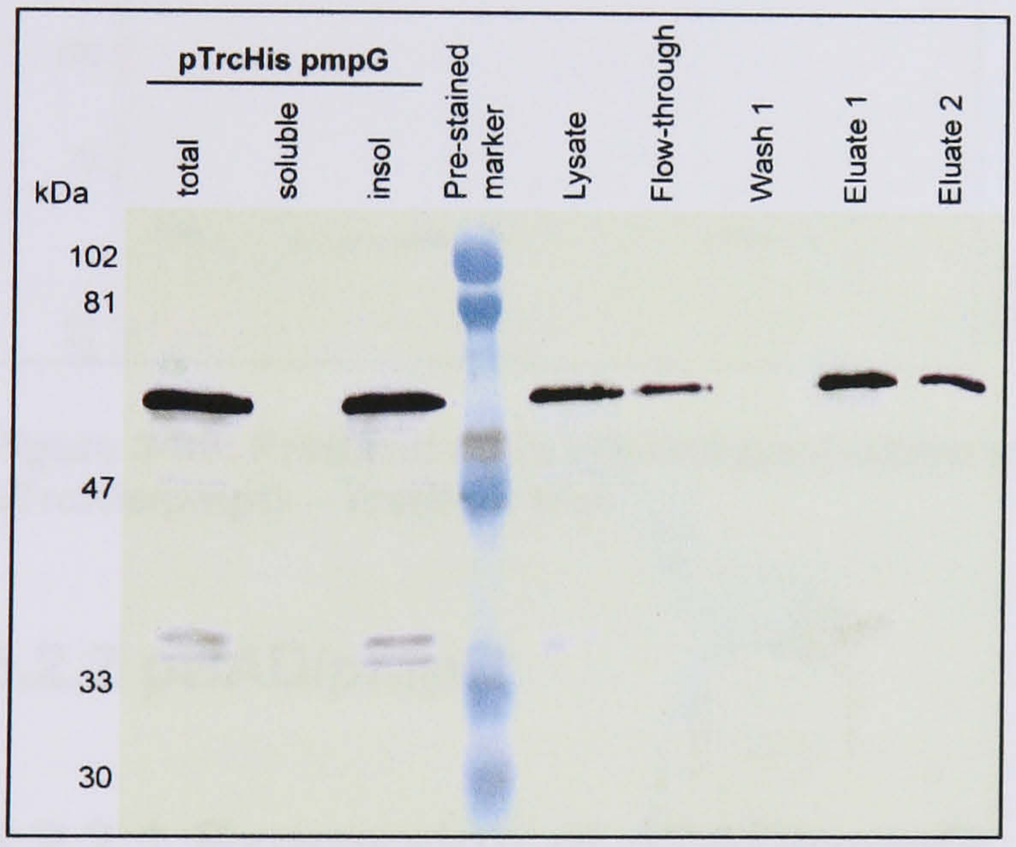


Figure 3-27: Ni-NTP column purification of recombinant protein from pTrcHis/pmpG - SDS-PAGE



Western blot probed with Anti-Xpress® antibody (Invitrogen)

Figure 3-28: Ni-NTP column purification of recombinant protein from pTrcHis/pmpG - Western blot

Purification was also undertaken using a Pro-Bond resin column as described in section 2.3.4. Using this method the recombinant protein band was observed on SDS-PAGE (Figure 3-29) and is clearly visible on the Western blot (Figure 3-30). The column was clearly saturated with the first aliquot of lysate with excess recombinant protein then appearing in the flow-through. No recombinant protein was lost in the wash and the majority eluted in the first two eluate volumes.

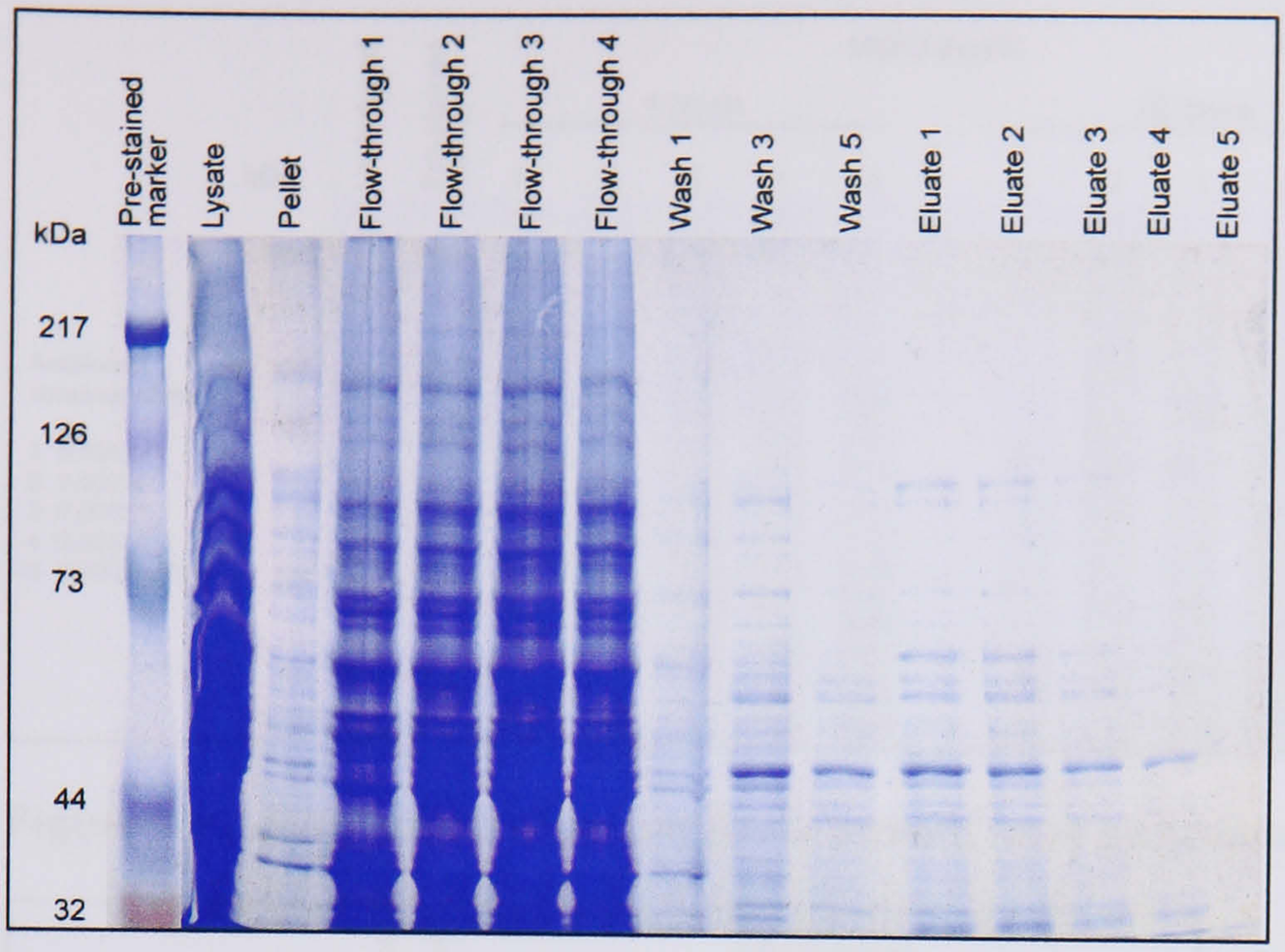
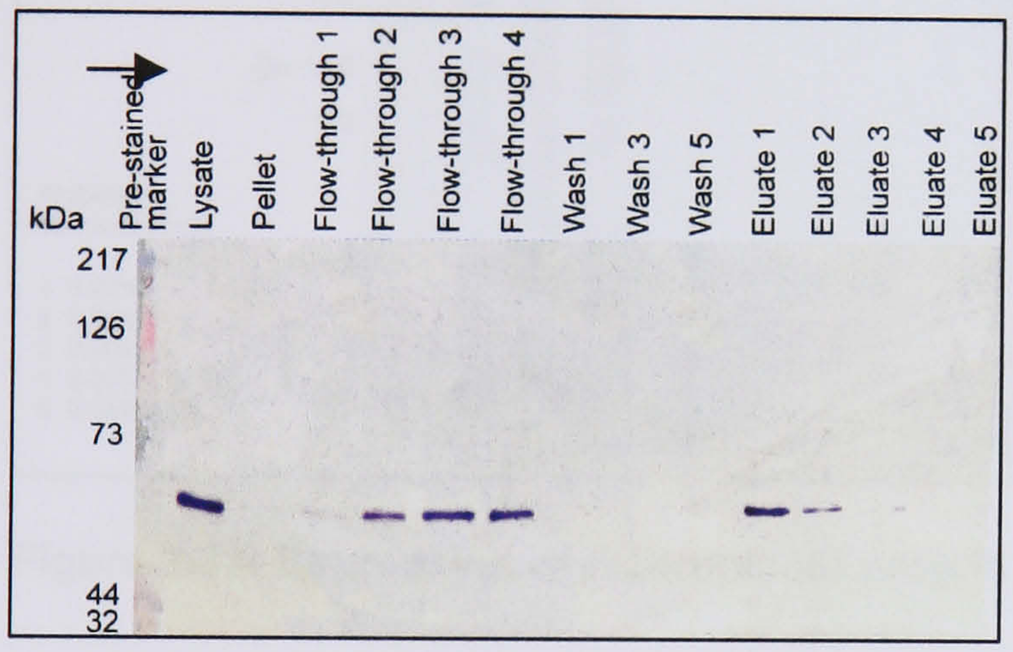


Figure 3-29: ProBond resin column purification of recombinant protein from pTrcHis/pmpG - SDS-PAGE



Western blot probed with Anti-Xpress® antibody (Invitrogen)

Figure 3-30: ProBond resin column purification of recombinant proteins from pTrcHis/pmpG - Western blot

3.2.2 pBAD/pmpG

3.2.2.1 Expression of pBAD/pmpG

Determination of expression conditions at both 25^{O} and 37^{O}C under varying concentrations of the arabinose inducer was undertaken as described in section 2.2.3. An aliquot of pelleted cells, equivalent to 50µl original culture, was lysed and analysed by SDS-PAGE electrophoresis and by Western blotting as described in section 2.2.1. No bands of recombinant protein were visible on SDS-PAGE analysis (Figure 3-31) . A recombinant band was observed after Western blotting using anti-V5 antibody (Figure 3-32), although only small quantities were evident together with numerous degradation products. Optimal expression conditions were determined to be 4 hours incubation at 25^{O}C in the presence of 0.002% arabinose.

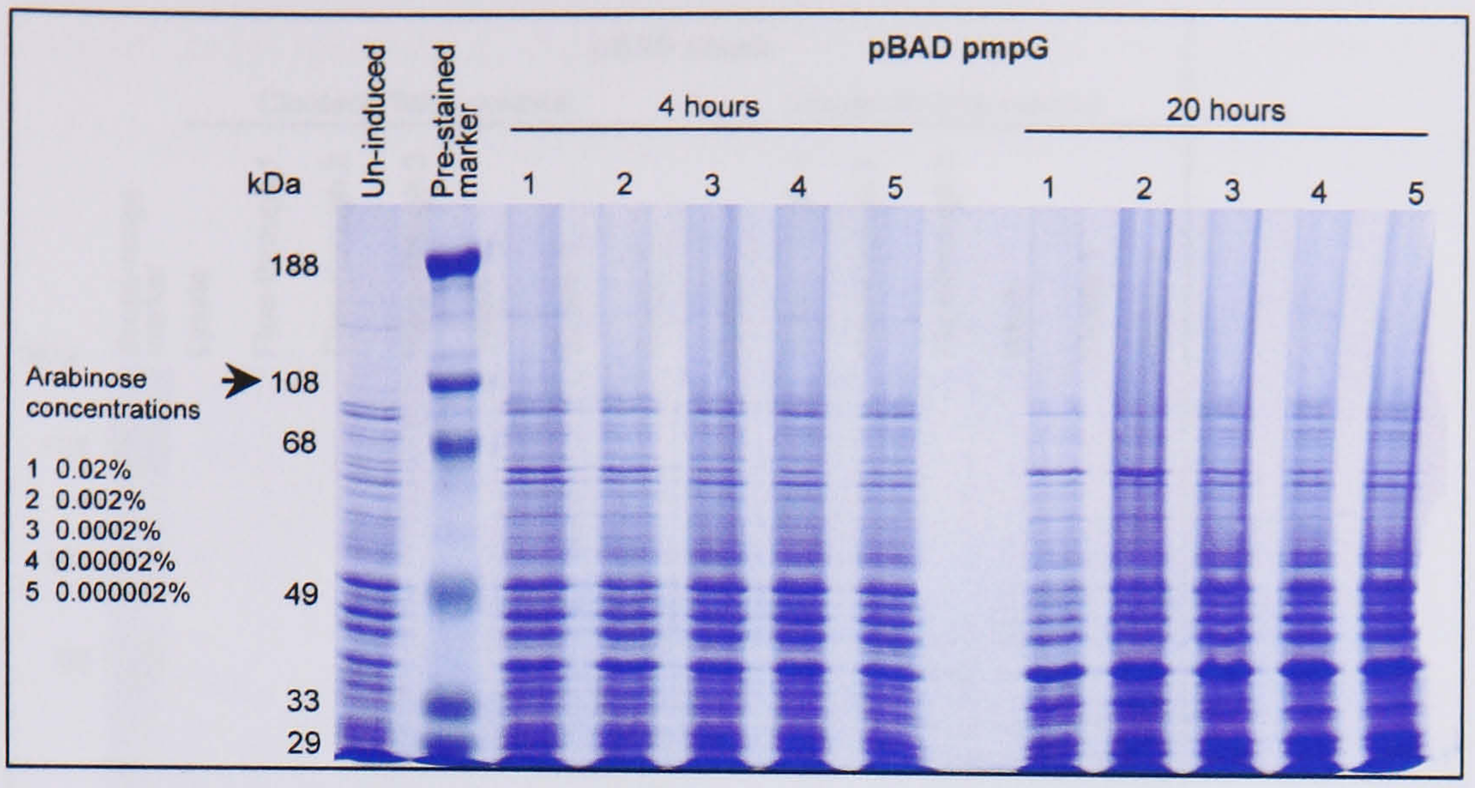
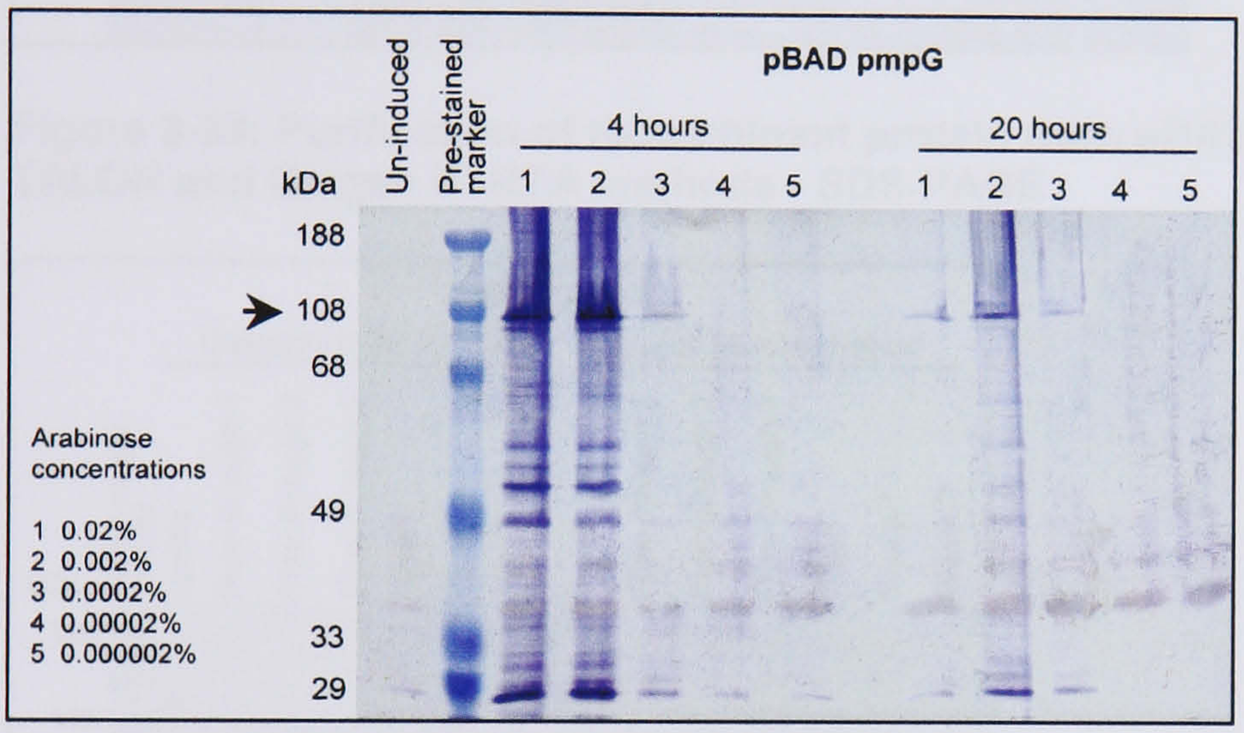


Figure 3-31: Expression of recombinant protein from pBAD/pmpG- SDS-PAGE



Western blot probed with Anti-V5® monoclonal antibody (Invitrogen) . Predicted size of recombinant protein marked by arrow.

Figure 3-32: Expression of recombinant protein from pBAD/pmpG- Western blot

3.2.2.2 Purification of recombinant proteins from pBAD/pmpG

Recombinant PMPG was purified from bulk culture using both the Ni-NTA columns and the TALON spin-columns for comparison using the methods described in section 2.3. Samples equivalent to 250µl original culture were analysed by SDS-PAGE and Western blotting. For the stained SDS-PAGE gel, eluates equivalent to 1000µl original culture were used to improve visualisation. Despite this adjustment, no recombinant protein bands could be seen on the Coomassie-stained SDS-PAGE gels (Figure 3-33), however recombinant PMPG was successfully observed by Western blotting and demonstrated that the TALON spin-columns were more efficient at binding the recombinant protein and also had higher concentrations of recombinant protein in the eluates (Figure 3-34).

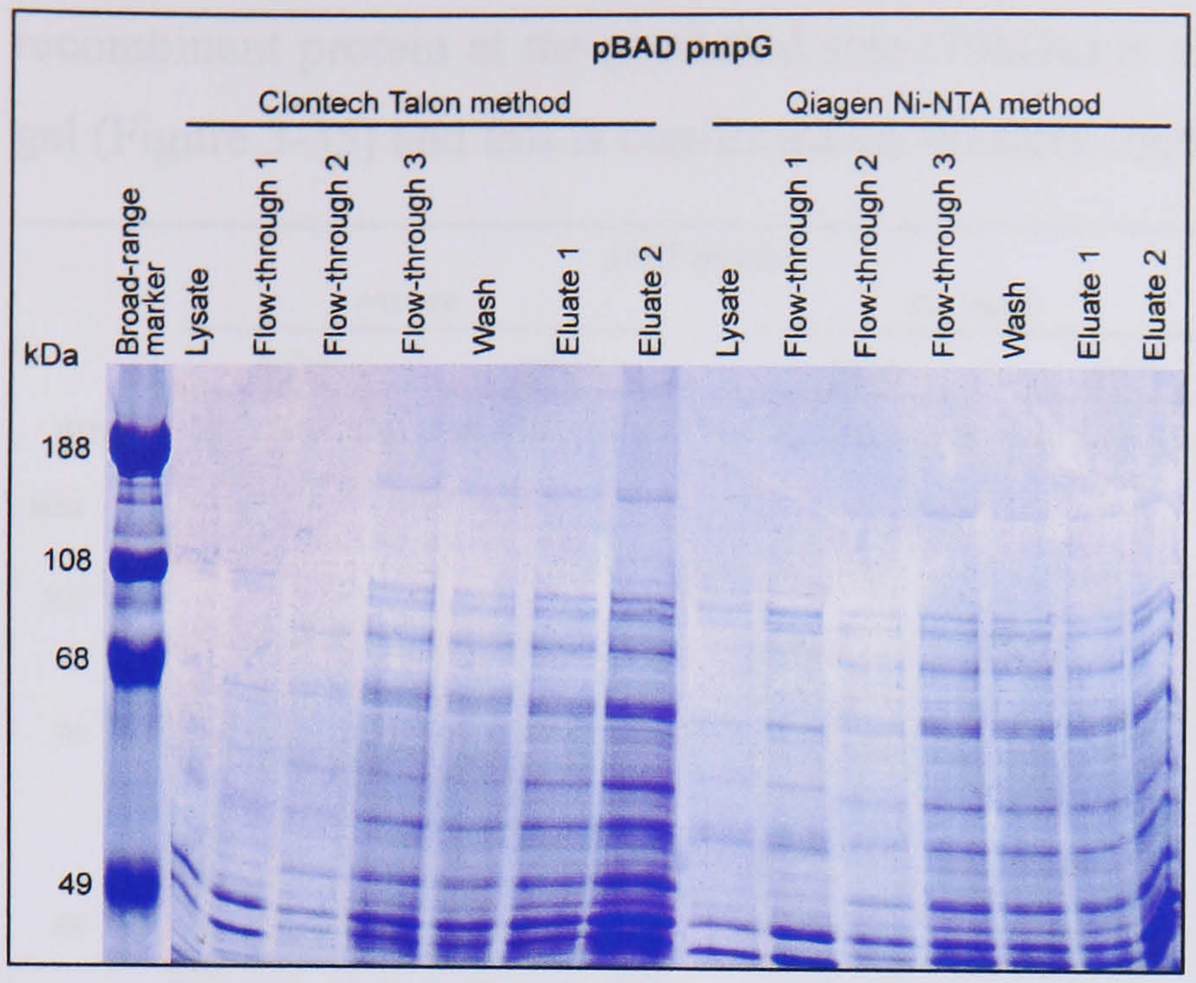
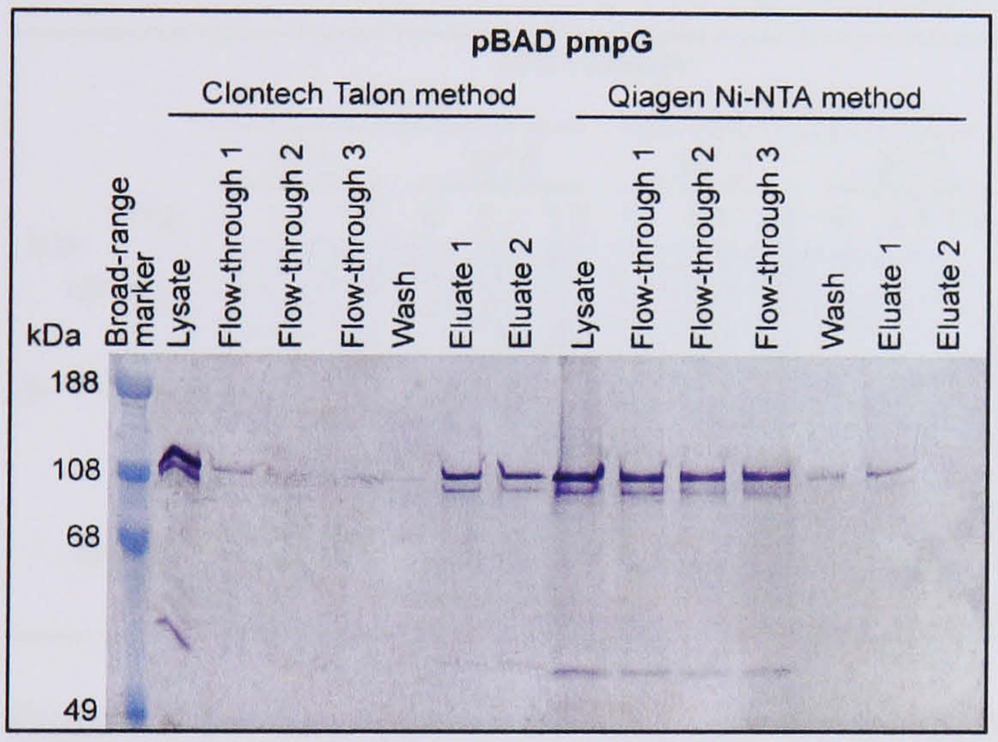


Figure 3-33: Purification of recombinant protein from pBAD/pmpG using Clontech TALON and Qiagen Ni-NTA methods - SDS-PAGE



Western blot probed with Anti-V5® monoclonal antibody (Invitrogen) .

Figure 3-34: Purification of recombinant protein from pBAD/pmpG using Clontech TALON and Qiagen Ni-NTA methods - Western blot

With such poor yields it was apparent that the pBAD construct would not generate adequate amounts of recombinant protein for development of assays for the fieldwork and a decision was made to generate a further construct in pPET.

3.2.3 pPET/pmpGc

3.2.3.1 Expression of pPET/pmpGc

Determination of expression conditions at both 25^{O} and $37^{O}C$ under varying concentrations of the inducer IPTG was undertaken as described in section 2.2.4. An aliquot of pelleted cells, equivalent to 50µl original culture, was lysed and analysed by SDS-PAGE electrophoresis and by Western blotting as described. A band of

recombinant protein at the predicted size (74kDa) is clearly visible on the SDS-PAGE gel (Figure 3-35) and this is confirmed on Western blotting (Figure 3-36).

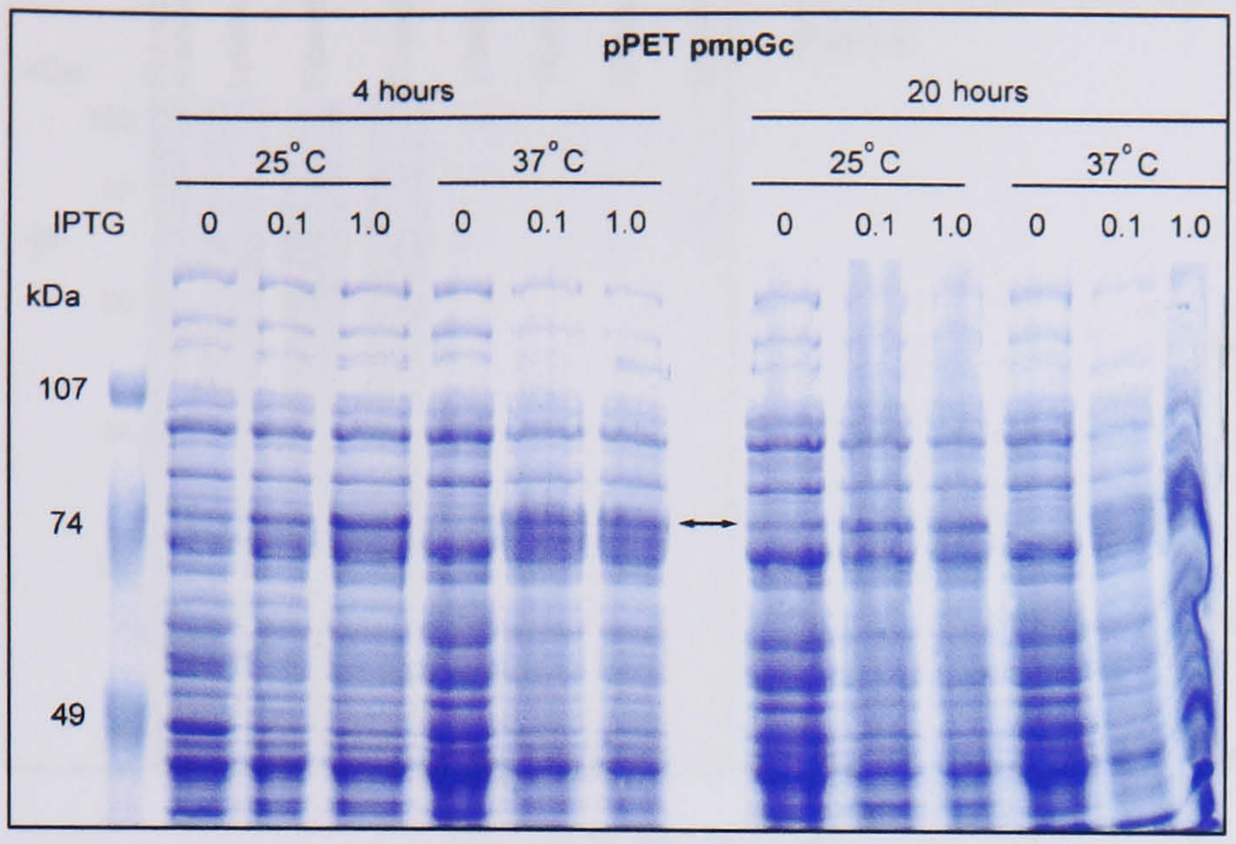
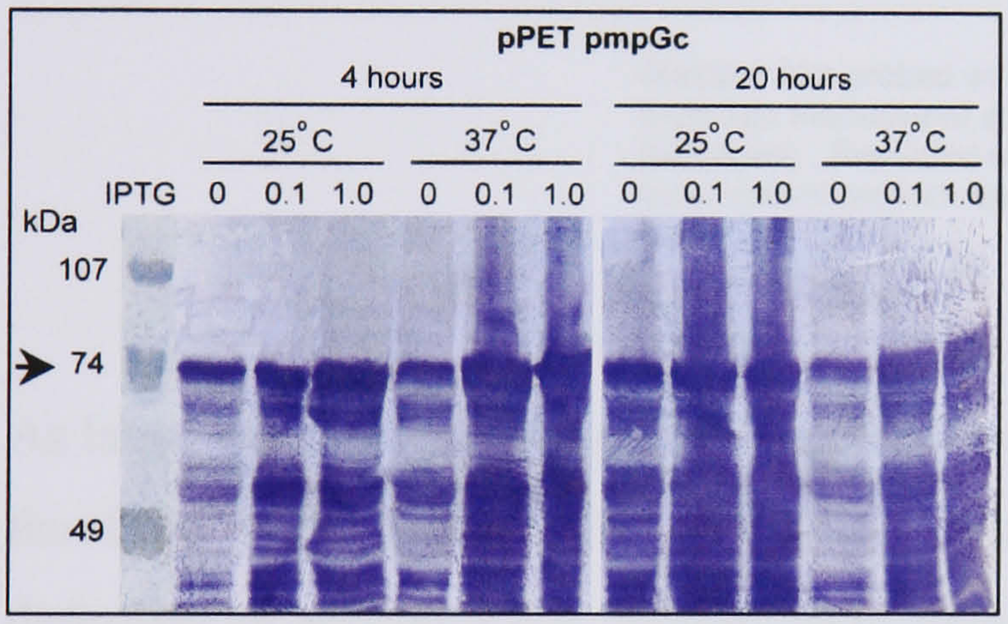


Figure 3-35: Expression of recombinant protein from pPET/pmpGc - SDS-PAGE



Western blot probed with Anti-S-protein monoclonal antibody (Novagen) . Predicted size of recombinant protein marked by arrow.

Figure 3-36: Expression of recombinant protein from pPET/pmpGc - Western blot

Optimal expression appeared to occur at 37°C for 4 hours in the presence of 0.1mM IPTG. These conditions were used for further larger-scale inductions.

3.2.3.2 Purification of recombinant proteins from pPET/pmpGc

Recombinant PMPGc was purified from bulk culture using TALON spin-columns as described in section 2.3.5. Samples equivalent to 75µl original culture were analysed by SDS-PAGE and Western blotting. The columns were fully saturated with recombinant protein as demonstrated by the excess appearing in the flow-through. Very little recombinant protein was lost in the washing stage and relatively moderate amounts recovered on elution (Figure 3-37) and (Figure 3-38)

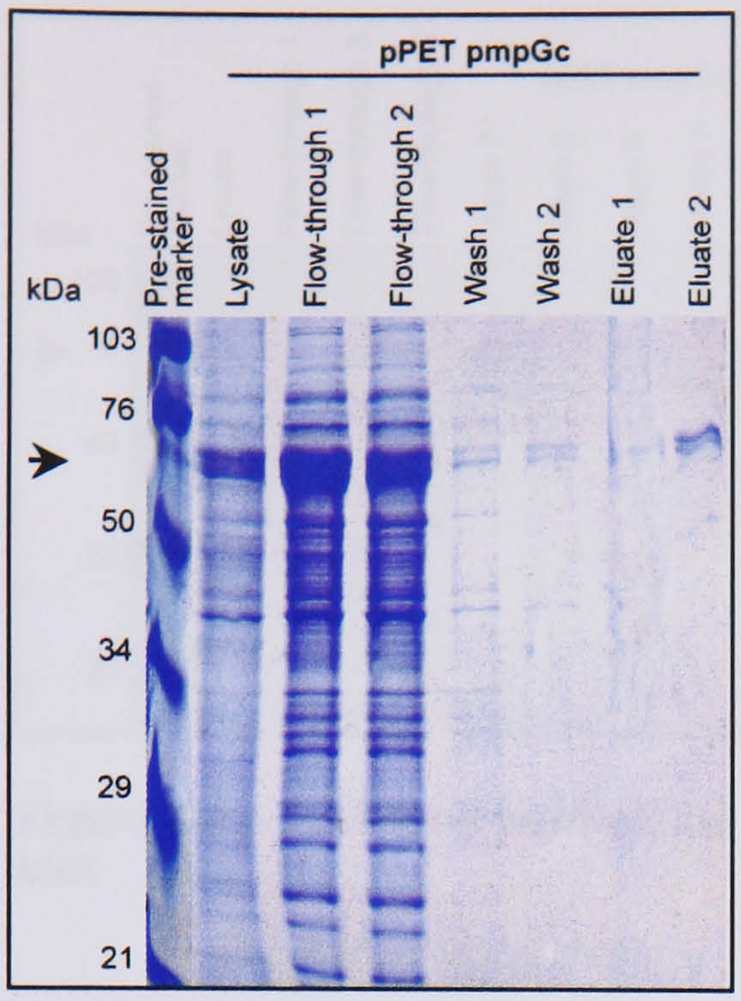


Figure 3-37: TALON spin-column purification of recombinant protein from pPET/pmpGc - SDS-PAGE

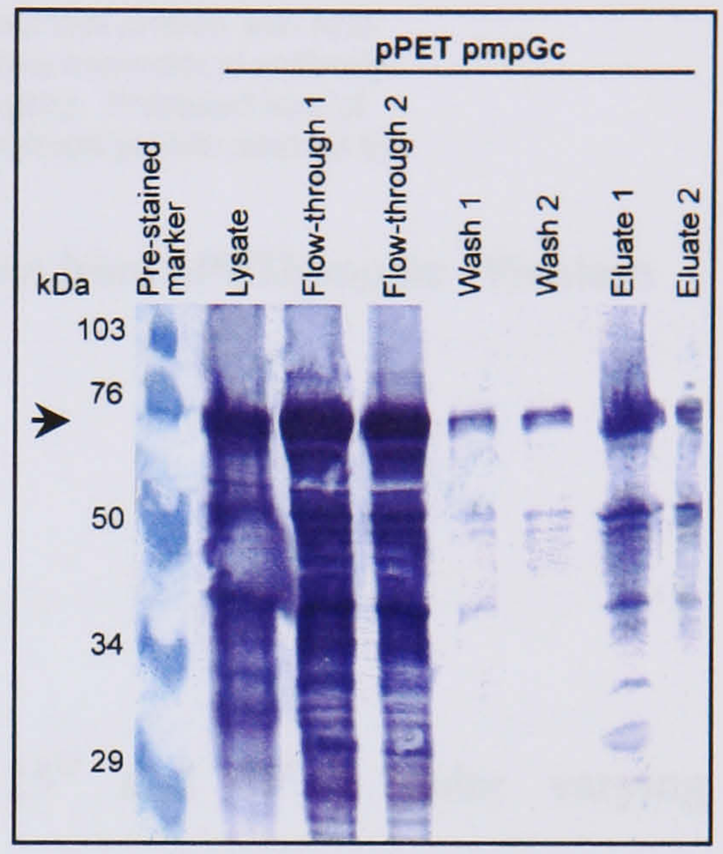


Figure 3-38: TALON spin-column purification of recombinant protein from pPET/pmpGc - Western blot

Western blot probed with Anti-S-protein monoclonal antibody (Novagen) . Predicted size of recombinant protein marked by arrow.

As larger quantities of recombinant protein were required for development of assays for the field work, larger-scale purification was undertaken using Pro-Bond® resin as described in section 2.3.4. Results were very similar to those seen with the TALON spin-columns method (Figure 3-39) and (Figure 3-40) in that once again excess recombinant protein was seen in the flow-through, very little protein was lost in the washing stage, and the majority of the protein was recovered in the first two eluates.

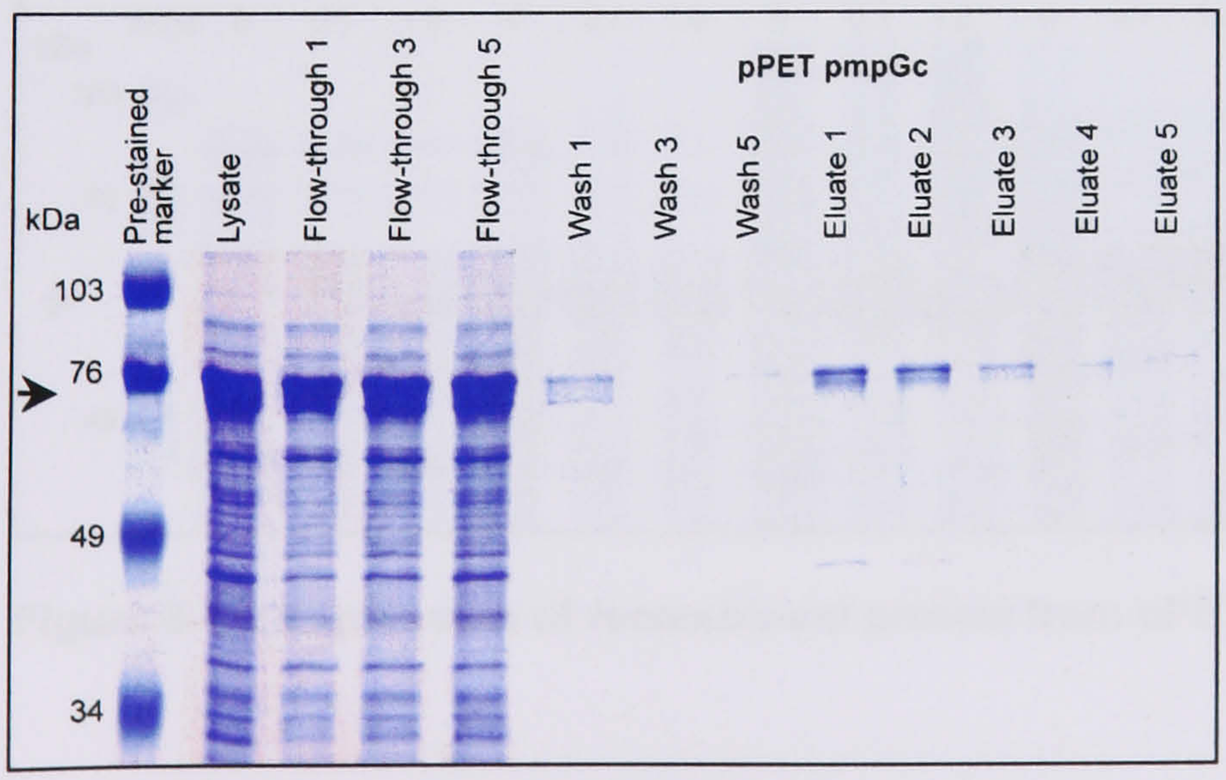
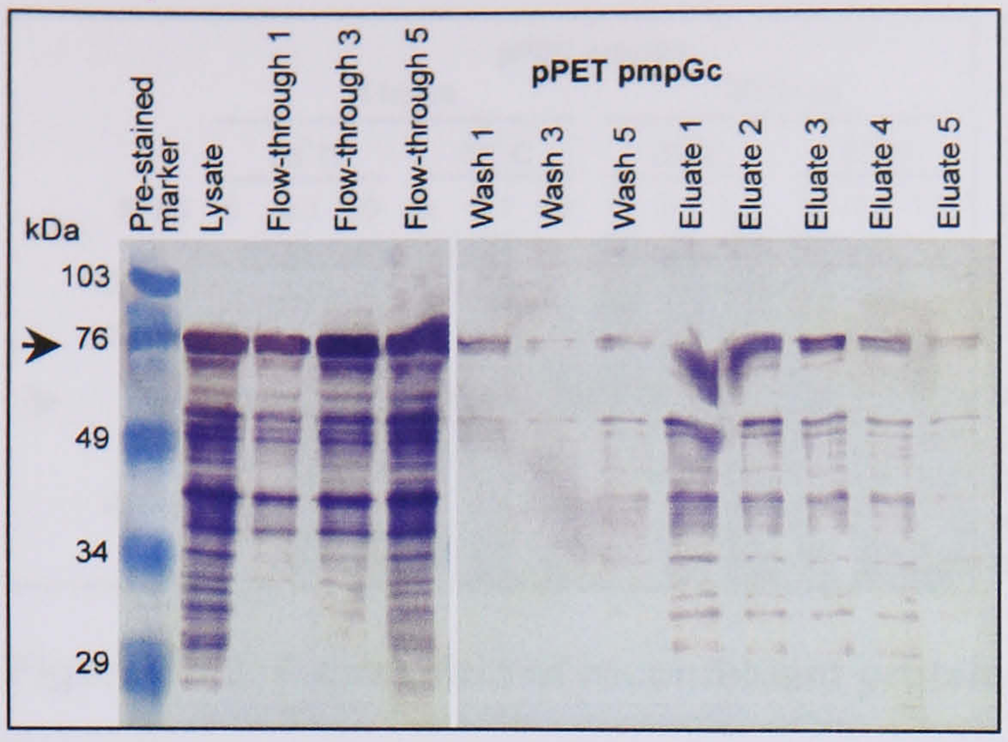


Figure 3-39: Pro-Bond purification of recombinant protein from pPET/pmpGc - SDS-PAGE



Western blot probed with Anti-S-protein monoclonal antibody (Novagen) . Predicted size of recombinant protein marked by arrow.

Figure 3-40: Pro-Bond purification of recombinant protein from pPET/pmpGc - Western blot

3.2.4 pPET/pmpGa

3.2.4.1 Expression of pPET/pmpGa

Determination of expression conditions at both 25^{O} and 37^{O}C under varying concentrations of the inducer IPTG was undertaken as described in section 2.2.4. An aliquot of pelleted cells, equivalent to 50µl original culture, was lysed and analysed by SDS-PAGE electrophoresis and by Western blotting as described in section 2.2.1. A band of recombinant protein of the predicted size (61kDa) was clearly visible on the SDS-PAGE gel (Figure 3-41) and this was confirmed on Western blotting (Figure 3-42).

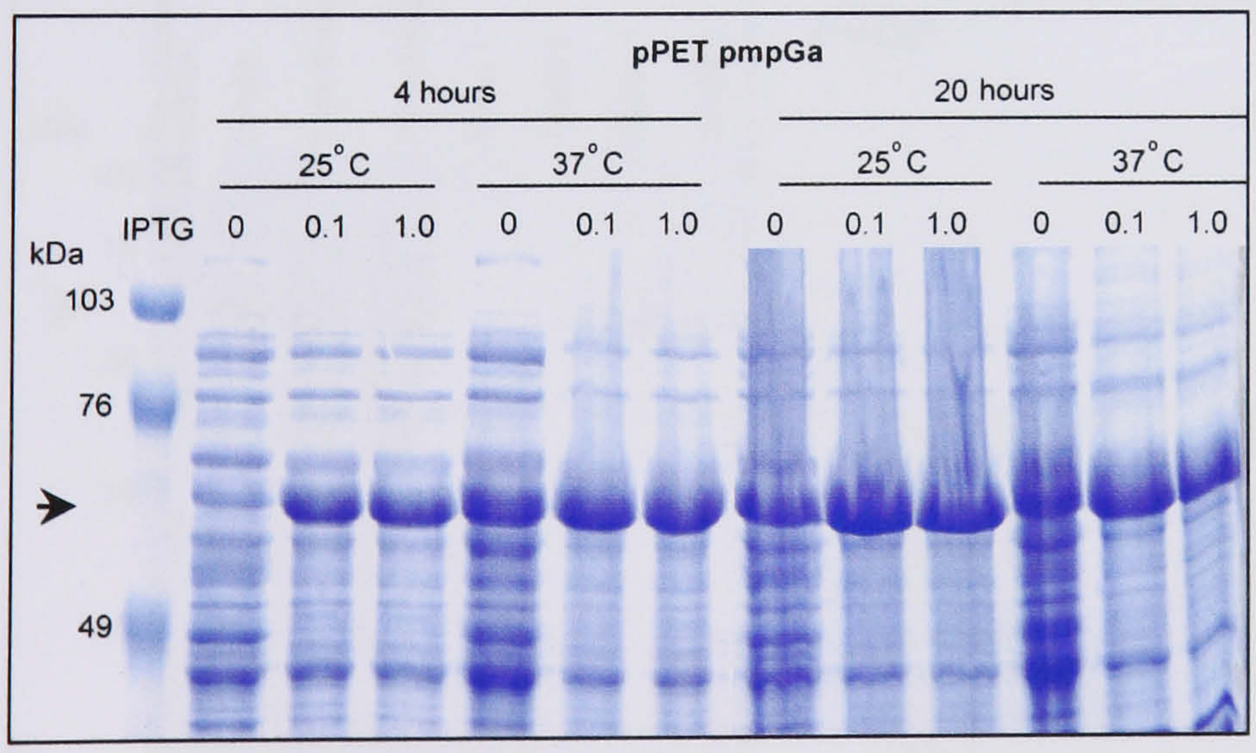
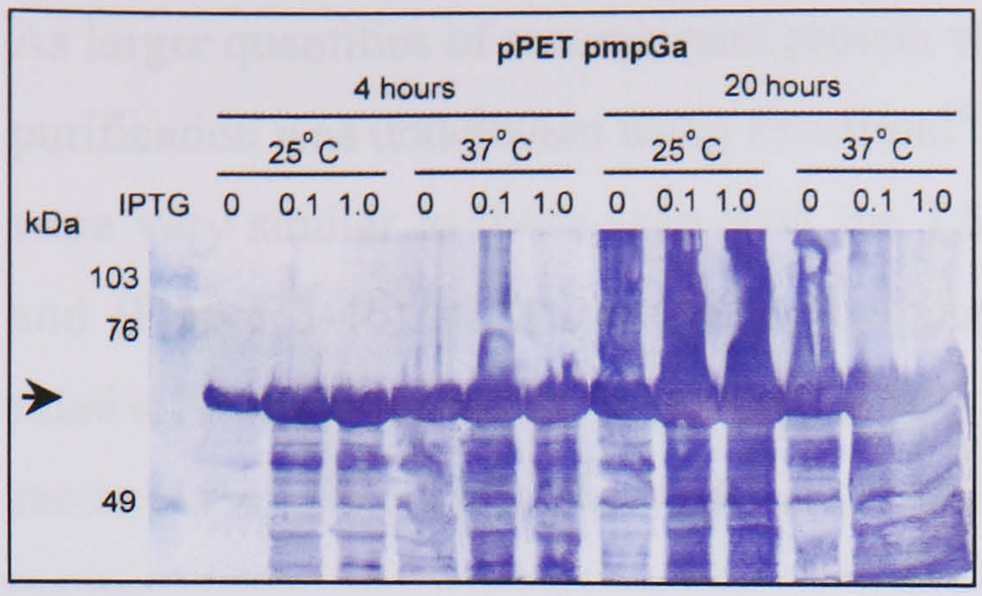


Figure 3-41: Expression of recombinant protein from pPET/pmpGa - SDS-PAGE



Western blot probed with Anti-S-protein monoclonal antibody (Novagen) . Predicted size of recombinant protein marked by arrow.

Figure 3-42: Expression of recombinant protein from pPET/pmpGa - Western blot

It is evident that production of recombinant protein occurred even in the absence of IPTG. Further larger-scale cultures were undertaken for 4 hours at 37°C in the presence of 0.1mM IPTG.

3.2.4.2 Purification of recombinant proteins from pPET/pmpGa

Recombinant PMPGa was purified from large-scale culture using TALON spin-columns as described in section 2.3.5. Samples equivalent to 75µl original culture were analysed by SDS-PAGE and Western blotting. The columns were again fully saturated as demonstrated by recombinant protein appearing in the flow-through. Relatively little recombinant protein was lost in the washing stage and acceptable amounts recovered on elution (Figure 3-43) and (Figure 3-44)

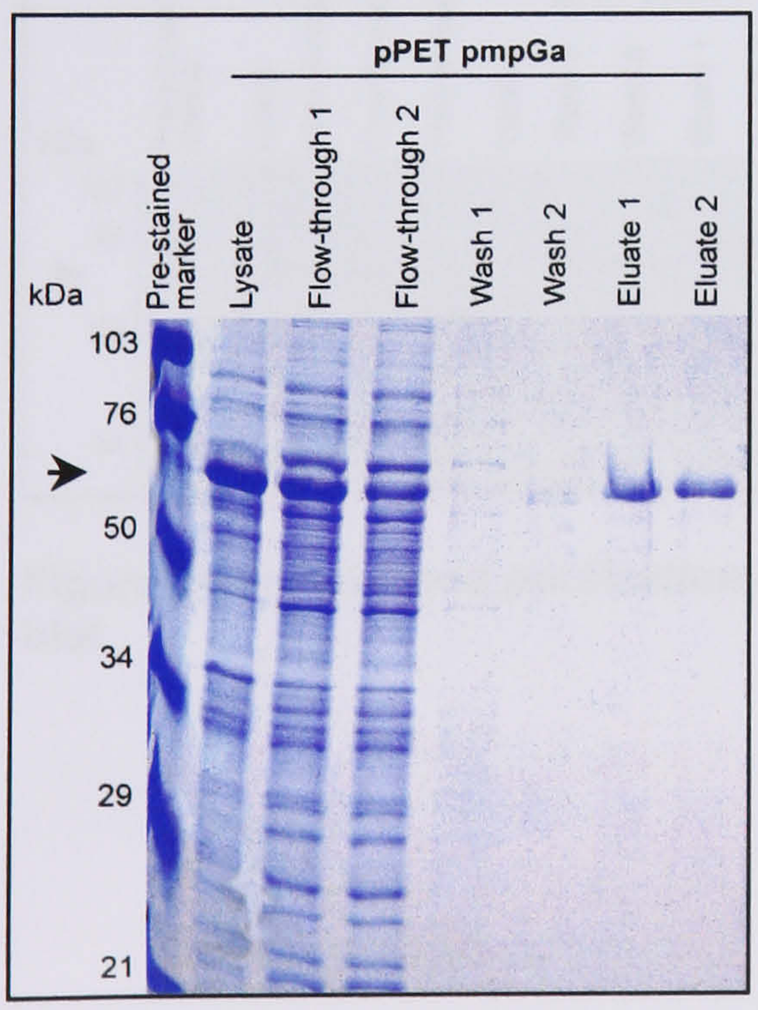


Figure 3-43: TALON spin-column purification of recombinant protein from pPET/pmpGa - SDS-PAGE

Western blot probed with Anti-S-protein monoclonal antibody (Novagen) . Predicted size of recombinant protein marked by arrow.

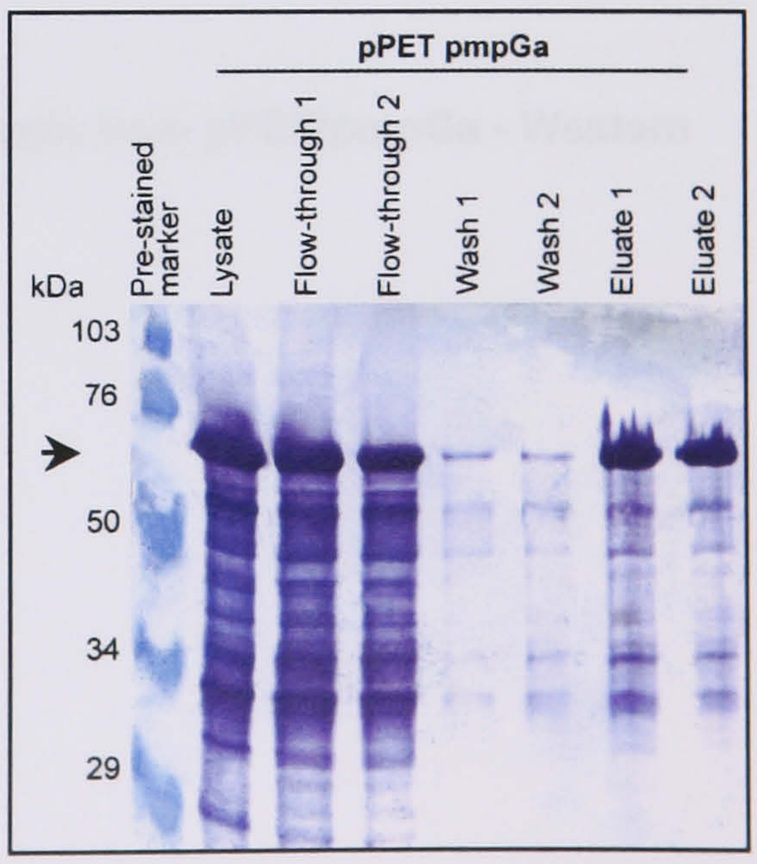


Figure 3-44: TALON spin-column purification of recombinant protein from pPET/pmpGa - Western blot

As larger quantities of recombinant protein were required for the field work, large-scale purification was undertaken using Pro-Bond® resin as described in section 2.3.4. Results were very similar to those seen with the TALON spin-columns method (Figure 3-45) and (Figure 3-46) in that excess recombinant protein was seen in the flow-through, relatively small amounts were lost in the washing stage and the majority of the recombinant protein was recovered in the first two eluates.

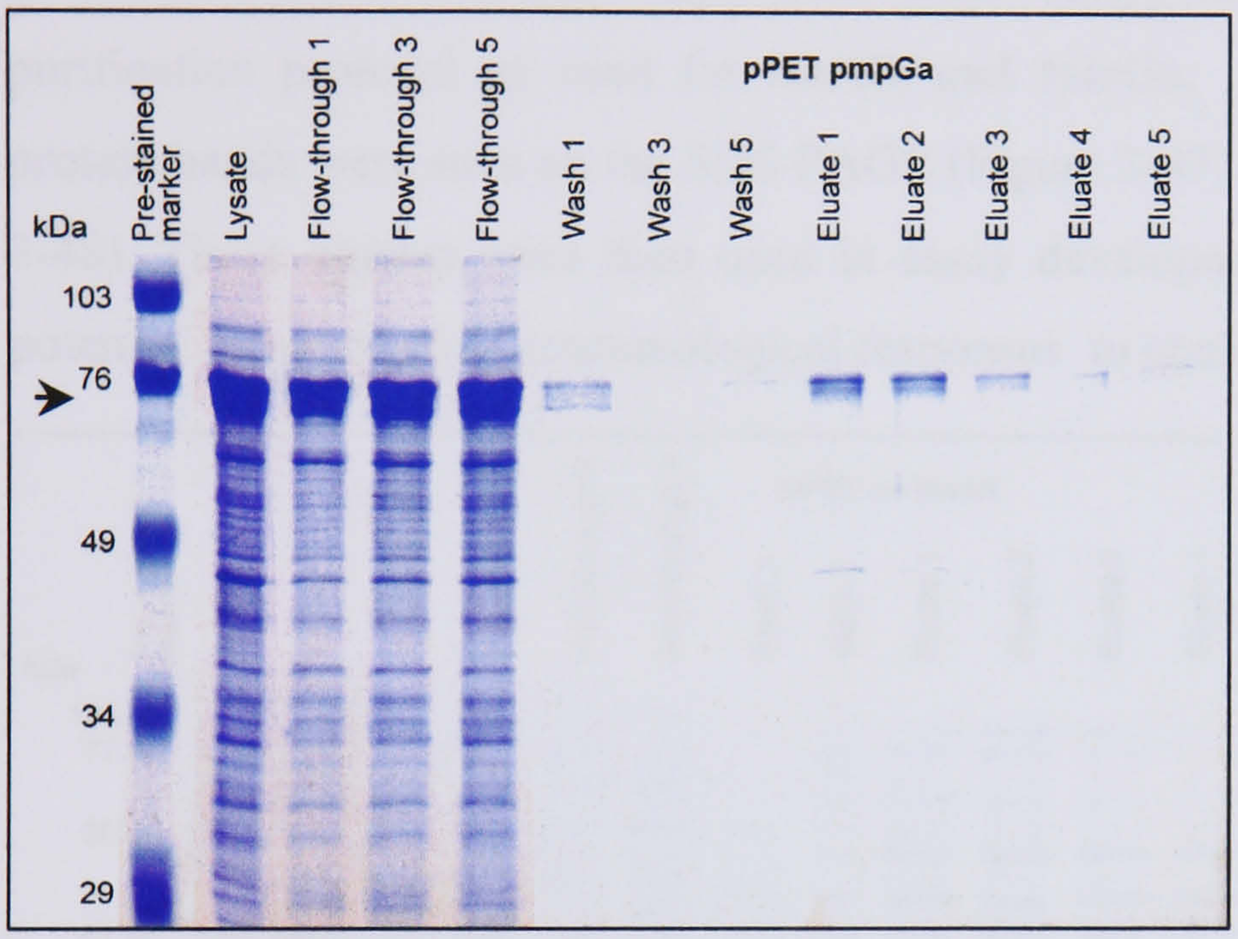
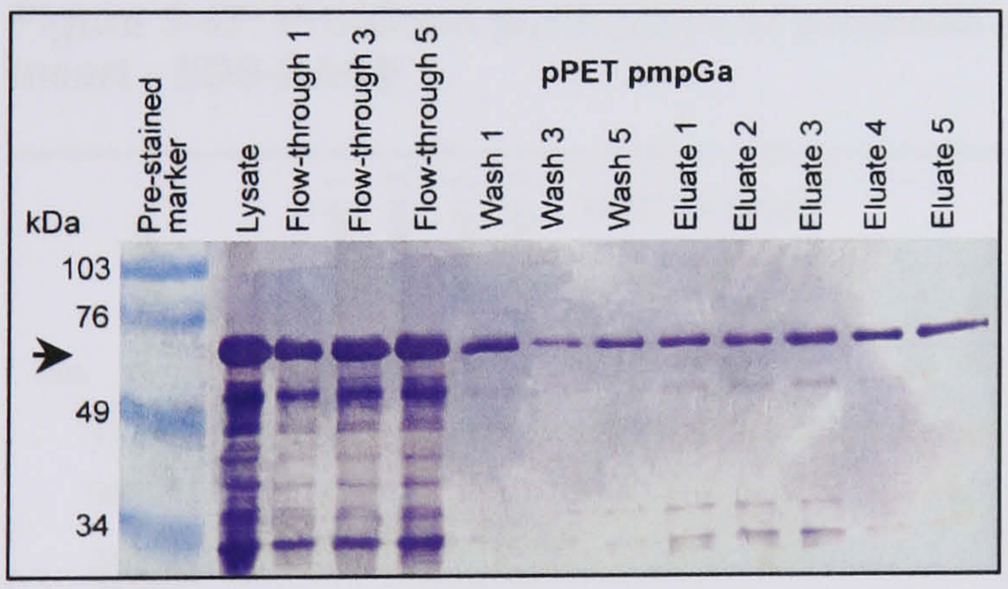


Figure 3-45: Pro-Bond purification of recombinant protein from pPET/pmpGa - SDS-PAGE



Western blot probed with Anti-S-protein monoclonal antibody (Novagen) . Predicted size of recombinant protein marked by arrow.

Figure 3-46: Pro-Bond purification of recombinant protein from pPET/pmpGa - Western blot

3.2.5 pPET without insert

3.2.5.1 Expression of pPET without insert and purification of induced products

Bacterial cells containing pPET without an insert were induced with IPTG as described in section 2.2.4. Pelleted cells were then subjected to an identical Pro-Bond® purification protocol as used for PMPGc and PMPGa. As expected no recombinant protein bands were seen on the SDS-PAGE (Figure 3-47) or on the Western blot (Figure 3-48). These eluates were then used in assay development as material to control for potential cross-reacting immunological responses to contaminating *E. coli* proteins.

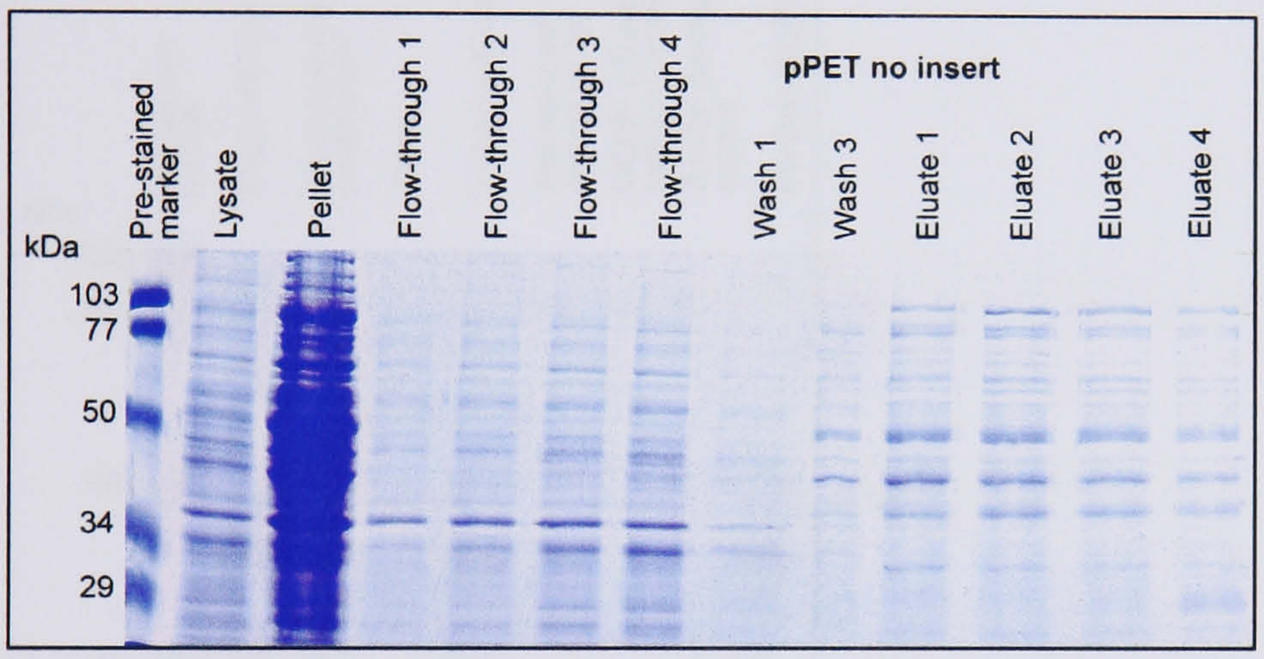
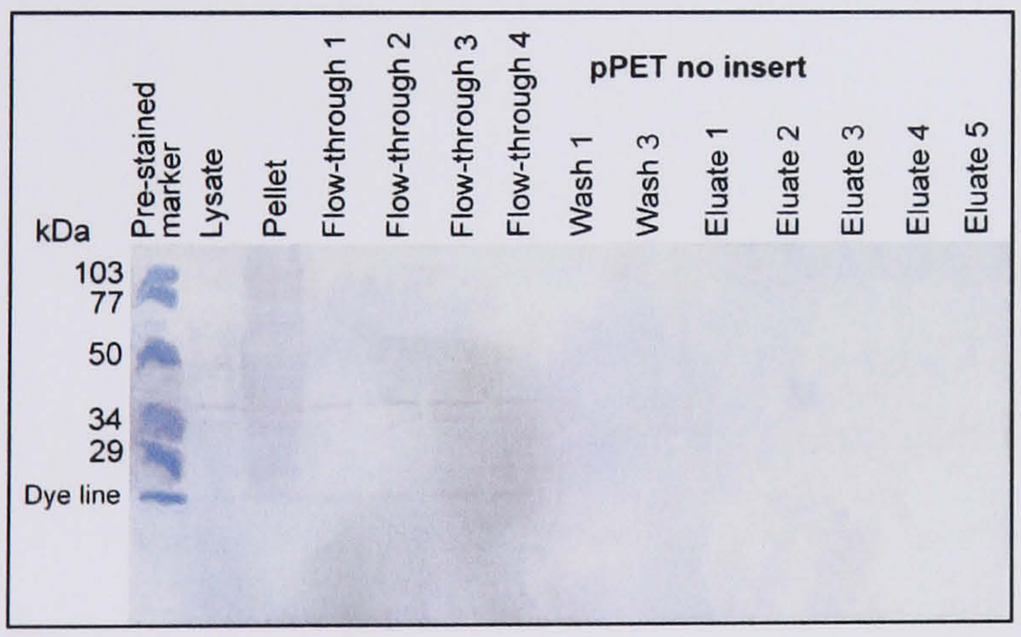


Figure 3-47: Pro-Bond purification of products of induction of pPET vector without an insert - SDS-PAGE



Western blot probed with Anti-S-protein monoclonal antibody (Novagen).

Figure 3-48: Pro-Bond purification of products of induction of pPET vector without an insert - Western blot

3.2.6 MOMP

3.2.6.1 Expression and purification of rMOMP

The MOMP expressing host was induced as described in section 2.2.5, inclusion bodies isolated and MOMP purified from those inclusion bodies as described in section 2.3.2. Aliquots equivalent to 150µl original culture (purified MOMP 300µl equivalent) were then analysed by SDS-PAGE (Figure 3-49). The recombinant protein band of MOMP was clearly visible.

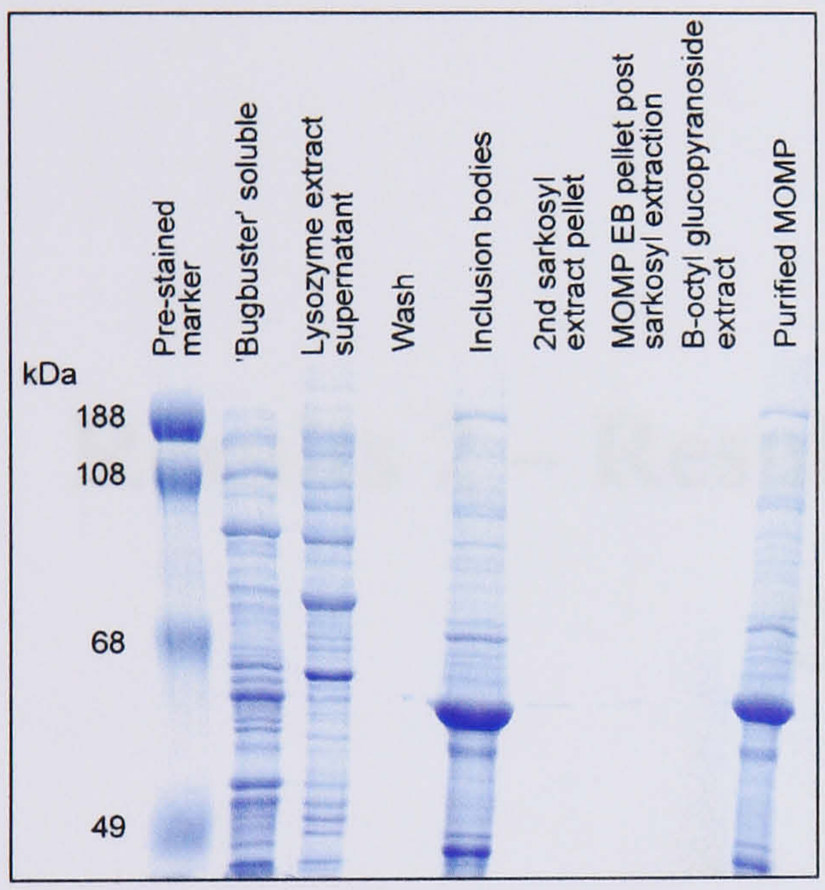


Figure 3-49: Purification of recombinant MOMP - SDS-PAGE

Chapter Four

Results 2 – Results of field work in The Gambia

4 Results 2 – Results of field work in The Gambia

4.1 Quality control issues

4.1.1 Quality control issues for immunoblots

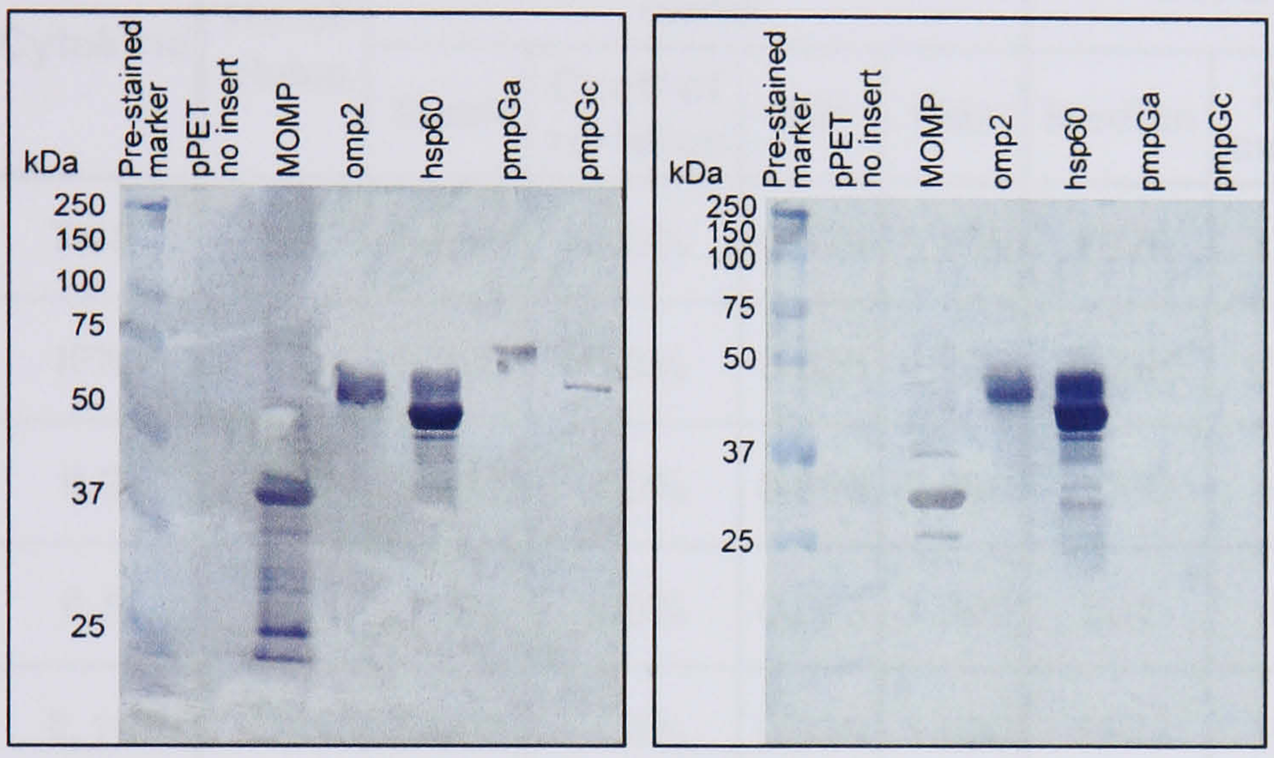


Figure 4-1: Two typical immunoblots

Antigen-specific IgG from patient sera, bound to chlamydial proteins on a Western blot and detected using goat anti-human

Two typical immunoblots are shown (Figure 4-1). The intensity of each individual result was scored by three independent observers as described in section 2.6. Correlation between the observers was good. Correlation coefficients for immunoblot scores between the three observers are given in Table 4-1 and the full dataset is provided in the appendix. No individuals demonstrated antibody responses to the sham antigen 'pPET without insert', this antigen is therefore excluded from the immunoblot result analyses which follow.

	Correlation between:		
	1 & 2	1 & 3	2 & 3
MOMP	0.76	0.68	0.77
omp2	0.84	0.83	0.85
hsp60	0.76	0.75	0.78
pmpGa	0.83	0.88	0.87
pmpGc	0.86	0.90	0.92

Table 4-1: Correlation coefficients between observers for immunoblot scores

4.1.2 Quality control issues for cytokine ELISAs

The cytokine assay used was proven to be robust and reliable in this setting. Standard curves were highly accurate as seen by the R^2 values in Table 4-2 where a value of 1 indicated a perfect correlation. Optical density values for the cytokine concentrations used for the standard curves were highly reproducible. The median values and inter-

quartile ranges for the positive control wells demonstrate that the variation was quite small, however there were a few outliers, a few of which lay well outside the interquartile ranges (Figure 4-2). There was insufficient supernatant to repeat the study ELISAs from these plates, however it was felt that results should not therefore be adjusted to control for the positive control well values. Any patient samples which gave results outside the standard curve were repeated at a higher dilution to give an accurate result.

Cytokine	No of plates	R-squared for standard curve				Control wells		
		Mean	Coeff of variation	Min	Max	Median	25th centile	75th centile
TNF	58	0.996	0.5%	0.979	1.000	1826	1716	1899
IFN	57	0.990	1.9%	0.876	1.000	14500	10800	18800
IL2	58	0.988	2.0%	0.899	1.000	1060	696	1140
IL5	56	0.994	0.6%	0.973	1.000	534	483	595
IL10	53	0.992	1.4%	0.929	1.000	1922	1794	2033
TGF	37	0.976	2.1%	0.929	1.000	1756	1280	2146

Table 4-2: Quality control statistics for cytokine ELISAs

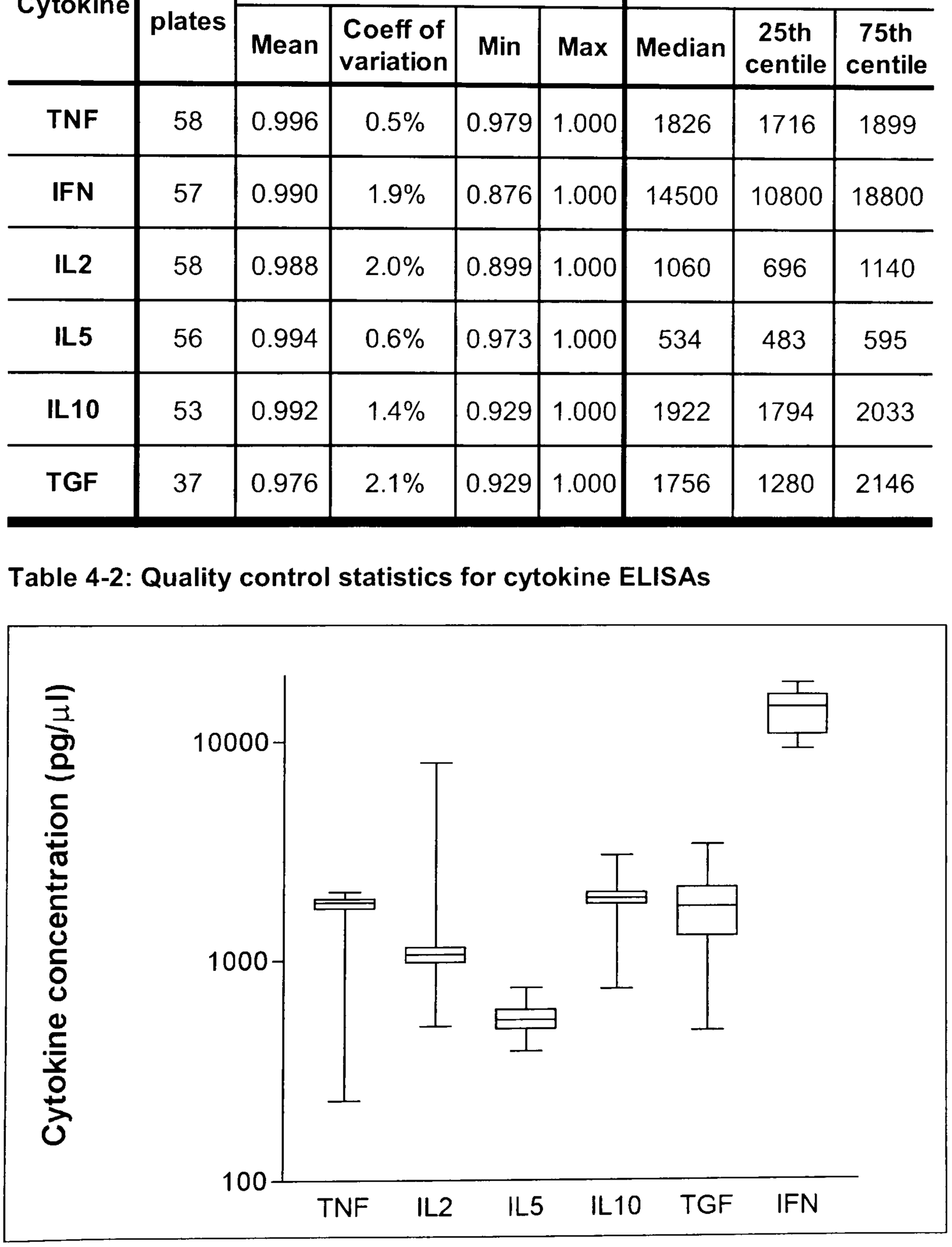


Figure 4-2: Distribution of values for cytokine positive control wells

Figure 4-2 shows median value and interquartile ranges for each cytokine in positive control supernatants. The error bars indicate the range of the outlying values.

There was some concern that this variation in the positive control wells might also reflect similar variation in the patient samples. Correlation coefficients were therefore calculated between the control well values and those of the patient samples on the same plate. The correlation coefficients range from 0.001 to 0.361 with a mean of 0.11 if TGFβ excluded and 0.23 for TGFβ. (The higher correlations between TGFβ results is due to a number of individuals with non-specific activation affecting all wells including the negative controls, see below.) These levels of correlation are not considered significant[242] and their effects are very small, for example the mean correlation coefficient of 0.11 indicates that only 1.2% of the variance is due to this correlation.

	TNF	IFN	IL-2	IL-5	IL-10	TGF
PHA	0.175	0.155	0.015	0.088	0.328	0.243
No antigen	0.044	0.006	-0.082	0.035	0.243	-0.001
Candida	0.122	0.179	-0.046	0.066	0.172	0.237
PPD	0.065	0.120	-0.061	0.213	0.080	0.178
PET - no antigen	0.008	0.037	-0.123	0.117	-0.101	0.176
EBs	0.076	0.181	0.063	0.044	0.182	0.140
MOMP	0.067	0.310	0.050	0.084	-0.138	0.222
omp2	0.123	0.297	0.150	0.055	0.114	0.285
hsp60	0.043	0.254	-0.026	-0.004	0.076	0.327
pmpGa	0.090	0.143	0.055	0.099	0.121	0.361
pmpGc	0.159	0.078	0.055	0.081	0.169	0.359

Table 4-3: Correlation coefficients between values for the positive control wells and those for patient samples on the same plate

TGFβ was the only cytokine to provide results which were difficult to interpret. This was because in many cases there was non-specific TGFβ secretion, unrelated to the specific antigen, producing high results for all wells including the negative controls. Those samples showing non-specific high-level secretion were unrelated to any of the clinical groups by χ^2 analysis (active trachoma $p = 1.00$, scarred trachoma $p = 0.91$, infertile women $p = 0.66$) and also unrelated to age, gender or BCG status. On reviewing the data it was apparent that cases with non-specific TGFβ production had negative control values of greater than 130 pg/ml and thus all individuals generating more than 130 pg/ml of TGFβ with the negative control were removed from the analysis. In this way 41 of 106 samples were excluded. Figure 4-3 illustrates the variation in values in the total sample set (upper) and those used for analysis (lower).

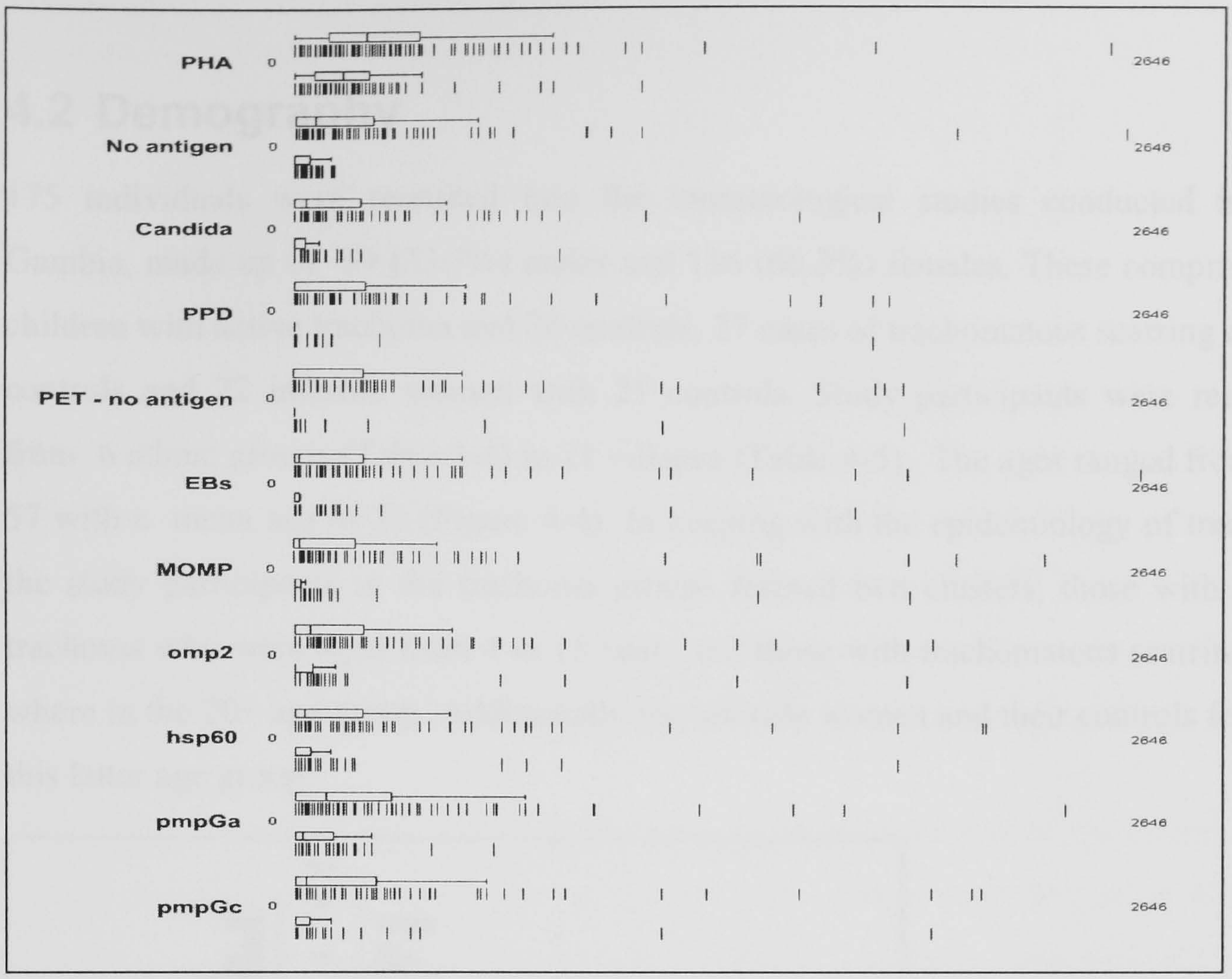


Figure 4-3: Spread of values for TGFβ by ELISA, for each antigen or control (upper distribution of each pair is the entire sample set, lower of each pair is after removal of results for all individuals who generated >130 pg/ml TGFβ in the negative control well)

4.2 Demography

175 individuals were recruited into the immunological studies conducted in The Gambia, made up of 59 (33.7%) males and 116 (66.3%) females. These comprised 52 children with active trachoma and 26 controls, 27 cases of trachomatous scarring and 27 controls and 22 infertile women with 21 controls. Study participants were recruited from 6 ethnic groups (Table 4-4) in 21 villages (Table 4-5) . The ages ranged from 4 to 57 with a mean age of 20 (Figure 4-4). In keeping with the epidemiology of trachoma the study participants in the trachoma groups formed two clusters, those with active trachoma who were aged from 4 to 15 years and those with trachomatous scarring who where in the 20+ age group. Additionally the infertile women and their controls fell into this latter age group.

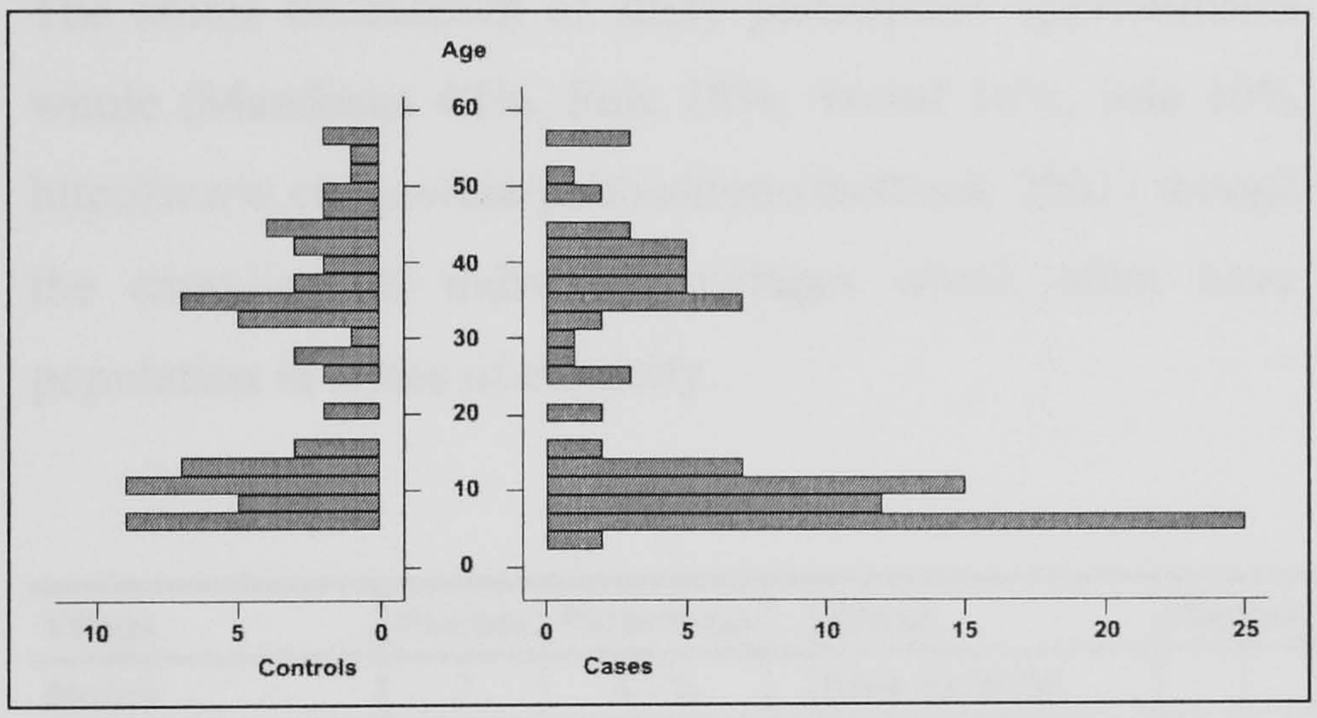


Figure 4-4: Age distribution graph of all cases and controls

Ethnic group	Cases		Controls	
	Number	Percentage	Number	Percentage
Fula	9	8.8 %	8	11 %
Jola	15	14.7 %	15	20.6 %
Mandinka	57	55.9 %	46	63 %
Manjago	8	7.8 %	0	0 %
Serere	1	1 %	0	0 %
Wolof	8	7.8 %	2	2.7 %
Not known	4	3.9	2	2.7 %

Table 4-4: Distribution of study participants by ethnicity

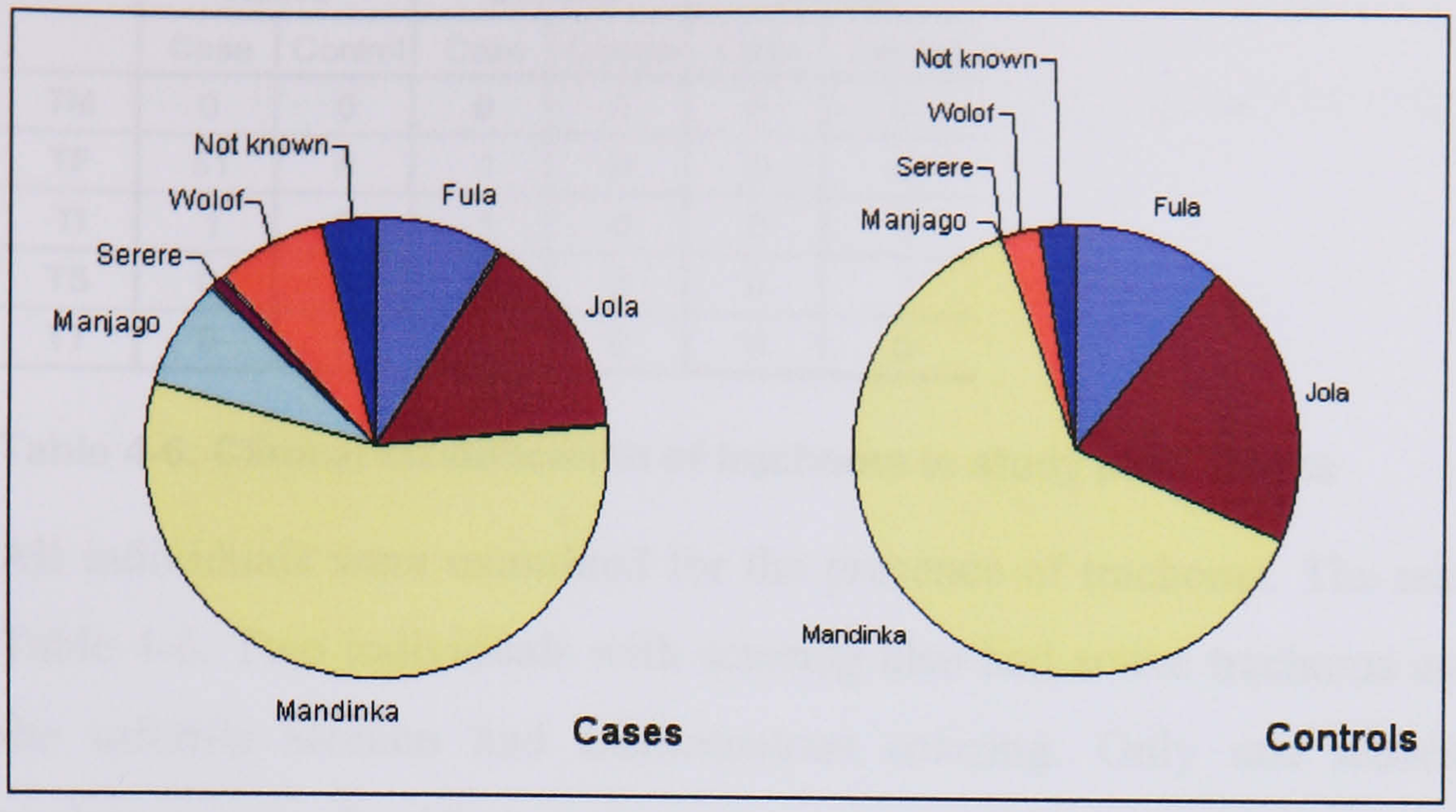


Figure 4-5: Distribution of study participants by ethnicity

The ethnic breakdown of study participants approximates to that of the country as a whole (Mandinka 42%, Fula 18%, Wolof 16%, Jola 10%, Serere 9%, other 4% - ref http://www.cia.gov/cia/publications/factbook 2001) though with some variation due to the sampling of individual villages which often have a somewhat homogenous population in terms of ethnicity.

Village	Number	Percentage		Village	Number	Percentage
Bambali	7	4.1 %		Konte Kunda Niji	1	0.6 %
Bassik	1	0.6 %		Konteh Kunda Sukoto	4	2.3 %
Berending	30	17.4 %		Kumbija	2	1.2 %
Dai Mandinka	3	1.7 %		Medina Kiaf	6	3.5 %
Dibba Kunda Wollof	1	0.6 %		Nyoro Angeleh	8	4.7 %
India Badibu	2	1.2 %		Palen Fula	4	2.3 %
Jajari	6	3.5 %		Sandaly	5	2.9 %
Jali	63	36.6 %		Sare Sajo	5	2.9 %
Jasobo	8	4.7 %		Sinchu Sanjal	1	0.6 %
Kani Kunda Suba	5	2.9 %		Yallal	3	1.7 %
Kanton Kunda	4	2.3 %		Not known	6	3.4 %

Table 4-5: Distribution of study participants by village of residence

The locations of the study villages are indicated on 1.7.3.

	Active		Scarred		Infertile	
	Case	Control	Case	Control	Case	Control
TM	0	0	0	0	0	0
TF	51	0	1	0	0	0
TI	1	0	1	0	0	0
TS	0	0	27	0	0	1
TT	0	0	5	0	0	0

Table 4-6: Clinical classification of trachoma in study participants

All individuals were examined for the presence of trachoma. The results are shown in Table 4-6. Two individuals with scarring also had active trachoma and one control for the infertile women had trachomatous scarring. Only one individual with active trachoma was classified as intense active trachoma (TI) making comparison of the different classifications of trachoma impossible.

4.3 Results of studies on individuals with trachoma

4.3.1 Immunoblot results

4.3.1.1 Active trachoma

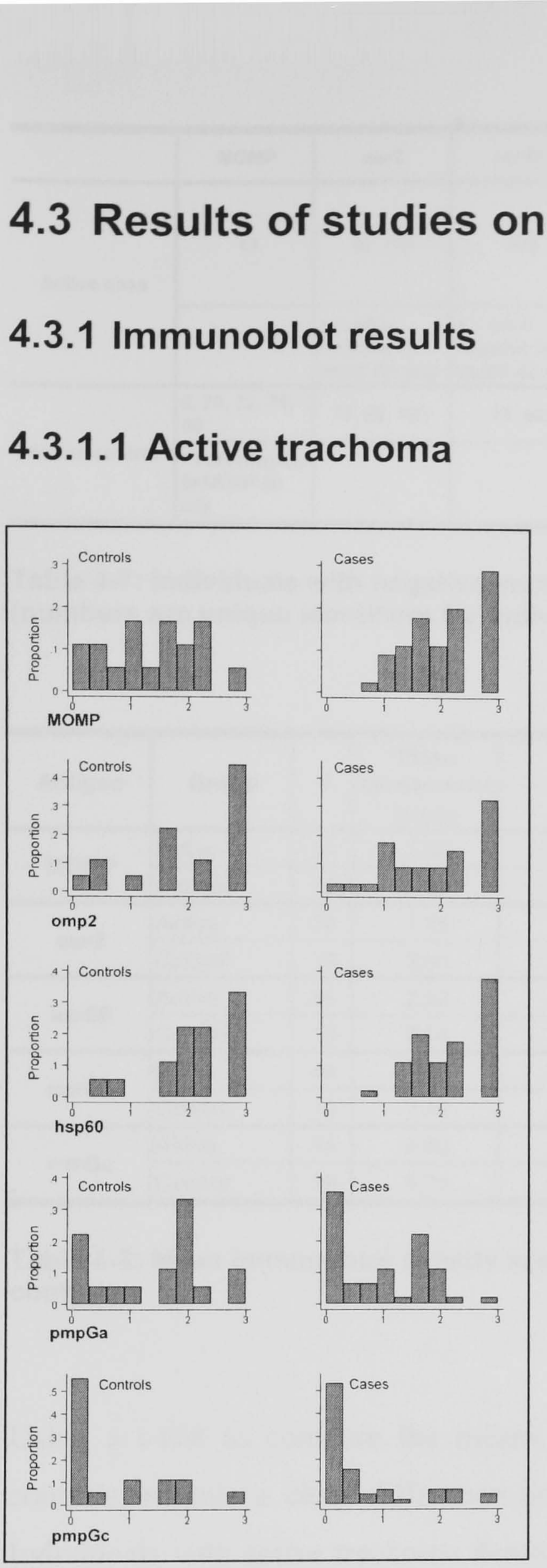


Figure 4-6: Frequency-distribution graphs of immunoblot density scores for children with active trachoma and their controls

The frequency distribution graphs clearly show that the children with active trachoma have higher immunoblot density scores than their controls. The graphs also demonstrate that many of the children have antibodies to the recombinant PMP proteins. 12/18 (67%) of controls and 22/44 (50%) of cases have detectable antibodies to PMPGa whilst 7/18 (39%) of controls and 12/44 (27%) of cases have antibodies to PMPGc.

Additionally 5/18 controls (28%) had no detectable antibodies to MOMP suggesting they may never have been exposed to *Chlamydia* compared to only 1/50 (2%) of cases ($p < 0.002$). However three of these individuals did have antibodies to other chlamydial antigens. Two individuals, a six year old boy and an eight year old girl, both from Jali, had no antibodies to any chlamydial antigens and presumably had either never been exposed or there were technical problems with the Western blots.

	MOMP	OMP2	HSP60	PMPGA	PMPGC
Active case	13	48, 115	103	11, 12, 42, 66, 92, 93, 100, 101, 102, 103, 104, 105, 106, 112, 114, 115, 120	11, 12, 13, 42, 48, 66, 92, 93, 100, 101, 102, 103, 104, 105, 106, 112, 114, 115, 120
		+ 1 other negative for omp2 Ab only	*+ 1 other negative for hsp60 Ab only*		*+ 5 others negative for pmpGc Ab only*
Active control	9, 70, 72, **79**, **80**	**79**, **80**, 129,	**79**, **80**,	17, 20, **79**, **80**, 111, 129,	9, **17**, 20, 72, **79**, **80**, 111
	+ 1 other negative for MOMP Ab only				*+ 4 others negative for pmpGc Ab only*

Table 4-7: Individuals with negative immunoblot responses to chlamydial antigens (numbers are unique identifiers for individual study participants)

Antigen	Group	n	Mean immunoblot score	t-test		Mann-Whitney
				95% C.I.	p <	p <
MOMP	Active	44	2.08	1.88 - 2.28	0.0003	0.0018
	Control	19	1.33	0.93 - 1.73		
OMP2	Active	34	1.95	1.63 - 2.26	0.83	0.76
	Control	19	2.01	1.52 - 2.49		
HSP60	Active	44	2.22	2.03 - 2.42	0.66	0.89
	Control	19	2.14	1.80 - 2.49		
PMPGa	Active	44	0.92	0.66 - 1.19	0.06	0.04
	Control	19	1.41	0.90 - 1.91		
PMPGc	Active	44	0.60	0.33 - 0.87	0.46	0.73
	Control	19	0.79	0.30 - 1.28		

Table 4-8: Mean immunoblot density scores for children with active trachoma and their controls

Using a t-test to compare the means of the two populations as a whole, cases vs. controls, reveals a clear difference in antibody responses to MOMP and to PMPGa. Individuals with active trachoma demonstrate a mean immunoblot response of 2.08 vs. 1.33 for the control population ($p < 0.0003$) to MOMP and 0.92 vs. 1.41 ($p < 0.06$) to PMPGa. The frequency plots of antibody responses (Figure 4-6) show that these data are generally not normally distributed, they were therefore also investigated using the non-parametric Mann-Whitney U test. This test confirmed the significance of the differing responses to MOMP and to PMPGa.

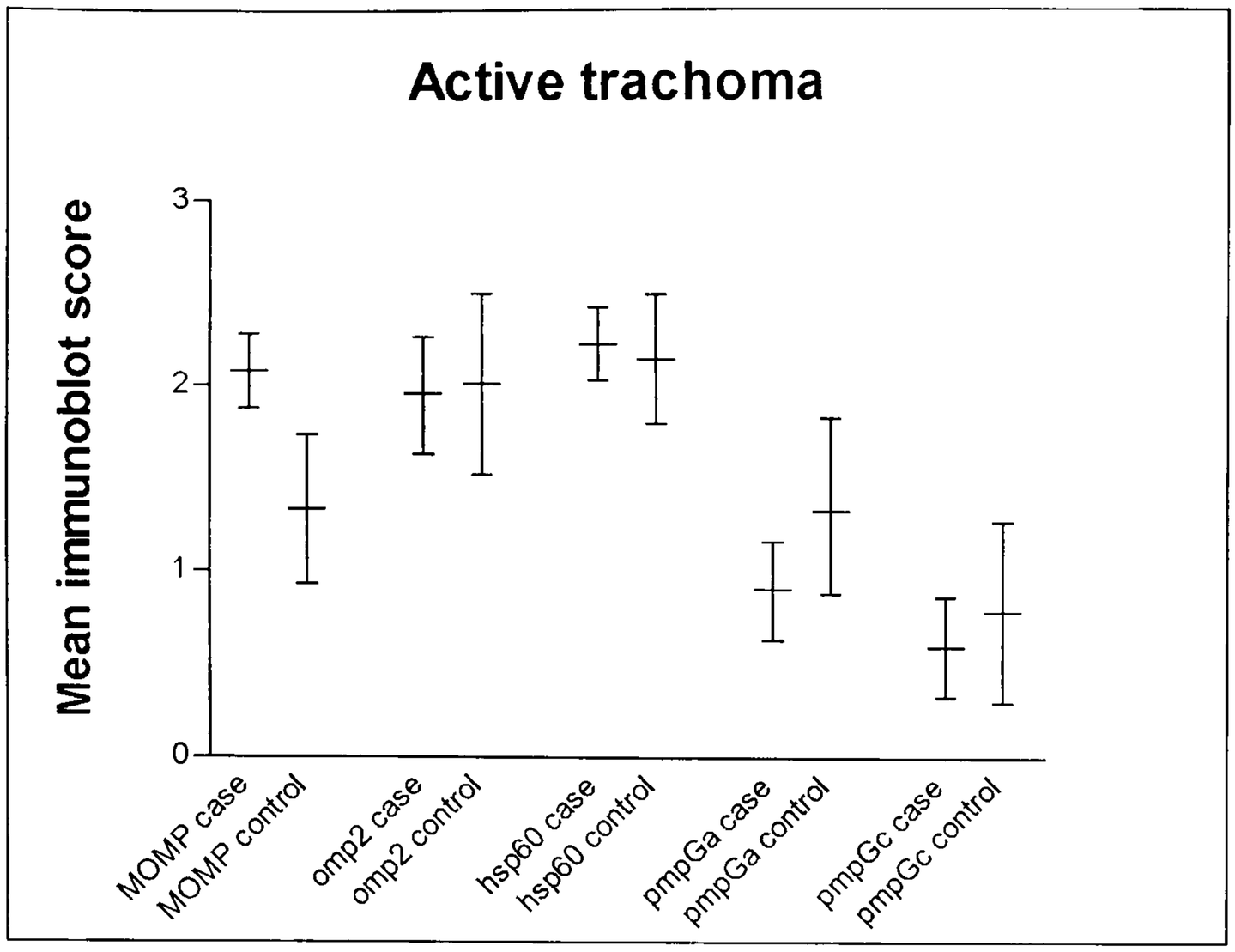


Figure 4-7: Mean immunoblot density scores and 95% confidence intervals for children with active trachoma and their controls

Figure 4-7, showing the mean and 95% confidence intervals, clearly illustrates the differing responses of children with active trachoma and matched controls towards MOMP and suggests that there may be a trend towards a lower response to PMPGa in those with trachoma.

Antigen	O.R.	logistic regression	
		95% C.I.	p <
MOMP	5.79	1.68 - 20.02	0.006
OMP2	0.96	0.43 - 2.16	0.92
HSP60	1.24	0.52 - 2.91	0.63
PMPGa	0.65	0.36 - 1.14	0.13
PMPGc	0.75	0.41 - 1.34	0.33

Table 4-9: Conditional logistic regression of immunoblot scores from children with active trachoma and their controls

Conditional logistic regression was used to maximise the power given by the case-control matched data. This again confirms the strong association between higher immunoblot density scores to MOMP and the presence of active trachoma. Each incremental rise in the arbitrary immunoblot density score is associated with an odds ratio of 5.79 of being a case (95% C.I. 1.68-20.02, $p<0.006$). χ^2 analysis for trend

enables a further estimation of the odds ratio of being a case for each level of MOMP immunoblot density.

Immunoblot score	Group	n	Odds ratio	95% C.I.	p <
MOMP < 1	Active	1	1.00	-	0.01
	Control	5			
MOMP 1 - 1.99	Active	17	12.1	0.9 - 169	
	Control	7			
MOMP >=2	Active	35	13.5	1.2 - 154	
	Control	13			

Table 4-10: χ^2analysis for trend for immunoblot scores to MOMP in children with active trachoma and their controls

A χ^2 test for trend was also used to look again at the relationship between antibody responses to PMPGa having active trachoma. This again picks up a trend towards a lower likelihood of being a case with increasing responses to PMPGa significant at the 6% level.

Immunoblot score	Group	n	Odds Ratio	95% C.I.	p <
PMPGa < 0.4	Active	19	1.00	-	0.06
	Control	6			
PMPGa 0.4 - 0.99	Active	8	1.26	0.20 - 7.87	
	Control	2			
PMPGa 1.0 - 1.99	Active	18	0.59	0.16 - 2.13	
	Control	8			
PMPGa 2.0 - 3.0	Active	10	0.32	0.08 - 1.20	
	Control	10			

Table 4-11: χ^2 analysis for trend for immunoblot scores to PMPGa in children with active trachoma and their controls

4.3.1.2 Scarred trachoma

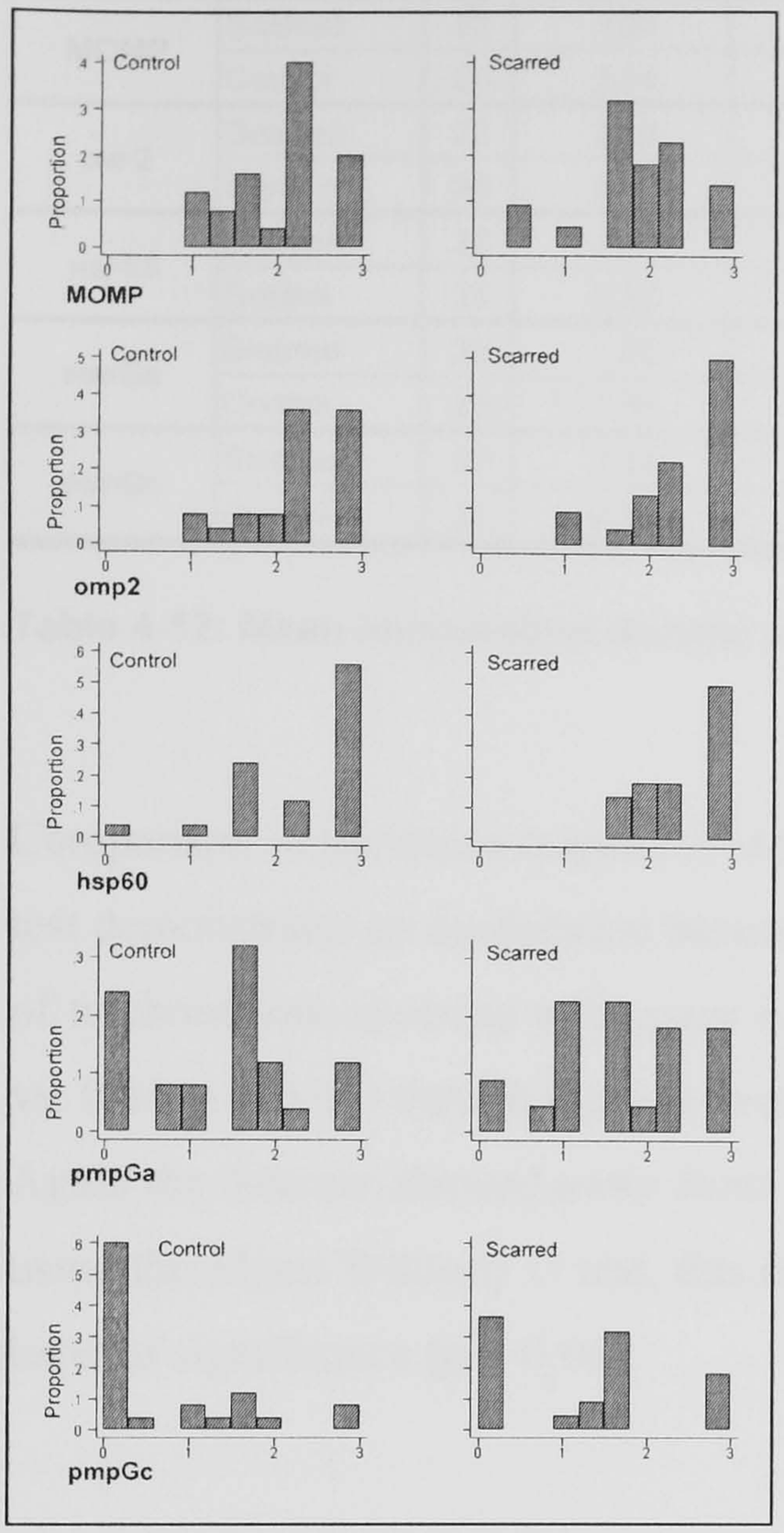


Figure 4-8: Frequency distribution of immunoblot scores for individuals with scarring and their controls

In contrast to the situation seen in children with active trachoma and their controls, in the case of scarred trachoma 0/25 controls and 2/22 (9%) of cases have no detectable antibodies to MOMP suggesting that in this group almost all individuals have been exposed to chlamydial infection.

In contrast to previous studies, no association is demonstrated between detectable antibodies to HSP60 and the presence of scarring; 24/25 (96%) of controls and 22/22 of scarred individuals have antibodies to HSP60. There is perhaps some evidence of a trend at higher levels of response; 17/25 (68%) of controls vs. 19/22 (86%) of scarred individuals have immunoblot density scores of 2 or higher ($p < 0.14$).

Again these graphs demonstrate that the majority of individuals have detectable antibodies to the PMPG recombinant proteins. 17/25 (68%) of the controls and 19/22 (86%) of those with scarring have antibodies to PMPGa whilst 9/25 (36%) of controls and 14/22 (64%) of those with scarring have antibodies to PMPGc, a 'significantly' higher proportion in those with scarring for PMPGc ($p < 0.06$).

Antigen	Group	n	Mean immunoblot score	t-test		Mann-Whitney
				95% C.I.	p	p
MOMP	Scarred	22	1.91	1.59 - 2.23	0.40	0.37
	Control	25	2.01	1.82 - 2.33		
OMP2	Scarred	22	2.42	2.15 - 2.70	0.59	0.60
	Control	25	2.32	2.07 - 2.58		
HSP60	Scarred	22	2.46	2.24 - 2.67	0.60	0.96
	Control	25	2.36	2.03 - 2.68		
PMPGa	Scarred	22	1.65	1.27 - 2.04	0.25	0.27
	Control	25	1.35	0.96 - 1.74		
PMPGc	Scarred	22	1.24	0.75 - 1.72	0.05	0.06
	Control	25	0.64	0.26 - 1.03		

Table 4-12: Mean immunoblot density scores for scarred individuals and their controls

Comparison of the mean responses of the scarred case and control populations using a t-test demonstrates an association between antibody responses to PMPGc and the presence of trachomatous scarring with cases responding at twice the intensity of controls (1.24 vs. 0.64, $p < 0.05$) though there is overlap in the 95% confidence intervals (Figure 4-9). Again the data are skewed away from the normal distribution and so were also analysed using the Mann-Whitney U test, this confirmed the association but at a slightly reduced level of significance ($p < 0.06$).

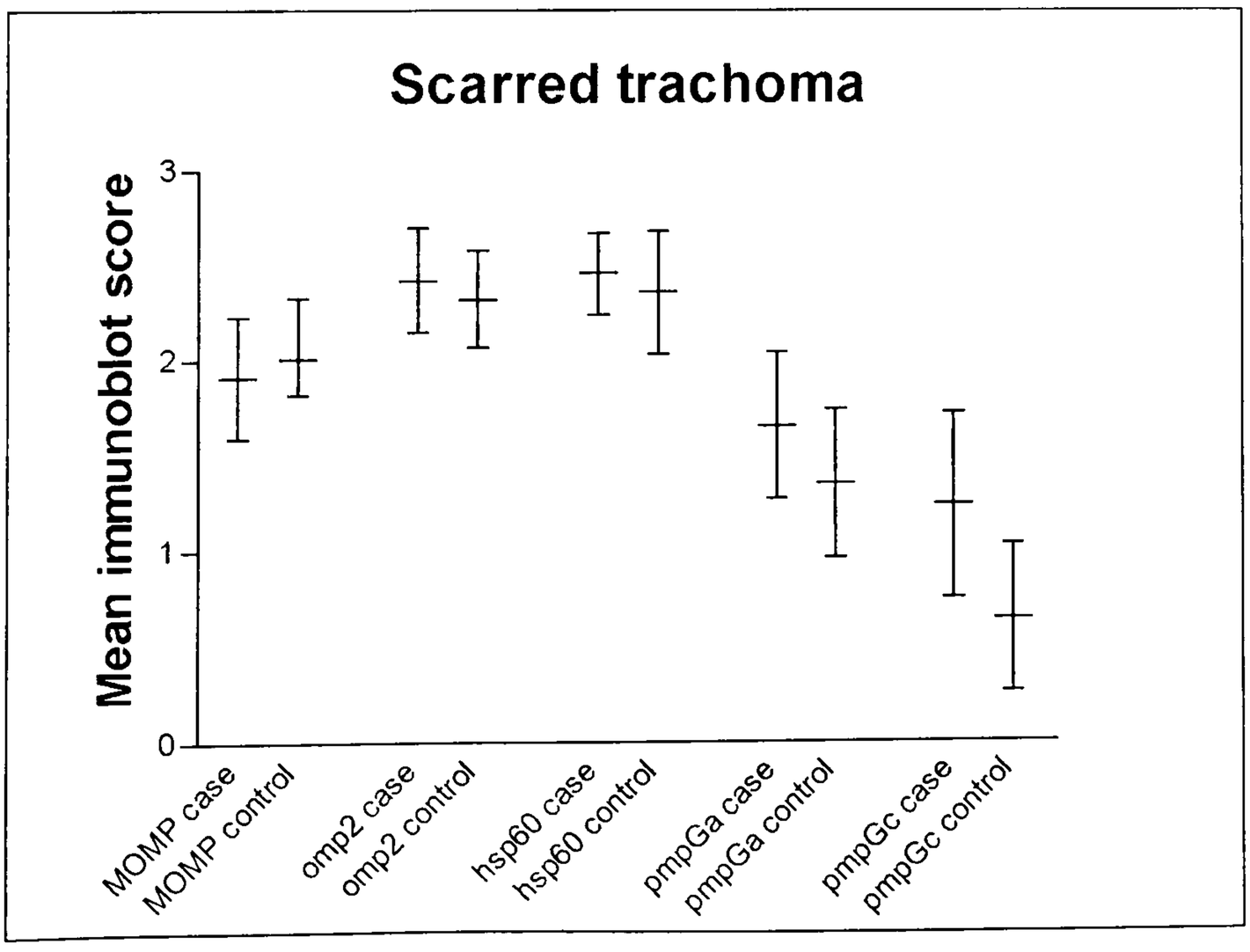


Figure 4-9: Mean immunoblot density scores for scarred individuals and their controls

Antigen	O.R.	logistic regression	
		95% C.I.	p <
MOMP	0.70	0.28 - 1.73	0.44
OMP2	1.26	0.47 - 3.36	0.65
HSP60	1.22	0.50 - 2.95	0.66
PMPGa	1.65	0.79 - 3.45	0.18
PMPGc	2.03	0.99 - 4.19	0.05

Table 4-13: Conditional logistic regression of immunoblot scores for scarred individuals and their controls

Conditional logistic regression analysis, using pair-wise analysis, again highlights the association between response to PMPGc and scarring with each incremental rise in response to PMPGc being associated with an odds ratio of 2.03 for being scarred ($p < 0.05$) though the odds ratio does just include 1. Using a χ^2 analysis for trend to calculate the relationship for each level of response again shows a rise in the odds ratio of being a case with rising responses to PMPGc with individuals demonstrating immunoblot density scores in the highest quartile being 4.5 times more likely to be scarred than those without demonstrable anti-PMPGc IgG ($p < 0.02$ - Table 4-14).

Immunoblot score	Group	n	Odds Ratio	95% C.I.	p <
PMPGc < 0.4	Scarred	8	1.00	-	0.02
	Control	16			
PMPGc 0.4 - 0.99	Scarred	1	1.00	0.07 - 13.39	
	Control	2			
PMPGc 1.0 - 1.99	Scarred	9	3.60	0.83 - 15.66	
	Control	5			
PMPGc 2.0 - 3.0	Scarred	9	4.50	0.94 - 21.57	
	Control	4			

Table 4-14: χ^2 analysis for trend for immunoblot scores to PMPGc in scarred individuals and their controls

4.3.2 Cytokine ELISAs from whole blood assays

4.3.2.1 Active trachoma

4.3.2.1.1 TNF-α

The frequency-distribution plots of TNFα concentrations (Figure 4-10) demonstrate some responses for which the spread of concentrations is approximately normally distributed, such as PHA, and EB's and others for which the distribution was much more strongly skewed, such as PPD. Accordingly both the t-test and Mann-Whitney U test were used to compare the population means for cases and controls.

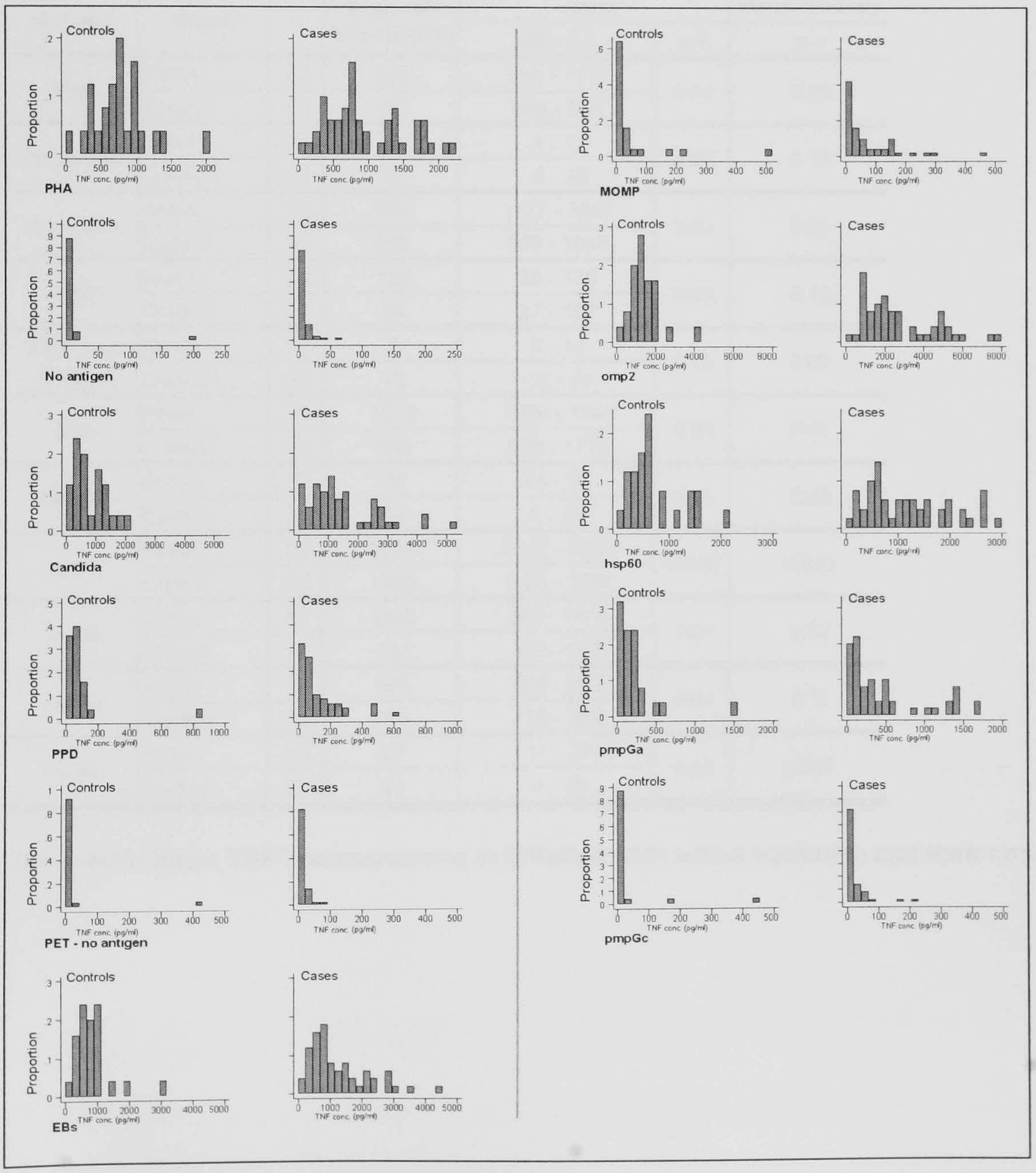


Figure 4-10: Frequency distribution of TNFα responses in children with active trachoma and their controls

Analysis of the mean TNFα concentrations for cases of active trachoma and controls using a t-test reveals a higher TNFα response in children with active trachoma to Candida (1467 pg/ml vs. 823, $p < 0.01$), to chlamydial elementary bodies (1273 vs. 868, $p > 0.06$), to OMP2 (2726 vs. 1455, $p > 0.002$). to HSP60 (1188 vs. 731, $p < 0.01$) and to PMPGa (463 vs. 235, $p < 0.04$). Use of the Mann-Whitney U test reduces the level of significance for the difference in response to EBs and PMPGa and certainly there is some overlapping of the 95% confidence intervals for these antigens. Conditional logistic regression was therefore used to exploit the additional power provided by examining each case-control pair in turn.

Antigen	Group	n	Mean TNF level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Active	50	953	794 - 1112	0.14	0.29
	Control	25	767	600 - 934		
No antigen	Active	50	7	4 - 11	0.65	0.13
	Control	25	10	-6 - 26		
Candida	Active	50	1467	1127 - 1807	0.01	0.03
	Control	25	823	598 - 1048		
PPD	Active	50	130	89 - 170	0.33	0.19
	Control	25	94	27 - 162		
PET - no antigen	Active	50	9	5 - 14	0.40	0.02
	Control	25	19	-15 - 54		
EBs	Active	50	1273	1000 - 1547	0.06	0.11
	Control	25	868	622 - 1114		
MOMP	Active	50	70	44 - 96	0.45	0.06
	Control	25	52	5 - 98		
OMP2	Active	50	2726	2192 - 3260	0.002	0.003
	Control	25	1455	1126 - 1784		
HSP60	Active	50	1188	960 - 1415	0.01	0.02
	Control	25	731	520 - 942		
PMPGa	Active	50	463	321 - 606	0.04	0.11
	Control	25	235	116 - 354		
PMPGc	Active	50	23	11 - 34	0.64	0.007
	Control	25	30	-9 - 69		

Table 4-15: Mean TNF concentrations in children with active trachoma and their controls

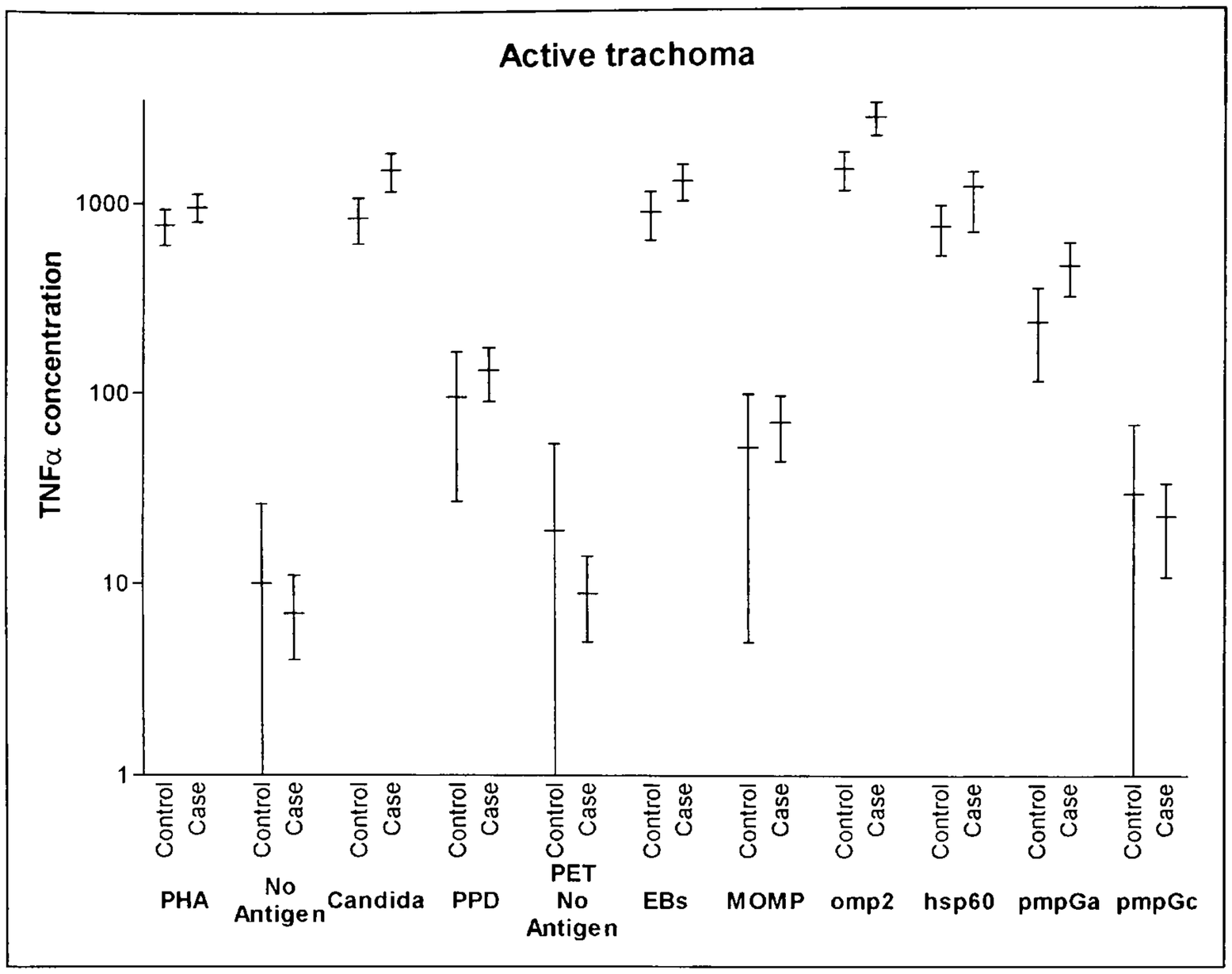


Figure 4-11: Mean TNFα concentrations in children with active trachoma and their controls

Antigen	logistic regression		
	O.R. for 25% rise in TNF	95% C.I.	p <
PHA	1.26	0.97 - 1.62	0.08
No antigen	1.00	0.95 - 1.04	0.86
Candida	1.31	1.05 - 1.65	0.02
PPD	1.12	0.95 - 1.31	0.17
PET - No antigen	0.99	0.96 - 1.03	0.65
EB's	1.31	1.01 - 1.70	0.04
MOMP	1.05	0.96 - 1.15	0.29
OMP2	1.45	1.08 - 1.96	0.01
HSP60	1.29	1.04 - 1.61	0.02
PMPGa	1.18	1.00 - 1.39	0.06
PMPGc	1.00	0.95 - 1.04	0.85

Table 4-16: Conditional logistic regression of TNFα concentrations in children with active trachoma and their controls

Conditional logistic regression confirms the higher responses in those with active disease to Candida ($p < 0.02$), EBs ($p < 0.04$), OMP2 ($p < 0.004$), HSP60 ($p < 0.02$) and PMPGa ($p < 0.06$) and also demonstrates an association for PHA ($p < 0.08$) (Table 4-16).

Quartile	Group	n	Odds ratio	95% C.I.	p <
TNF < 25th cent	Active	9	1.00	-	0.03
	Control	9			
TNF 25 - 50%	Active	12	1.71	0.45 - 6.56	
	Control	7			
TNF 50 - 75%	Active	13	2.17	0.54 - 8.62	
	Control	6			
TNF > 75th cent	Active	16	5.33	1.00 - 28.56	
	Control	3			

Table 4-17: χ^2 test for trend for TNFα responses to *Candida* in children with active trachoma and their controls

Here the χ^2 test clearly demonstrates an increasing likelihood of being a case with increasing TNFα responses to the common recall antigen Candida.

Quartile	Group	n	Odds ratio	95% C.I.	p <
TNF < 25th cent	Active	11	1.00	-	0.19
	Control	7			
TNF 25 - 50%	Active	12	1.09	0.28 - 4.20	
	Control	7			
TNF 50 - 75%	Active	11	0.88	0.23 - 3.32	
	Control	8			
TNF > 75th cent	Active	16	3.39	0.66 - 17.37	
	Control	3			

Table 4-18: χ^2 test for trend for TNFα responses to chlamydial EBs in children with active trachoma and their controls

The χ^2 test for trend does not demonstrate a consistent trend although those demonstrating the highest quartile of TNFα response to chlamydial EBs are 3.39 times more likely to be cases than controls.

Quartile	Group	n	Odds ratio	95% C.I.	p <
TNF < 25th cent	Active	10	1.00	-	0.004
	Control	8			
TNF 25 - 50%	Active	9	0.72	0.19 - 2.69	
	Control	10			
TNF 50 - 75%	Active	13	1.73	0.44 - 6.84	
	Control	6			
TNF > 75th cent	Active	18	14.4	1.15 - 180.25	
	Control	1			

Table 4-19: test for trend for TNFα responses to OMP2 in children with active trachoma and their controls

A clear relationship between the odds ratio for having active trachoma and the concentration of TNFα generated in response to OMP2 is demonstrated by the χ^2 test for trend .

Quartile	Group	n	Odds ratio	95% C.I.	p <
TNF < 25th cent	Active	9	1.00	-	0.03
	Control	9			
TNF 25 - 50%	Active	11	1.38	0.37 - 5.14	
	Control	8			
TNF 50 - 75%	Active	14	3.50	0.76 - 16.18	
	Control	4			
TNF > 75th cent	Active	15	3.75	0.81 - 17.33	
	Control	4			

Table 4-20: χ^2 test for trend for TNFα responses to HSP60 in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
TNF < 25th cent	Active	10	1.00	-	0.05
	Control	8			
TNF 25 - 50%	Active	11	1.10	0.29 - 4.12	
	Control	8			
TNF 50 - 75%	Active	13	1.73	0.44 - 5.84	
	Control	6			
TNF > 75th cent	Active	16	4.27	0.82 - 22.19	
	Control	3			

Table 4-21: χ^2 test for trend for TNFα responses to PMPGa in children with active trachoma and their controls

The association between the odds ratio of being a case and the concentration of TNFα generated in response to HSP60 and PMPGa are similar as shown in the two tables.

4.3.2.1.2 IFNγ

The frequency distribution plots for IFNγ again demonstrate marked skewing in the distribution of the data, such that, in general, the Mann-Whitney U test is more appropriate for assessing the differences between cases and controls.

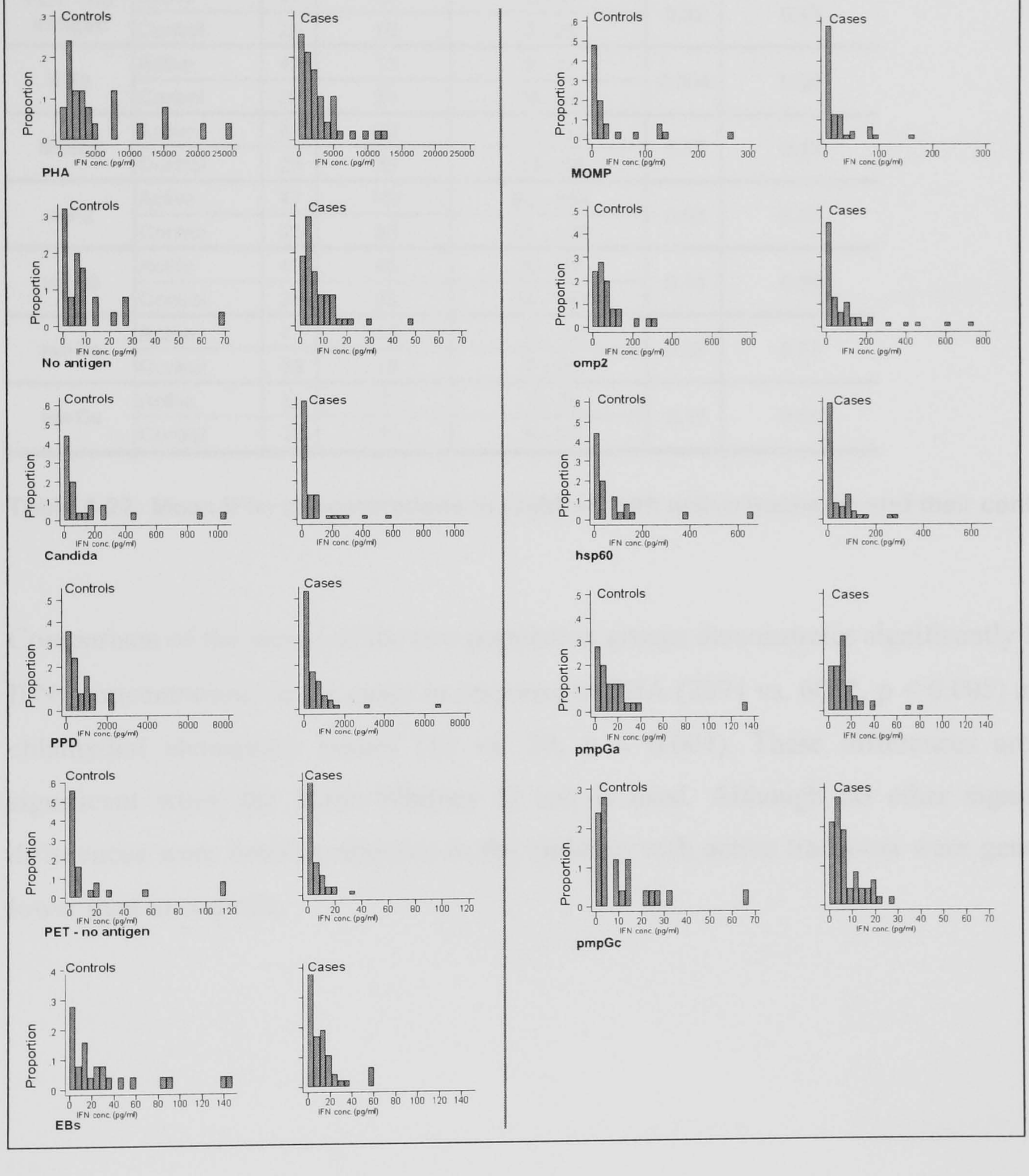


Figure 4-12: Frequency-distribution graphs of IFNγ responses in children with active trachoma and their controls

Antigen	Group	n	Mean IFN level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Active	47	2894	2085 - 3703	0.005	0.03
	Control	25	6032	3344 - 8719		
No antigen	Active	47	8	6 - 11	0.47	0.92
	Control	25	10	4 - 16		
Candida	Active	47	73	41 - 105	0.06	0.21
	Control	25	159	48 - 269		
PPD	Active	47	590	275 - 906	0.79	0.14
	Control	25	653	398 - 909		
PET - no antigen	Active	47	5	3 - 7	0.02	0.43
	Control	25	16	3 - 29		
EBs	Active	47	13	9 - 17	0.004	0.05
	Control	25	33	16 - 50		
MOMP	Active	47	22	13 - 31	0.12	0.49
	Control	25	39	13 - 65		
OMP2	Active	47	107	60 - 154	0.53	0.47
	Control	25	85	51 - 120		
HSP60	Active	47	48	30 - 66	0.14	0.68
	Control	25	85	24 - 144		
PMPGa	Active	47	15	11 - 20	0.59	0.77
	Control	25	18	7 - 29		
PMPGc	Active	47	7	5 - 9	0.11	0.56
	Control	25	11	5 - 17		

Table 4-22: Mean IFNγ concentrations in children with active trachoma and their controls

Comparison of the means of the two population groups demonstrates significantly lower IFNγ concentrations in the cases in response to PHA (2894 vs. 6032, $p < 0.005$) and to chlamydial elementary bodies (13 vs. 39, $p < 0.004$). These differences are still significant when the Mann-Whitney U test is used. Although no other significant differences were noted, responses in the children with active trachoma were generally lower than in controls.

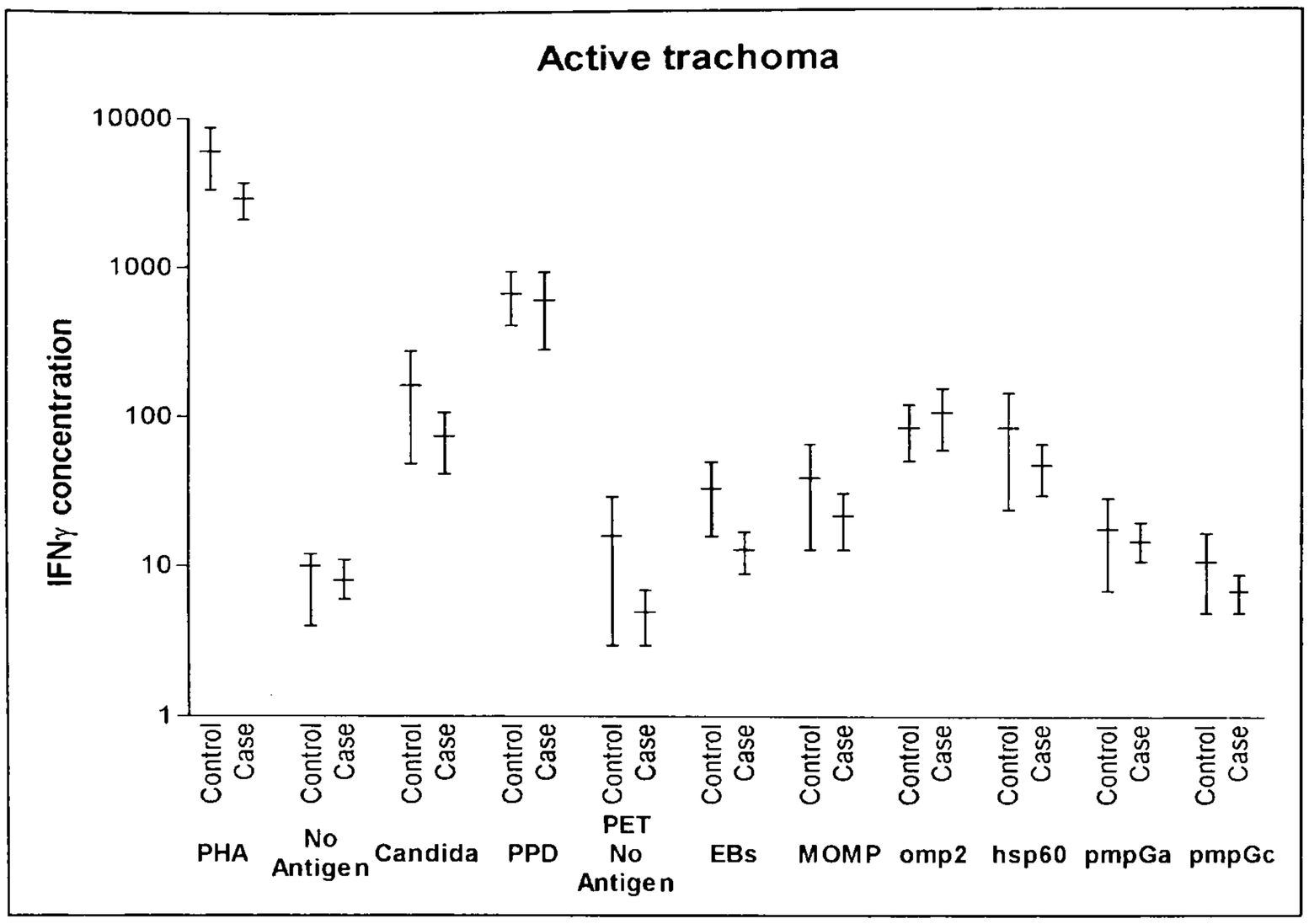


Figure 4-13: Mean IFNγ concentrations in children with active trachoma and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IFN	95% C.I.	p <
PHA	0.83	0.69 - 0.99	0.04
No antigen	0.98	0.90 - 1.07	0.65
Candida	0.94	0.86 - 1.02	0.14
PPD	0.99	0.91 - 1.07	0.74
PET - No antigen	0.94	0.86 - 1.02	0.14
EB's	0.89	0.80 - 1.00	0.06
MOMP	0.95	0.88 - 1.03	0.20
OMP2	1.03	0.93 - 1.14	0.52
HSP60	0.95	0.88 - 1.04	0.27
PMPGa	0.99	0.89 - 1.09	0.77
PMPGc	0.94	0.84 - 1.05	0.27

Table 4-23: Conditional logistic regression of IFNγ concentrations for children with active trachoma and their controls

Using a conditional logistic regression method it can be seen that there is indeed a significantly lower response in children with active trachoma to PHA and to chlamydial EBs. A χ^2 test for trend shows that individuals with IFNγ responses in the highest quartile have an odds ratio for disease of 0.2 against those in the lowest quartile for PHA and an odds ratio of 0.1 for EBs.

Quartile	Group	n	Odds ratio	95% C.I.	p <
IFN < 25th cent	Active	15	1.00	-	0.06
	Control	3			
IFN 25 - 50%	Active	11	0.31	0.08 - 1.61	
	Control	7			
IFN 50 - 75%	Active	12	0.40	0.08 - 2.05	
	Control	6			
IFN > 75th cent	Active	9	0.2	0-04 - 1.07	
	Control	9			

Table 4-24: χ^2 test for trend for IFNγ responses to PHA in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IFN < 25th cent	Active	18	1.00	-	0.03
	Control	7			
IFN 25 - 50%	Active	19	1.23	0.34 - 4.43	
	Control	6			
IFN 50 - 75%	Active	9	0.44	0.12 - 1.66	
	Control	8			
IFN > 75th cent	Active	1	0.10	0.01 - 1.34	
	Control	4			

Table 4-25: χ^2 test for trend for IFNγ responses to chlamydial EBs in children with active trachoma and their controls

4.3.2.1.3 IL-2

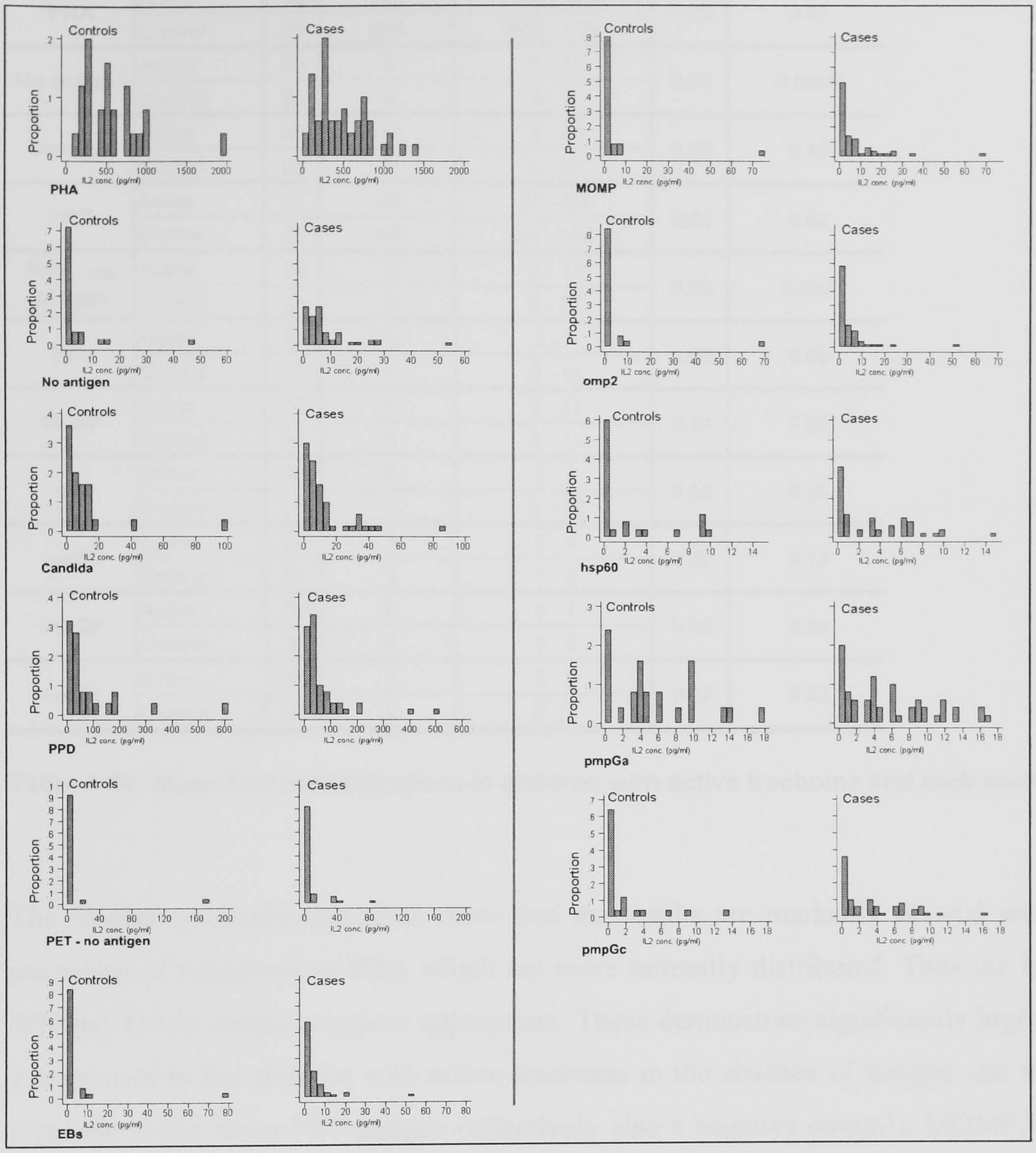


Figure 4-14: Frequency-distribution graphs of IL-2 responses in children with active trachoma and their controls

These frequency-distribution plots demonstrate that a higher proportion of the cases produce detectable IL-2 in response to antigen than controls. This is also noticeable where the blood was incubated without antigen when 11/25 (56%) of controls produced IL-2 vs. 43/50 (86%) of children with active trachoma ($p < 0.0005$). This would be in keeping with a higher level of general T-cell activation associated with an active infection. Similar effects are seen with chlamydial antigens, for example 10/25 (40%) of controls vs. 31/50 (62%) of cases produced IL-2 in response to chlamydial EBs ($p < 0.07$), similarly 10/25 (40%) of controls vs. 32/50 (64%%) of cases produced IL-2 in response to chlamydial HSP60 ($p < 0.05$).

Antigen	Group	n	Mean IL2 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Active	50	490	398 - 582	0.39	0.47
	Control	25	564	403 - 725		
No antigen	Active	50	8	6 - 11	0.08	0.0005
	Control	25	4	0 - 8		
Candida	Active	50	12	8 - 17	0.83	0.49
	Control	25	11	3 - 20		
PPD	Active	50	68	41 - 94	0.42	0.62
	Control	25	89	34 - 144		
PET - no antigen	Active	50	7	3 - 11	0.89	0.007
	Control	25	8	0 - 22		
EBs	Active	50	5	2 - 7	0.96	0.06
	Control	25	5	0 - 11		
MOMP	Active	50	8	4 - 11	0.31	0.02
	Control	25	5	0 - 11		
OMP2	Active	50	5	2 - 7	0.82	0.05
	Control	25	4	0 - 10		
HSP60	Active	50	3	2 - 4	0.29	0.13
	Control	25	2	1 - 4		
PMPGa	Active	50	6	4 - 7	0.96	0.98
	Control	25	6	4 - 8		
PMPGc	Active	50	3	2 - 4	0.07	0.02
	Control	25	2	0 - 3		

Table 4-26: Mean IL-2 concentrations in children with active trachoma and their controls

The frequency-distribution plots show that the results are markedly skewed with the exception of responses to PHA which are more normally distributed. Thus the Mann-Whitney U-test results are more appropriate. These demonstrate significantly higher IL-2 responses in the children with active trachoma in the absence of antigen and also in response to the sham PET antigen (effectively also a negative control), MOMP, OMP2 and PMPGc. However, with the exception of the true negative control, the confidence intervals overlap to a high degree as can be seen from Figure 4-15, making these differences unconvincing. More importantly, most of these results lie below the lower limit of accurate detection (8 pg/ml). Conditional logistic regression fails to confirm any of these associations.

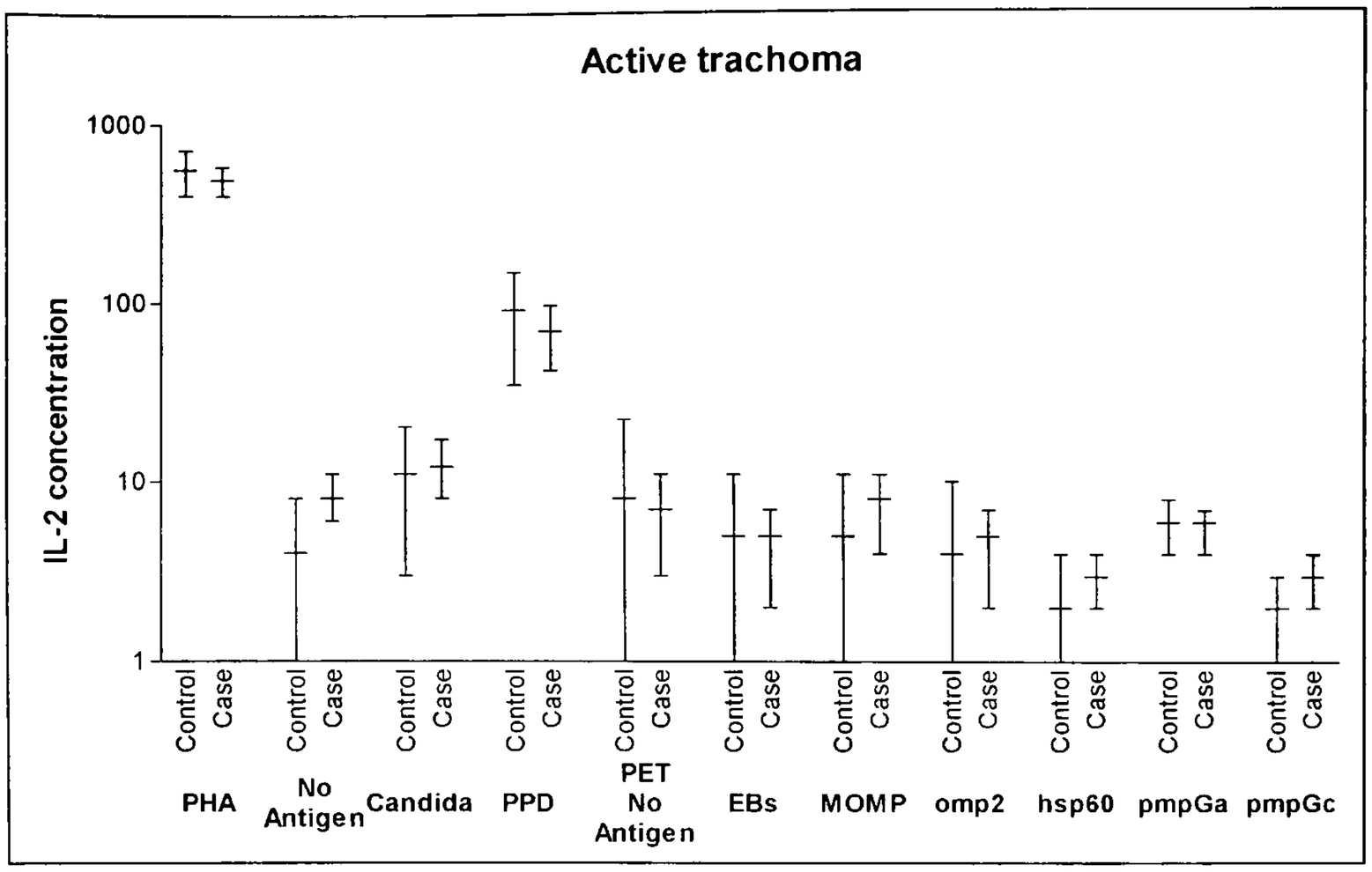


Figure 4-15: Mean IL-2 concentrations in children with active trachoma and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-2	95% C.I.	p <
PHA	0.95	0.76 - 1.18	0.63
No antigen	1.11	0.97 - 1.27	0.14
Candida	1.01	0.94 - 1.10	0.76
PPD	0.96	0.88 - 1.04	0.31
PET - No antigen	0.99	0.96 - 1.03	0.73
EB's	1.00	0.95 - 1.06	0.91
MOMP	1.04	0.96 - 1.12	0.38
OMP2	1.01	0.96 - 1.05	0.79
HSP60	1.06	0.94 - 1.20	0.33
PMPGa	1.00	0.86 - 1.16	0.98
PMPGc	1.13	0.97 - 1.31	0.11

Table 4-27: Conditional logistic regression of IL-2 concentrations for children with active trachoma and their controls

4.3.2.1.4 IL-5

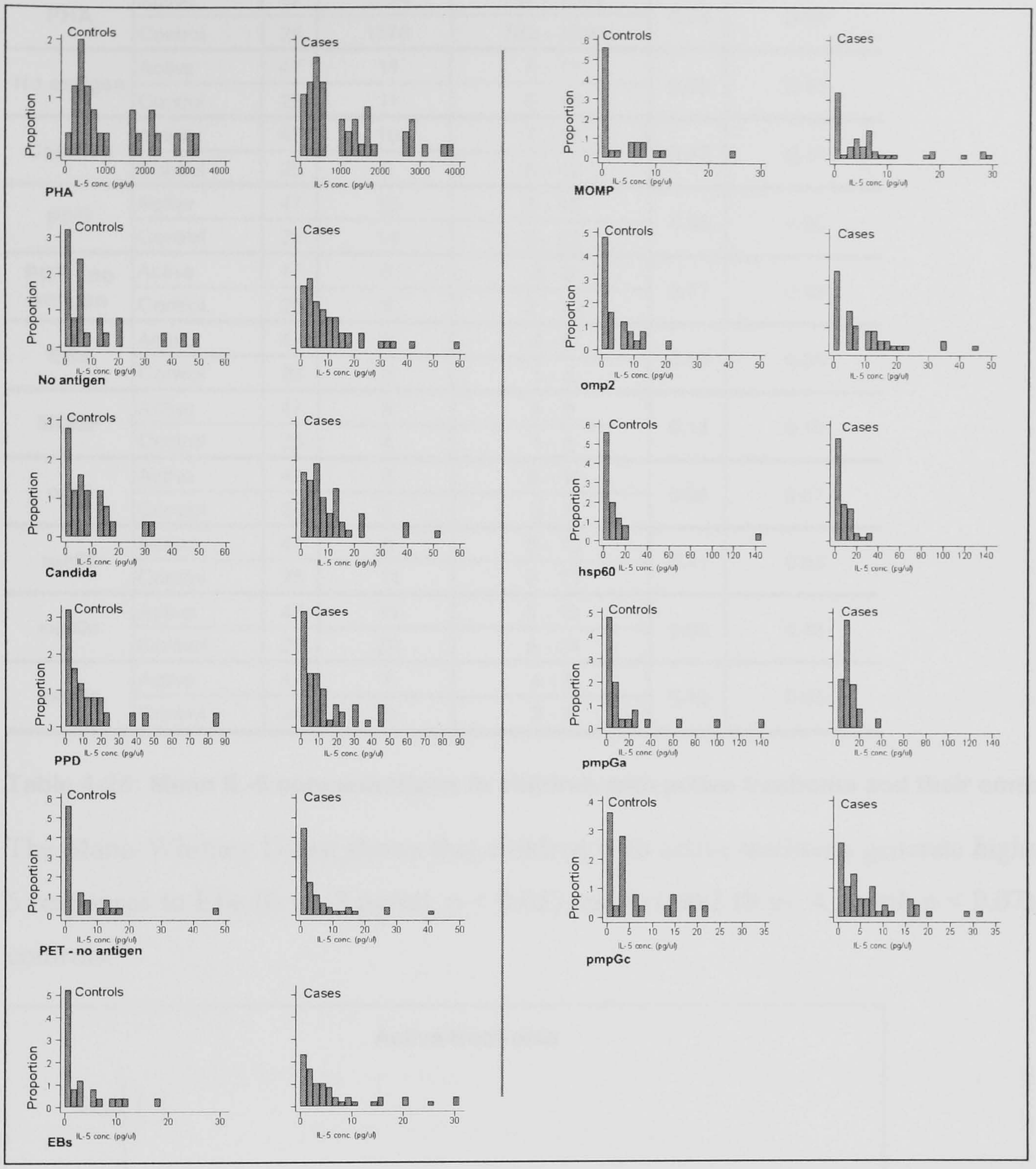


Figure 4-16: Frequency-distribution graphs of IL-5 responses in children with active trachoma and their controls

The frequency-distribution graphs for IL-5 indicate that only the responses to PHA approximate to a normal distribution, thus the Mann-Whitney U-test provides the most accurate comparison of the means.

Antigen	Group	n	Mean IL-5 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Active	47	1094	792 - 1396	0.74	0.46
	Control	25	1176	762 - 1591		
No antigen	Active	47	11	8 - 15	0.90	0.41
	Control	25	11	5 - 16		
Candida	Active	47	10	7 - 13	0.47	0.44
	Control	25	9	5 - 12		
PPD	Active	47	12	8 - 16	0.68	0.90
	Control	25	14	6 - 22		
PET - no antigen	Active	47	5	3 - 8	0.77	0.92
	Control	25	6	2 - 10		
EBs	Active	47	6	4 - 9	0.09	0.05
	Control	25	3	1 - 5		
MOMP	Active	47	6	4 - 9	0.13	0.15
	Control	25	4	1 - 6		
OMP2	Active	47	8	5 - 11	0.05	0.07
	Control	25	4	2 - 6		
HSP60	Active	47	8	5 - 10	0.41	0.53
	Control	25	11	0 - 23		
PMPGa	Active	47	11	8 - 13	0.08	0.43
	Control	25	20	6 - 34		
PMPGc	Active	47	7	4 - 9	0.40	0.35
	Control	25	5	3 - 8		

Table 4-28: Mean IL-5 concentrations in children with active trachoma and their controls

The Mann-Whitney U-test shows that children with active trachoma generate higher IL-5 responses to EBs (6 vs. 3 pg/ml, $p < 0.05$) and to OMP2 (8 vs. 4 pg/ml, $p < 0.07$) than controls.

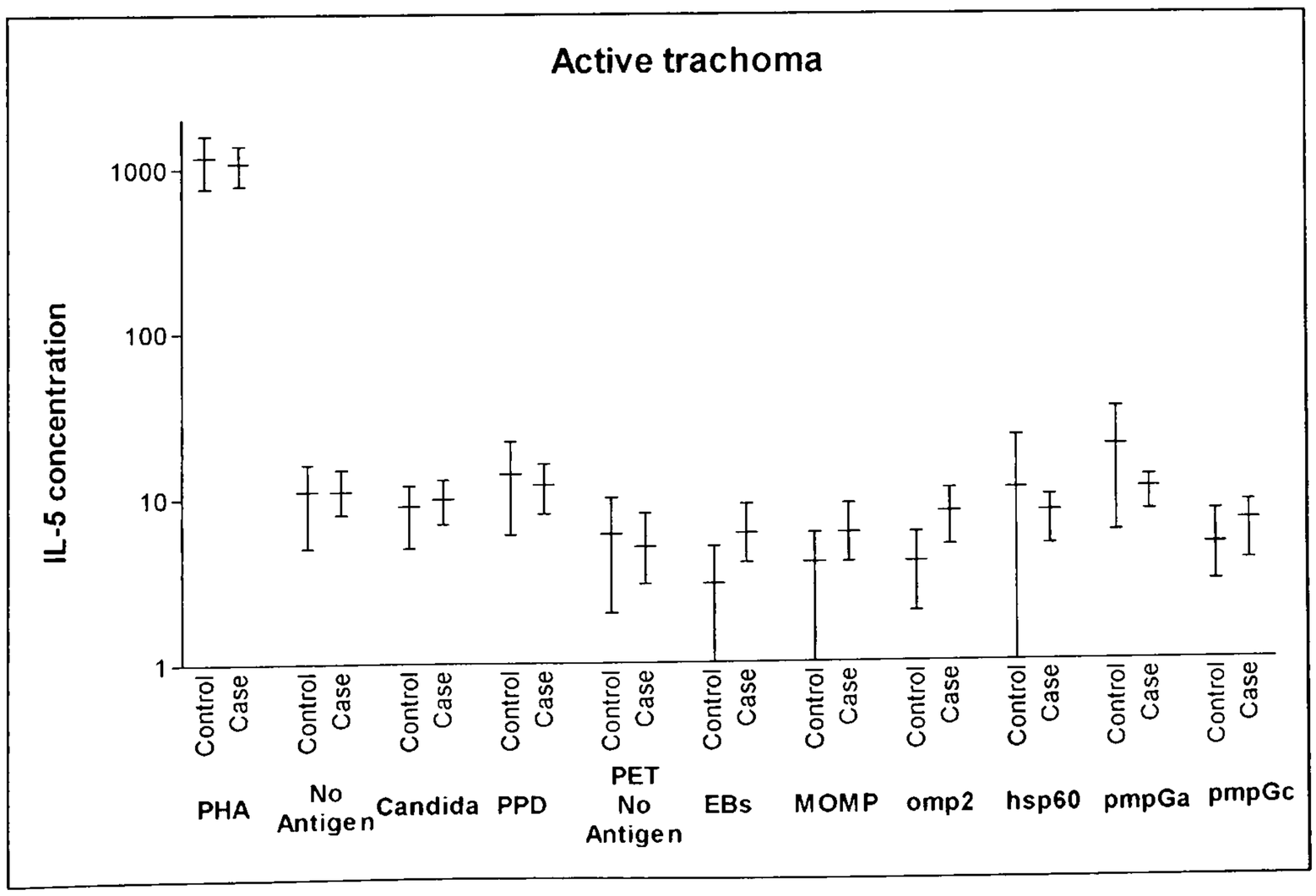


Figure 4-17: Mean IL-5 concentrations in children with active trachoma and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-5	95% C.I.	p <
PHA	1.01	0.87 - 1.17	0.89
No antigen	1.01	0.91 - 1.13	0.81
Candida	1.08	0.93 - 1.24	0.31
PPD	0.98	0.88 - 1.10	0.73
PET - No antigen	1.00	0.93 - 1.07	0.93
EB's	1.13	0.99 - 1.29	0.07
MOMP	1.12	0.97 - 1.28	0.11
OMP2	1.17	1.00 - 1.37	0.04
HSP60	0.97	0.90 - 1.04	0.42
PMPGa	0.93	0.84 - 1.03	0.17
PMPGc	1.07	0.95 - 2.21	0.27

Table 4-29: Conditional logistic regression of IL-5 concentrations for children with active trachoma and their controls

Conditional logistic regression confirms the association between having active trachoma and higher responses to EBs and chlamydial OMP2. However, there is no significant difference between the IL-5 responses to any of the chlamydial or common recall antigens and those seen in the negative control wells, and the IL-5 concentrations produced are less than the minimum level of reliable detection (8pg/ml), thus little if any confidence can be placed upon these associations.

4.3.2.1.5 IL-10

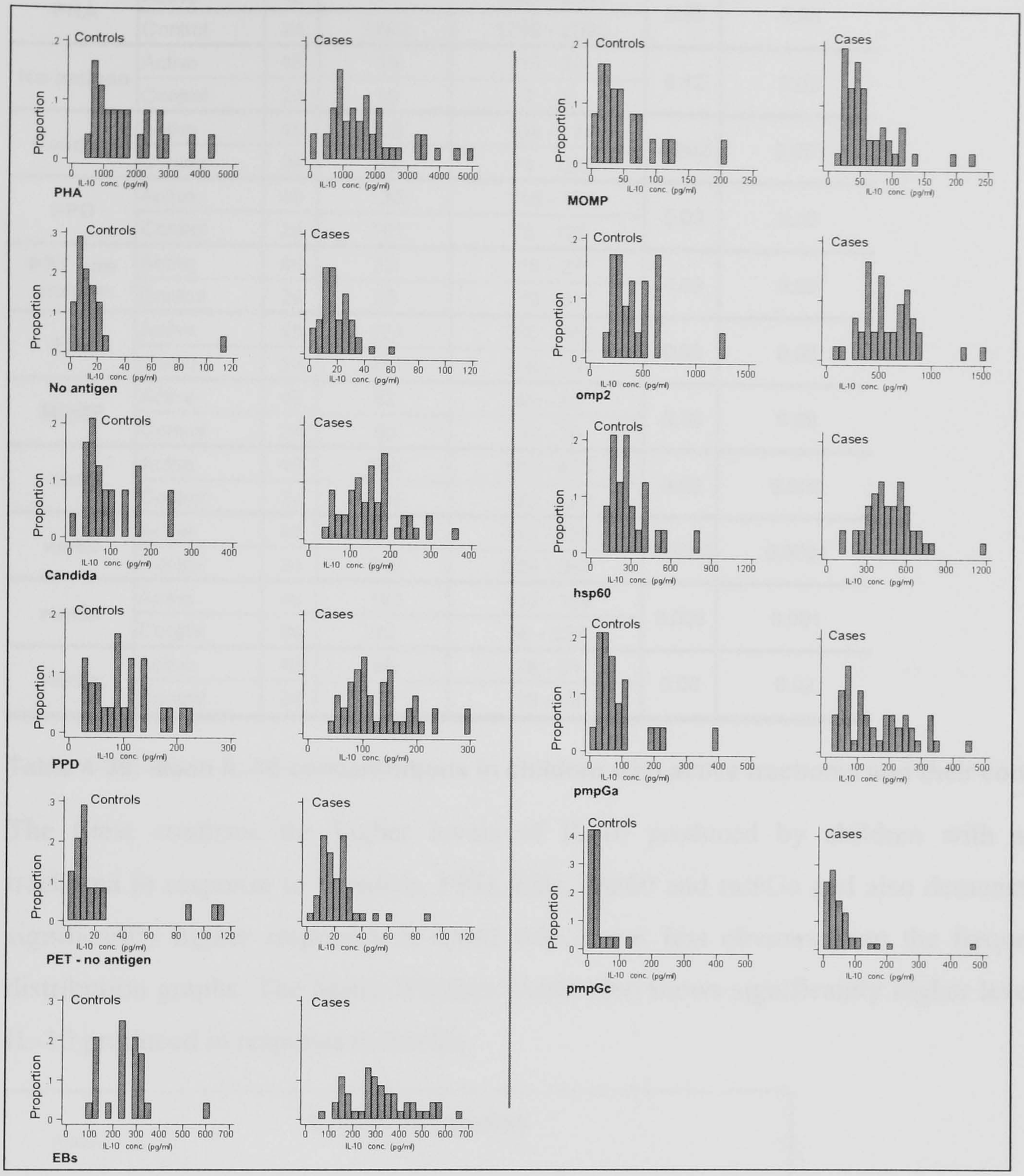


Figure 4-18: Frequency-distribution graphs of IL-10 responses in children with active trachoma and their controls

The frequency-distribution plots for IL-10 show approximately normally distributed data with the exception of responses to PMPGc, thus the t-tests provide the most reliable comparisons of the means. Quite marked differences in the distributions between cases and controls are apparent in the responses to *Candida*, PPD, EBs, HSP60 and PMPGa.

Antigen	Group	n	Mean IL-10 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Active	46	1717	1381 - 2052	0.85	0.86
	Control	24	1663	1236 - 2090		
No antigen	Active	46	19	16 - 23	0.42	0.02
	Control	24	16	7 - 25		
Candida	Active	46	153	132 - 174	0.002	0.001
	Control	24	98	71 - 125		
PPD	Active	46	133	115 - 151	0.03	0.03
	Control	24	101	78 - 125		
PET - no antigen	Active	46	23	18 - 27	0.89	0.02
	Control	24	23	10 - 37		
EBs	Active	46	321	280 - 362	0.03	0.03
	Control	24	251	205 - 297		
MOMP	Active	46	62	49 - 75	0.30	0.08
	Control	24	50	30 - 70		
OMP2	Active	46	588	500 - 675	0.02	0.006
	Control	24	124	320 - 528		
HSP60	Active	46	469	412 - 525	0.0003	0.0001
	Control	24	295	229 - 361		
PMPGa	Active	46	161	130 - 192	0.006	0.001
	Control	24	92	56 - 127		
PMPGc	Active	46	58	36 - 81	0.08	0.02
	Control	24	30	19 - 42		

Table 4-30: Mean IL-10 concentrations in children with active trachoma and their controls

The t-test confirms the higher levels of IL-10 produced by children with active trachoma in response to *Candida*, PPD, EBs, HSP60 and PMPGa and also demonstrates significantly higher responses to OMP2 which was less obvious from the frequency-distribution graphs. The Mann-Whitney U-test also shows significantly higher levels of IL-10 produced in response to PMPGc.

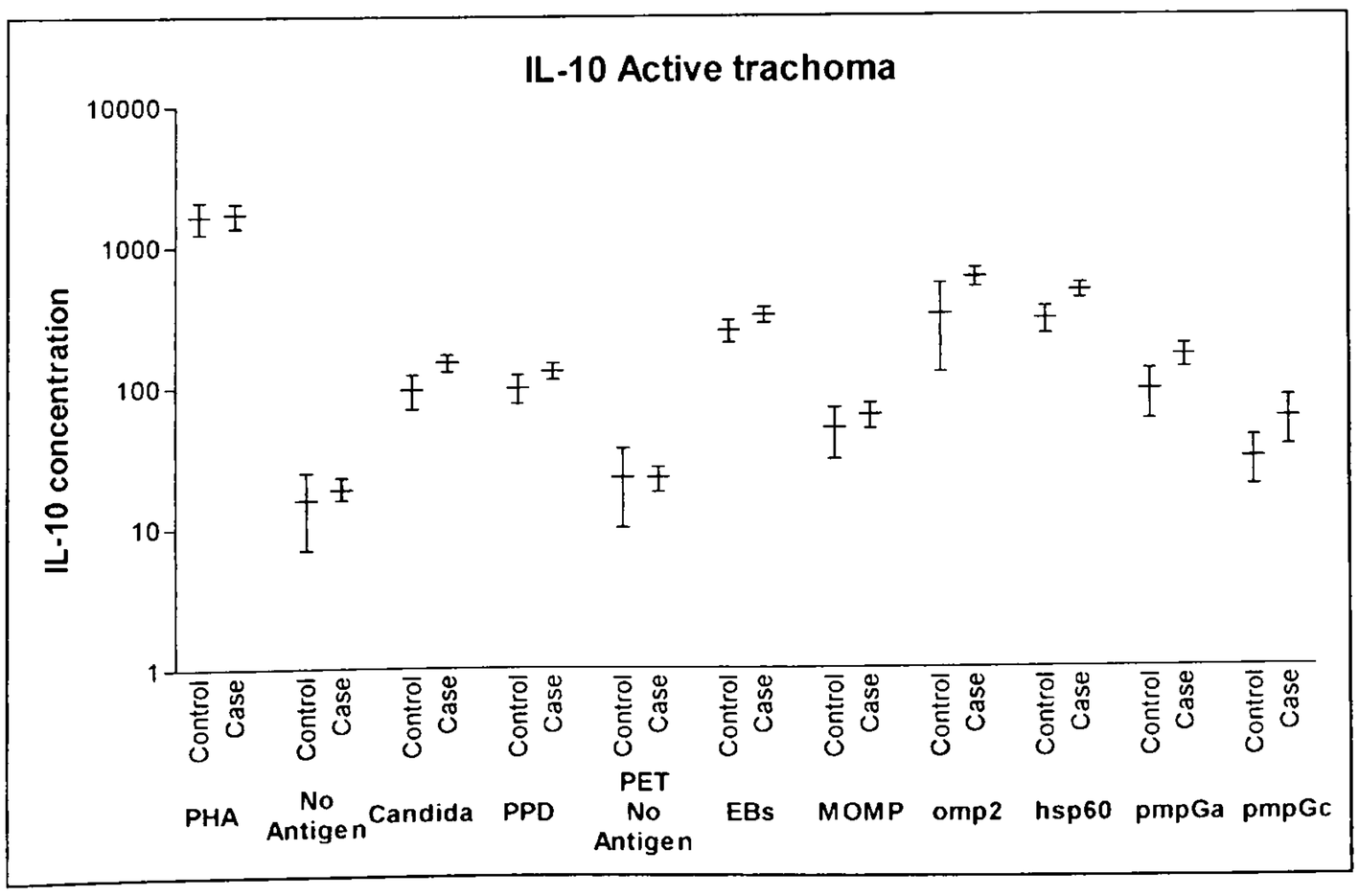


Figure 4-19: Mean IL-10 concentrations in children with active trachoma and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-10	95% C.I.	p <
PHA	1.06	0.87 - 1.29	0.59
No antigen	1.08	0.92 - 1.28	0.33
Candida	1.76	1.17 - 2.64	0.007
PPD	1.46	1.03 - 2.07	0.04
PET - No antigen	0.99	0.88 - 1.12	0.91
EB's	1.44	1.01 - 2.07	0.05
MOMP	1.10	0.92 - 1.33	0.30
OMP2	1.69	1.08 - 2.66	0.02
HSP60	1.95	1.27 - 2.98	0.002
PMPGa	1.35	1.05 - 1.74	0.02
PMPGc	1.22	0.97 - 1.55	0.09

Table 4-31: Conditional logistic regression of IL-10 concentrations for children with active trachoma and their controls

Conditional logistic regression analysis confirms the associations discussed above. The association for PMPGc is less strong with this analysis.

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	7	1.00	-	0.003
	Control	10			
IL-10 25 - 50%	Active	11	2.24	0.55 - 9.11	
	Control	7			
IL-10 50 - 75%	Active	12	3.43	0.76 - 15.53	
	Control	5			
IL-10 > 75th cent	Active	16	11.43	1.45 - 90.02	
	Control	2			

Table 4-32: χ^2 test for trend for IL-10 responses to *Candida* in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	8	1.00	-	0.10
	Control	9			
IL-10 25 - 50%	Active	13	2.93	0.67 - 12.77	
	Control	5			
IL-10 50 - 75%	Active	11	2.06	0.50 - 8.54	
	Control	6			
IL-10 > 75th cent	Active	14	3.94	0.82 - 18.85	
	Control	4			

Table 4-33: χ^2 test for trend for IL-10 responses to PPD in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	10	1.00	-	0.04
	Control	7			
IL-10 25 - 50%	Active	10	0.88	0.22 - 3.41	
	Control	8			
IL-10 50 - 75%	Active	9	0.79	0.20 - 3.13	
	Control	8			
IL-10 > 75th cent	Active	17	11.90	0.97 - 145.71	
	Control	1			

Table 4-34: χ^2 test for trend for IL-10 responses to chlamydial EBs in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	6	1.00	-	0.003
	Control	10			
IL-10 25 - 50%	Active	11	3.67	0.77 - 6.33	
	Control	5			
IL-10 50 - 75%	Active	9	2.14	0.50 - 9.25	
	Control	7			
IL-10 > 75th cent	Active	16	26.67	1.51 - 469.85	
	Control	1			

Table 4-35: χ^2 test for trend for IL-10 responses to OMP2 in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	6	1.00	-	0.0006
	Control	11			
IL-10 25 - 50%	Active	11	2.88	0.68 - 12.19	
	Control	7			
IL-10 50 - 75%	Active	13	5.96	1.12 - 31.70	
	Control	4			
IL-10 > 75th cent	Active	16	14.67	1.69 - 127.65	
	Control	2			

Table 4-36: χ^2 test for trend for IL-10 responses to HSP60 in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	6	1.00	-	0.002
	Control	10			
IL-10 25 - 50%	Active	11	2.29	0.56 - 9.39	
	Control	8			
IL-10 50 - 75%	Active	14	7.78	1.24 - 48.75	
	Control	3			
IL-10 > 75th cent	Active	15	8.33	1.32 - 52.53	
	Control	3			

Table 4-37: χ^2 test for trend for IL-10 responses to PMPGa in children with active trachoma and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Active	10	1.00	-	0.05
	Control	7			
IL-10 25 - 50%	Active	8	0.62	0.16 - 2.49	
	Control	9			
IL-10 50 - 75%	Active	13	1.82	0.43 - 7.74	
	Control	5			
IL-10 > 75th cent	Active	15	3.50	0.67 - 18.34	
	Control	3			

Table 4-38: χ^2 test for trend for IL-10 responses to PMPGc in children with active trachoma and their controls

χ^2 analysis for trend for each of the associations confirms the increasing likelihood of having active trachoma with increasing IL-10 levels generated in response to the antigens. This effect is most strikingly seen for OMP2 where those generating IL-10 in the highest quartile of concentrations are almost 27 times more likely to be cases than those in the lowest quartile. Corresponding figures for the other antigens are 11.4 for *Candida*, 3.9 for MOMP, 11.9 for EBs, 14.7 for HSP60, 8.3 for PMPGa and 3.5 for PMPGc.

4.3.2.1.6 TGF-β

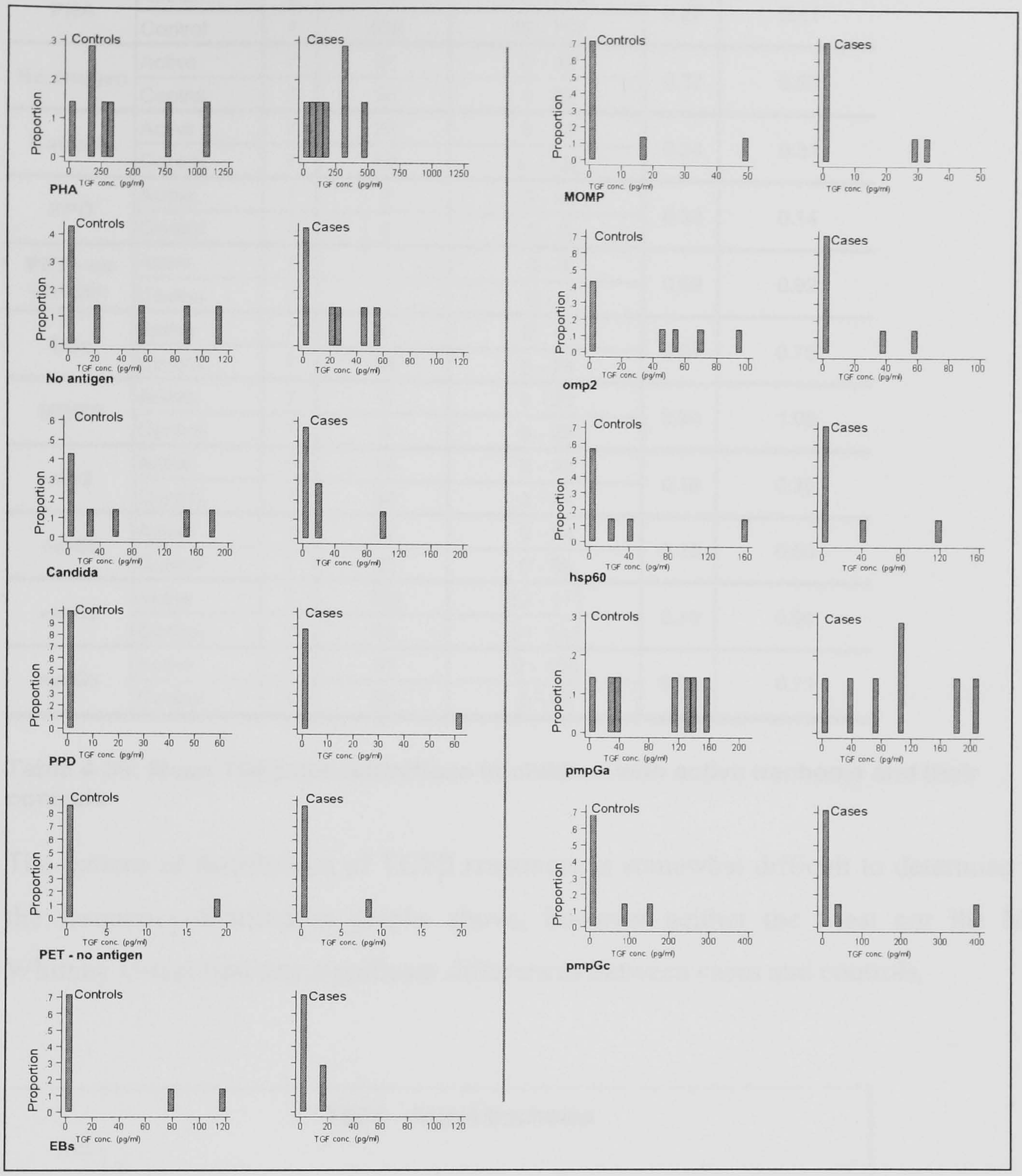


Figure 4-20: Frequency-distribution graphs of TGFβ responses in children with active trachoma and their controls

As explained in section 5.1.2, a significant number of TGFβ assays were discarded due to non-specific high levels of TGFβ production. Despite this a number of suspicious values remain which appear well outside the standard deviation and may represent spurious results. Two such examples from the graphs above are those at ~62 pg/ml for PPD and ~400 pg/ml for PMPGc.

Antigen	Group	n	Mean TGF level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Active	7	219	67 - 372	0.26	0.41
	Control	7	403	49 - 757		
No antigen	Active	7	21	0 - 42	0.37	0.59
	Control	7	39	0 - 82		
Candida	Active	7	20	0 - 54	0.24	0.31
	Control	7	59	0 - 128		
PPD	Active	7	9	0 - 31	0.33	0.14
	Control	7	0	0 - 0		
PET - no antigen	Active	7	1	0 - 4	0.60	0.92
	Control	7	3	0 - 9		
EBs	Active	7	4	0 - 11	0.24	0.75
	Control	7	28	0 - 74		
MOMP	Active	7	9	0 - 23	0.94	1.00
	Control	7	9	0 - 26		
OMP2	Active	7	14	0 - 38	0.18	0.20
	Control	7	38	2 - 74		
HSP60	Active	7	22	0 - 64	0.76	0.60
	Control	7	31	0 - 88		
PMPGa	Active	7	102	33 - 172	0.70	0.90
	Control	7	88	31 -144		
PMPGc	Active	7	61	0 - 195	0.65	0.71
	Control	7	34	0 - 88		

Table 4-39: Mean TGFβ concentrations in children with active trachoma and their controls

The pattern of distribution of TGFβ responses is somewhat difficult to determine from the frequency-distribution graphs above, however neither the t-test nor the Mann-Whitney U-test find any significant differences between cases and controls.

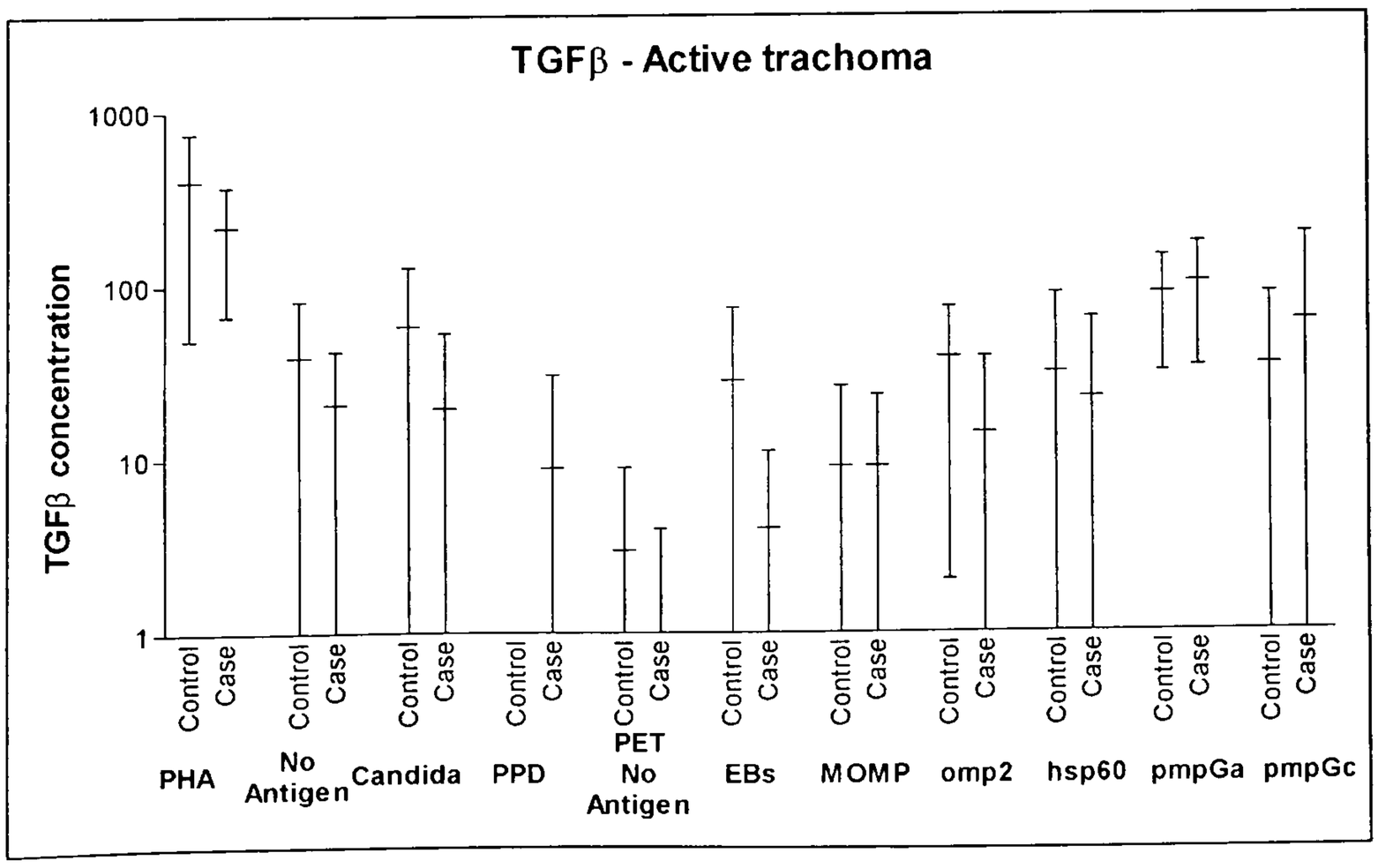


Figure 4-21: Mean TGFβ concentrations in children with active trachoma and their controls

Antigen	logistic regression		
	O.R. for 25% rise in TGF	95% C.I.	p <
PHA	0.78	0.49 - 1.23	0.28
No antigen	0.91	0.73 - 1.14	0.43
Candida	0.63	0.29 - 1.38	0.25
PPD	-	-	-
PET - No antigen	0.97	0.87 - 1.08	0.61
EB's	0.91	0.75 - 1.11	0.35
MOMP	0.98	0.78 - 1.24	0.90
OMP2	0.90	0.74 - 1.09	0.30
HSP60	0.98	0.85 - 1.12	0.76
PMPGa	1.11	0.70 - 1.76	0.65
PMPGc	1.03	0.91 - 1.17	0.65

Table 4-40: Conditional logistic regression of TGFβ concentrations for children with active trachoma and their controls

The conditional logistic regression confirms the earlier analyses and shows no association between TGFβ responses and active trachoma.

4.3.2.2 Scarred trachoma

4.3.2.2.1 TNF-α

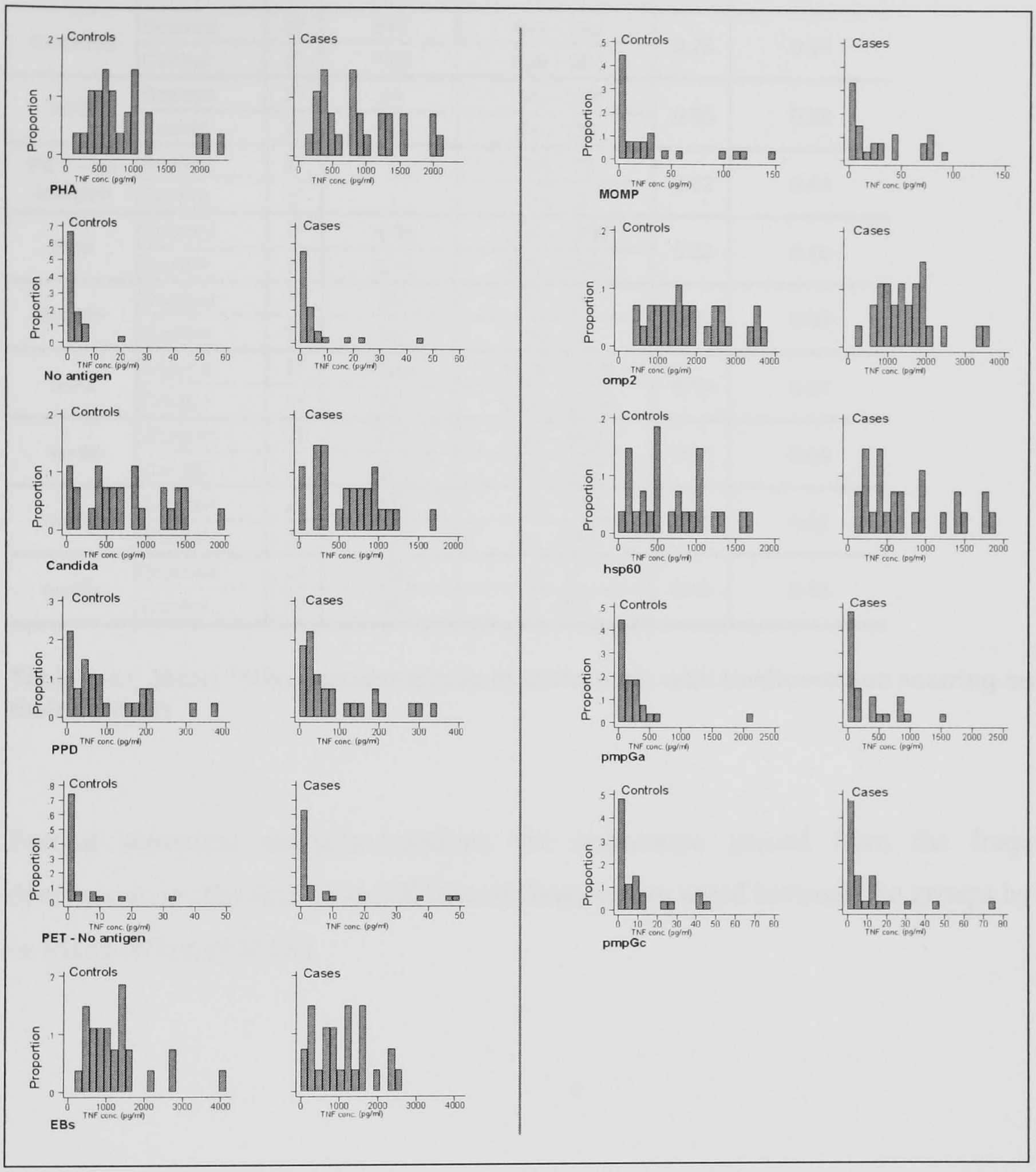


Figure 4-22: Frequency-distribution graphs of TNFα responses in individuals with trachomatous scarring and their controls

Examination of the frequency distribution plots for TNFα in response to the 11 antigens and controls reveals very similar responses in those individuals with trachomatous scarring and their controls.

Antigen	Group	n	Mean TNF level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Scarred	27	908	677 - 1138	0.71	0.82
	Control	27	851	635 - 1067		
No antigen	Scarred	27	5	1.2 - 9	0.18	0.16
	Control	27	2	0.6 - 4		
Candida	Scarred	27	617	451 - 782	0.28	0.44
	Control	27	758	529 - 967		
PPD	Scarred	27	94	55 - 133	0.85	0.69
	Control	27	99	59 - 138		
PET - no antigen	Scarred	27	7	1.5 - 13	0.22	0.44
	Control	27	3	0.5 - 6		
EBs	Scarred	27	1068	798 - 1339	0.33	0.44
	Control	27	1276	932 - 1620		
MOMP	Scarred	27	29	17 - 40	0.89	0.83
	Control	27	30	14 - 46		
OMP2	Scarred	27	1524	1220 - 1828	0.19	0.27
	Control	27	1833	1461 - 2206		
HSP60	Scarred	27	771	557 - 986	0.51	0.64
	Control	27	681	500 - 862		
PMPGa	Scarred	27	322	172 - 473	0.52	0.52
	Control	27	254	94 - 414		
PMPGc	Scarred	27	10	4 - 17	0.95	0.83
	Control	27	10	4 - 15		

Table 4-41: Mean TNFα concentrations in individuals with trachomatous scarring and their controls

Formal statistical analysis confirms the impression gained from the frequency-distribution graphs that no significant differences are noted between the groups by t-test or Mann-Whitney U test.

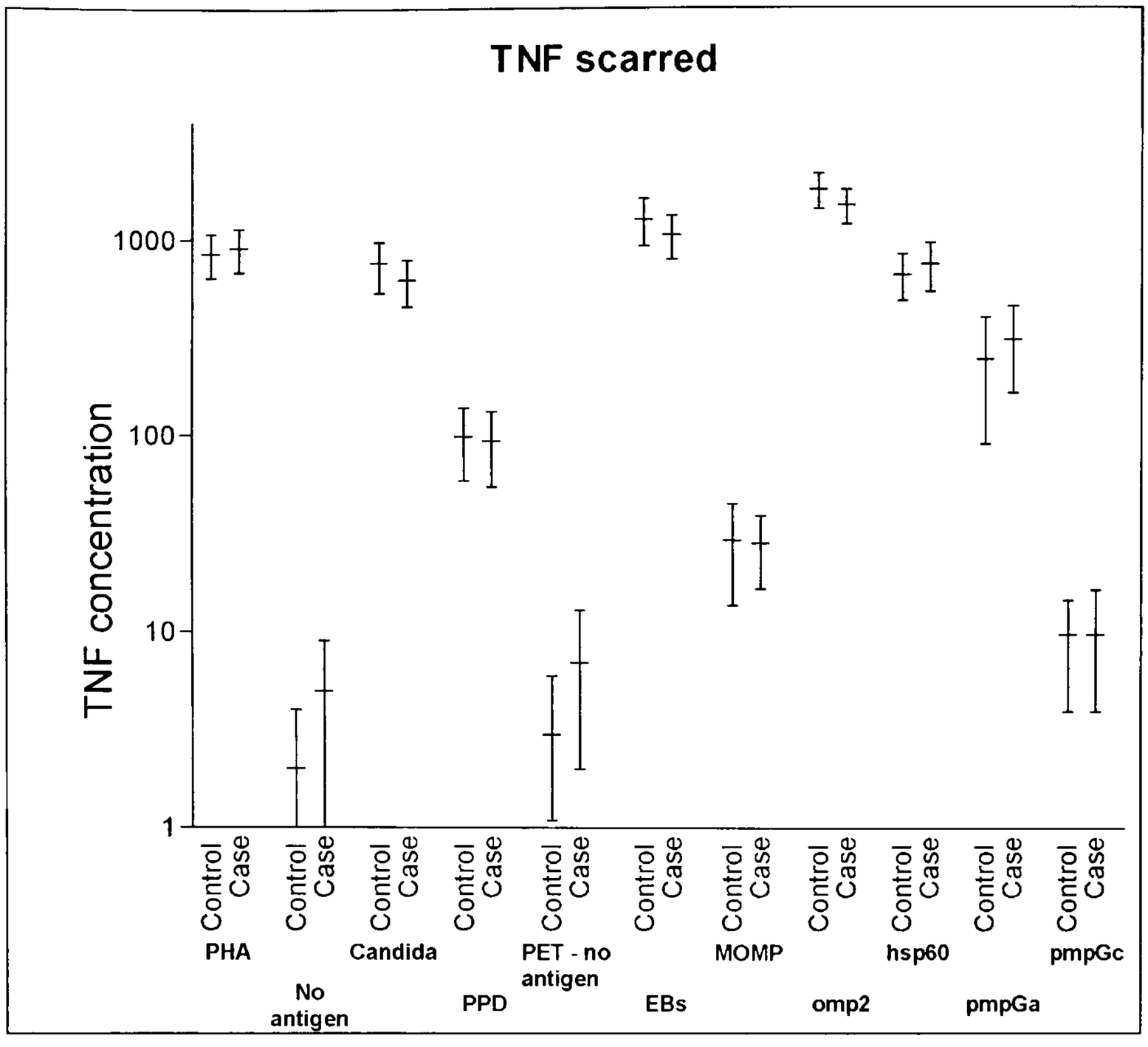


Figure 4-23: Mean TNFα concentrations in individuals with trachomatous scarring and their controls

Antigen	logistic regression		
	O.R. for 25% rise in TNF	95% C.I.	p <
PA	1.05	0.86 - 1.30	0.62
No antigen	1.05	0.96 - 1.15	0.26
Candida	0.91	0.74 - 1.11	0.33
PPD	1.00	0.86 - 1.15	0.95
PET - No antigen	1.04	0.97 - 1.11	0.27
EB's	0.91	0.73 - 1.13	0.40
MOMP	0.99	0.90 - 1.10	0.92
OMP2	0.85	0.64 - 1.12	0.25
HSP60	1.08	0.88 - 1.34	0.46
PMPGa	1.04	0.94 - 1.15	0.50
PMPGc	1.01	0.93 - 1.09	0.88

Table 4-42: Conditional logistic regression of TNFα concentrations for individuals with trachomatous scarring and their controls

Conditional logistic regression also fails to pick up any associations thus there appear to be no difference in the responses of cases and controls in respect of TNFα generation as measured by these assays.

4.3.2.2.2 IFNγ

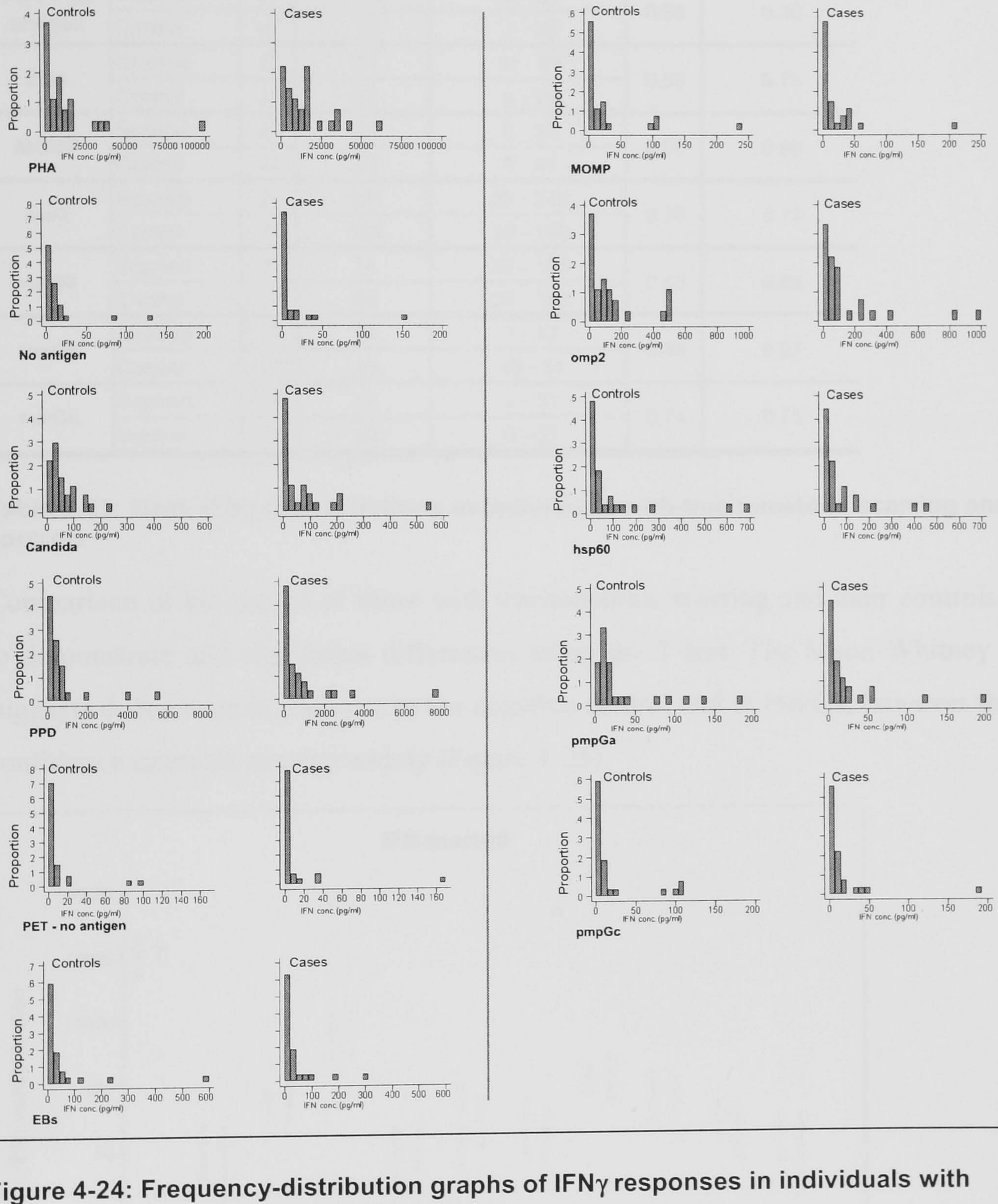


Figure 4-24: Frequency-distribution graphs of IFNγ responses in individuals with trachomatous scarring and their controls

The frequency distribution plots for IFNγ responses demonstrate markedly skewed results for scarred individuals as for children with active trachoma again making the Mann-Whitney U-test probably more accurate for comparing the cases and controls.

Antigen	Group	n	Mean IFN level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Scarred	27	15165	9195 - 21136	0.71	0.32
	Control	27	13361	5347 - 21375		
No antigen	Scarred	27	12	0.1 - 24	0.68	0.04
	Control	27	15	4 - 26		
Candida	Scarred	27	71	27 - 116	0.70	0.19
	Control	27	62	41 - 84		
PPD	Scarred	27	916	303 - 1529	0.64	0.93
	Control	27	739	251 - 1227		
PET - no antigen	Scarred	27	11	-2 - 24	0.96	0.38
	Control	27	11	1 - 20		
EBs	Scarred	27	38	11 - 64	0.58	0.70
	Control	27	52	5 - 99		
MOMP	Scarred	27	21	5 - 38	0.59	0.90
	Control	27	28	8 - 49		
OMP2	Scarred	27	151	56 - 246	0.76	0.73
	Control	27	134	70 - 198		
HSP60	Scarred	27	74	29 - 120	0.63	0.86
	Control	27	94	27 - 161		
PMPGa	Scarred	27	24	7 - 42	0.45	0.07
	Control	27	33	16 - 51		
PMPGc	Scarred	27	17	2 - 31	0.74	0.75
	Control	27	20	6 - 33		

Table 4-43: Mean IFNγ concentrations in individuals with trachomatous scarring and their controls

Comparison of the means of those with trachomatous scarring and their controls failed to demonstrate and significant differences using the T-test. The Mann-Whitney U test suggests differences in response to the negative control and to PMPGa, however the 95% confidence intervals overlap widely (Figure 4-25).

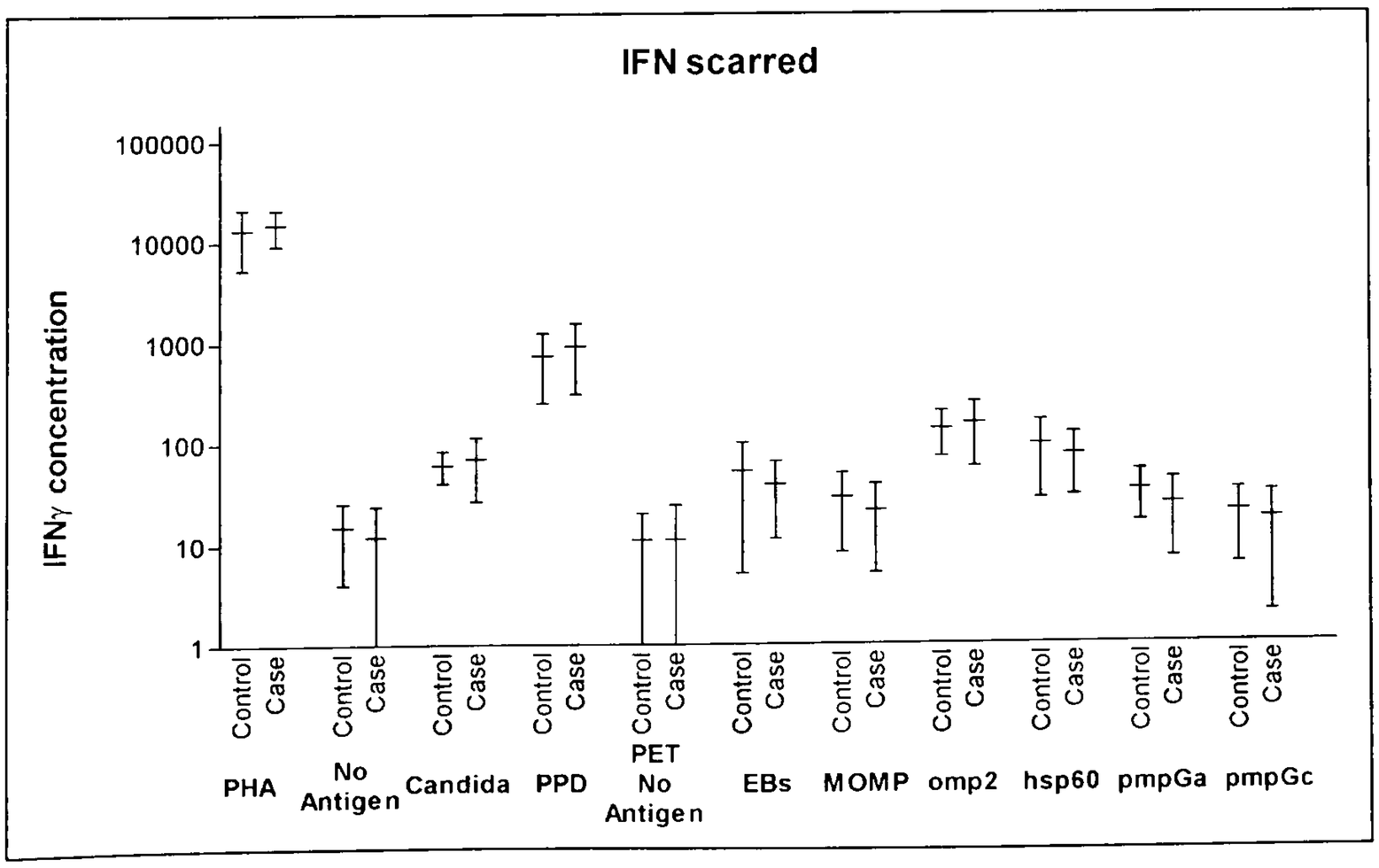


Figure 4-25: Mean IFNγ concentrations in individuals with trachomatous scarring and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IFN	95% C.I.	p <
PHA	1.07	0.88 - 1.30	0.47
No antigen	0.98	0.92 - 1.04	0.55
Candida	1.02	0.92 - 1.13	0.75
PPD	1.04	0.93 - 1.17	0.51
PET - No antigen	1.00	0.95 - 1.05	0.86
EB's	0.98	0.92 - 1.05	0.56
MOMP	0.98	0.91 - 1.05	0.54
OMP2	1.02	0.93 - 1.12	0.72
HSP60	0.98	0.90 - 1.06	0.62
PMPGa	0.97	0.89 - 1.05	0.44
PMPGc	0.98	0.92 - 1.05	0.63

Table 4-44: Conditional logistic regression of IFNγ concentrations for individuals with trachomatous scarring and their controls

Conditional logistic regression fails to demonstrate any statistically significant differences between those with trachomatous scarring and their controls with respect to IFNγ production.

4.3.2.2.3 IL-2

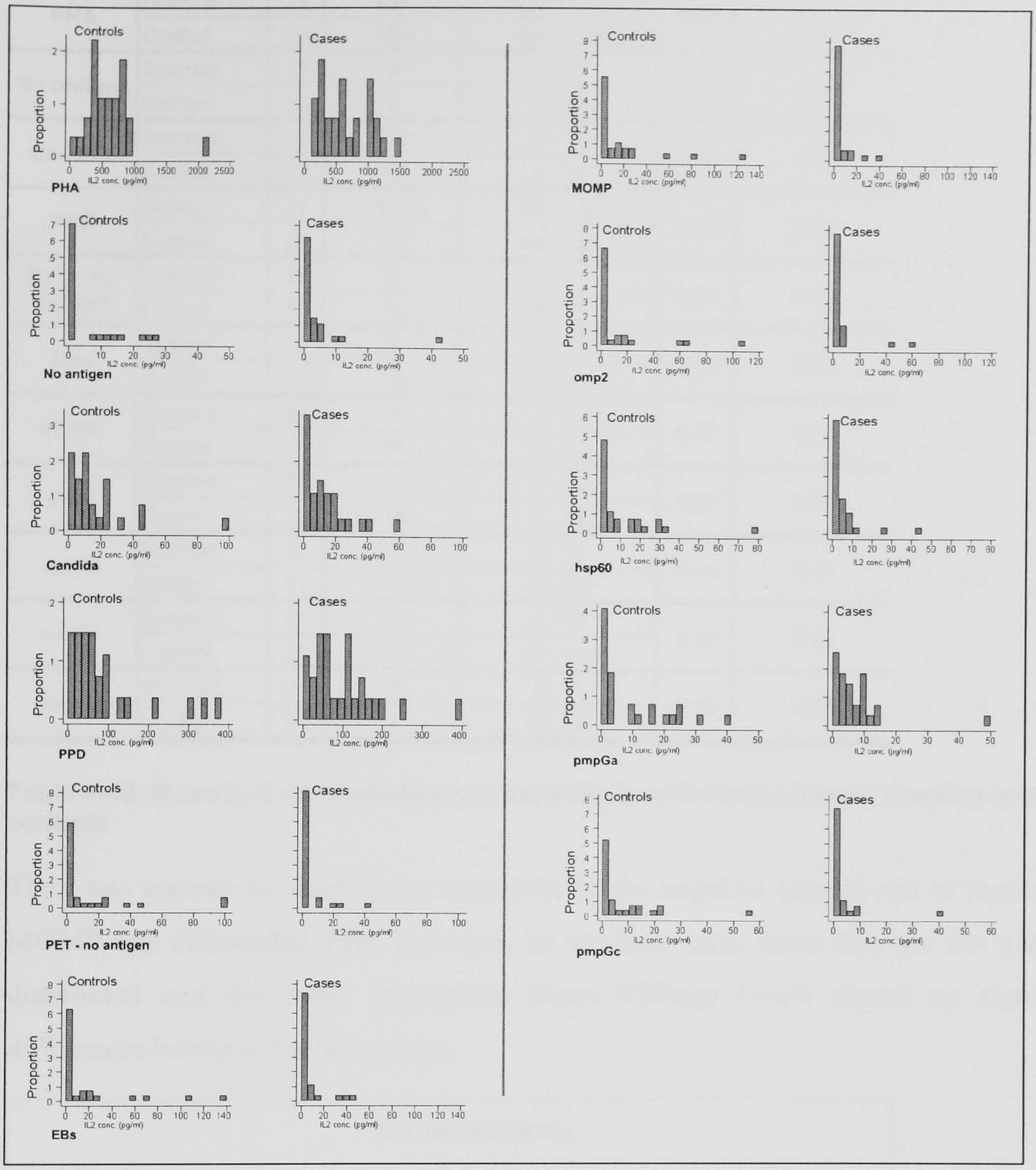


Figure 4-26: Frequency-distribution graphs of IL-2 responses in individuals with trachomatous scarring and their controls

Once again the frequency-distribution graphs show data which are all non-normally distributed except, perhaps, for the responses to PHA.

Antigen	Group	n	Mean IL2 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Scarred	27	644	495 - 793	0.67	0.67
	Control	27	599	450 - 748		
No antigen	Scarred	27	4	0 - 7	0.47	0.85
	Control	27	5	2 - 9		
Candida	Scarred	27	13	8 - 19	0.45	0.53
	Control	27	17	9 - 25		
PPD	Scarred	27	102	68 - 136	0.75	0.28
	Control	27	94	54 - 134		
PET - no antigen	Scarred	27	4	0 - 8	0.07	0.15
	Control	27	15	4 - 26		
EBs	Scarred	27	7	2 - 11	0.10	0.28
	Control	27	19	5 - 33		
MOMP	Scarred	27	5	2 - 9	0.07	0.29
	Control	27	16	5 - 28		
OMP2	Scarred	27	6	0 - 12	0.23	0.28
	Control	27	13	3 - 23		
HSP60	Scarred	27	5	1 - 9	0.14	0.45
	Control	27	11	4 - 18		
PMPGa	Scarred	27	7	4 - 11	0.48	0.99
	Control	27	9	5 - 14		
PMPGc	Scarred	27	3	0 - 6	0.12	0.05
	Control	27	7	3 - 12		

Table 4-45: Mean IL-2 concentrations in individuals with trachomatous scarring and their controls

The t-test appears to show lower responses in the negative control and in response to MOMP and chlamydial EBs. However, as we have seen, these data are not normally distributed and the more appropriate Mann-Whitney U-test shows no significant differences between the two groups.

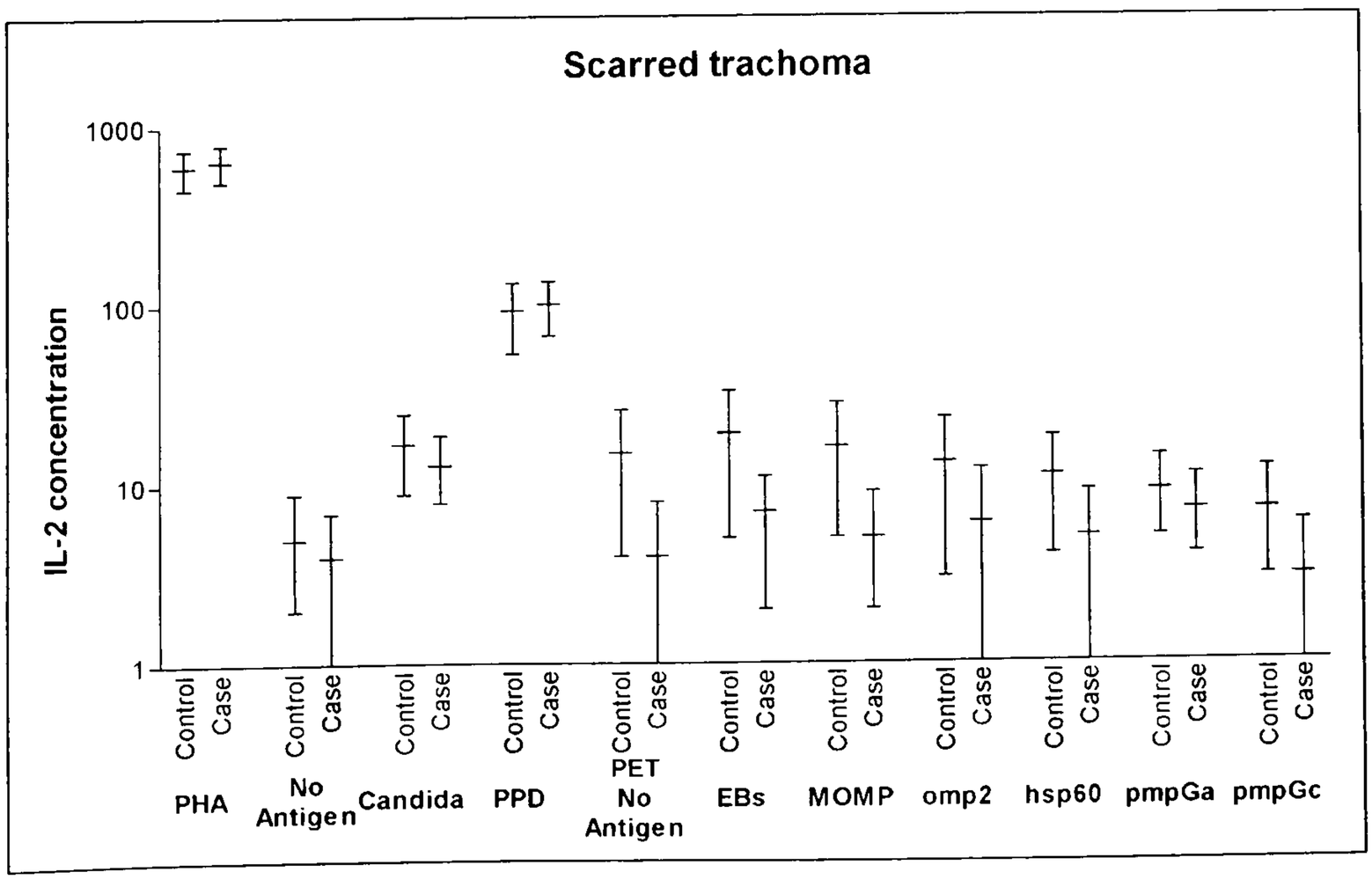


Figure 4-27: Mean IL-2 concentrations in individuals with trachomatous scarring and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-2	95% C.I.	p <
PHA	1.04	0.84 - 1.29	0.70
No antigen	0.98	0.92 - 1.04	0.58
Candida	0.94	0.81 - 1.08	0.35
PPD	1.02	0.89 - 1.16	0.81
PET - No antigen	0.91	0.82 - 1.02	0.11
EB's	0.94	0.86 - 1.03	0.17
MOMP	0.92	0.83 - 1.03	0.16
OMP2	0.96	0.90 - 1.03	0.29
HSP60	0.94	0.85 - 1.03	0.19
PMPGa	0.96	0.87 - 1.07	0.50
PMPGc	0.95	0.87 - 1.03	0.20

Table 4-46: Conditional logistic regression of IL-2 concentrations for individuals with trachomatous scarring and their controls

In keeping with the results of the Mann-Whitney U-test, conditional logistic regression shows no differences in the IL-2 responses between those with trachomatous scarring and their controls.

4.3.2.2.4 IL-5

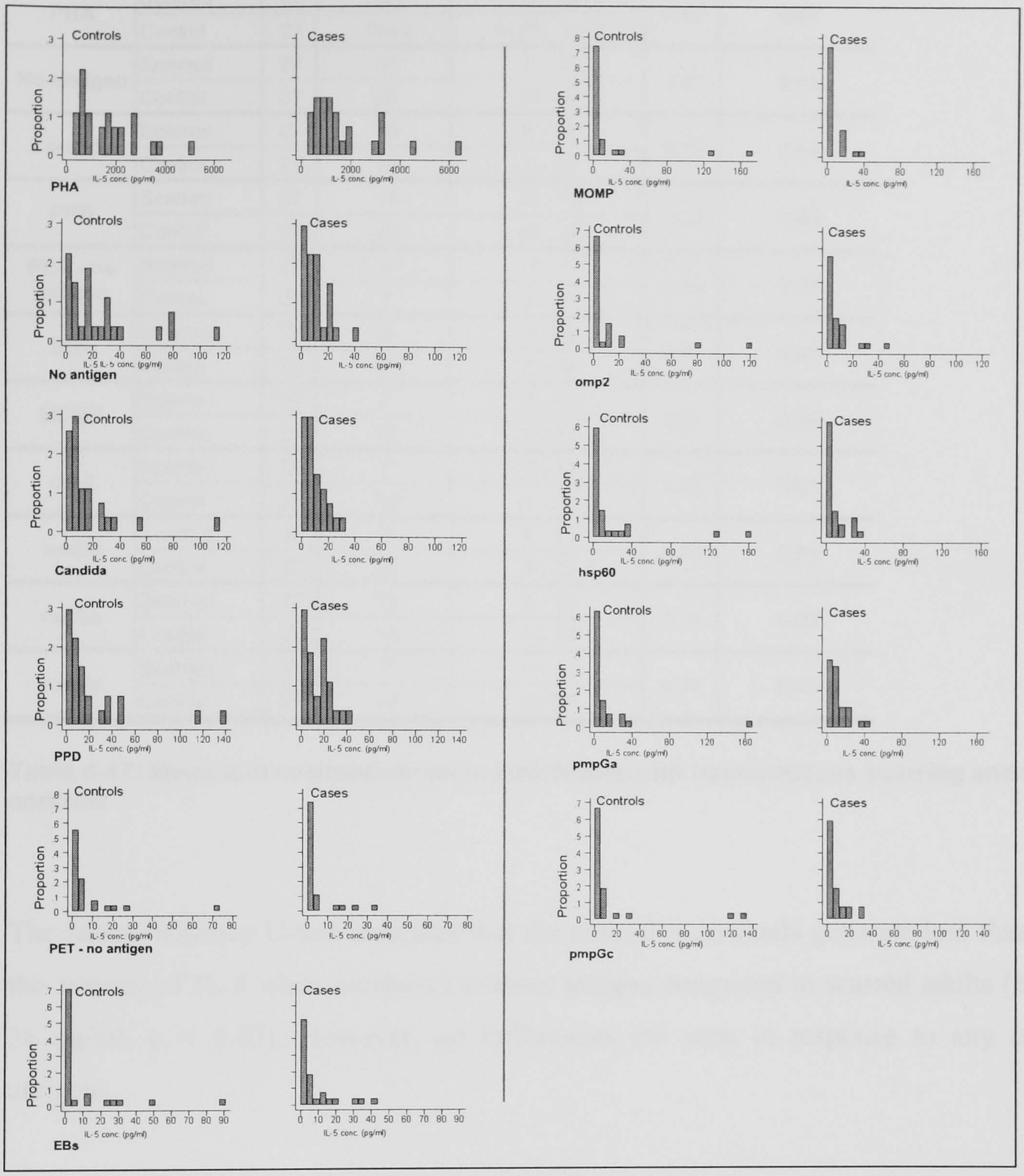


Figure 4-28: Frequency-distribution graphs of IL-5 responses in individuals with trachomatous scarring and their controls

Once again the IL-5 data are not normally distributed.

Antigen	Group	n	Mean IL-5 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Scarred	27	1676	1110 - 2242	0.93	0.88
	Control	27	1645	1179 - 2112		
No antigen	Scarred	27	11	7 - 15	0.01	0.03
	Control	27	26	15 - 37		
Candida	Scarred	27	10	6 - 13	0.18	0.53
	Control	27	16	7 - 25		
PPD	Scarred	27	15	10 - 19	0.23	0.99
	Control	27	23	10 - 36		
PET - no antigen	Scarred	27	4	1 - 7	0.35	0.26
	Control	27	7	2 - 13		
EBs	Scarred	27	8	3 - 12	0.67	0.47
	Control	27	10	2 - 18		
MOMP	Scarred	27	7	3 - 11	0.31	0.46
	Control	27	15	0 - 31		
OMP2	Scarred	27	7	3 - 11	0.40	0.81
	Control	27	12	1 - 23		
HSP60	Scarred	27	8	4 - 13	0.23	0.84
	Control	27	18	3 - 33		
PMPGa	Scarred	27	12	8 - 16	0.78	0.05
	Control	27	14	1 - 27		
PMPGc	Scarred	27	7	4 - 11	0.34	0.40
	Control	27	14	0 - 27		

Table 4-47: Mean IL-5 concentrations in individuals with trachomatous scarring and their controls

The Mann-Whitney U-test indicates that the control individuals generate less than half the amount of IL-5 when incubated without antigen compared to scarred adults (11 vs. 26 pg/ml, $p < 0.03$). However, no differences are seen in response to any of the antigens.

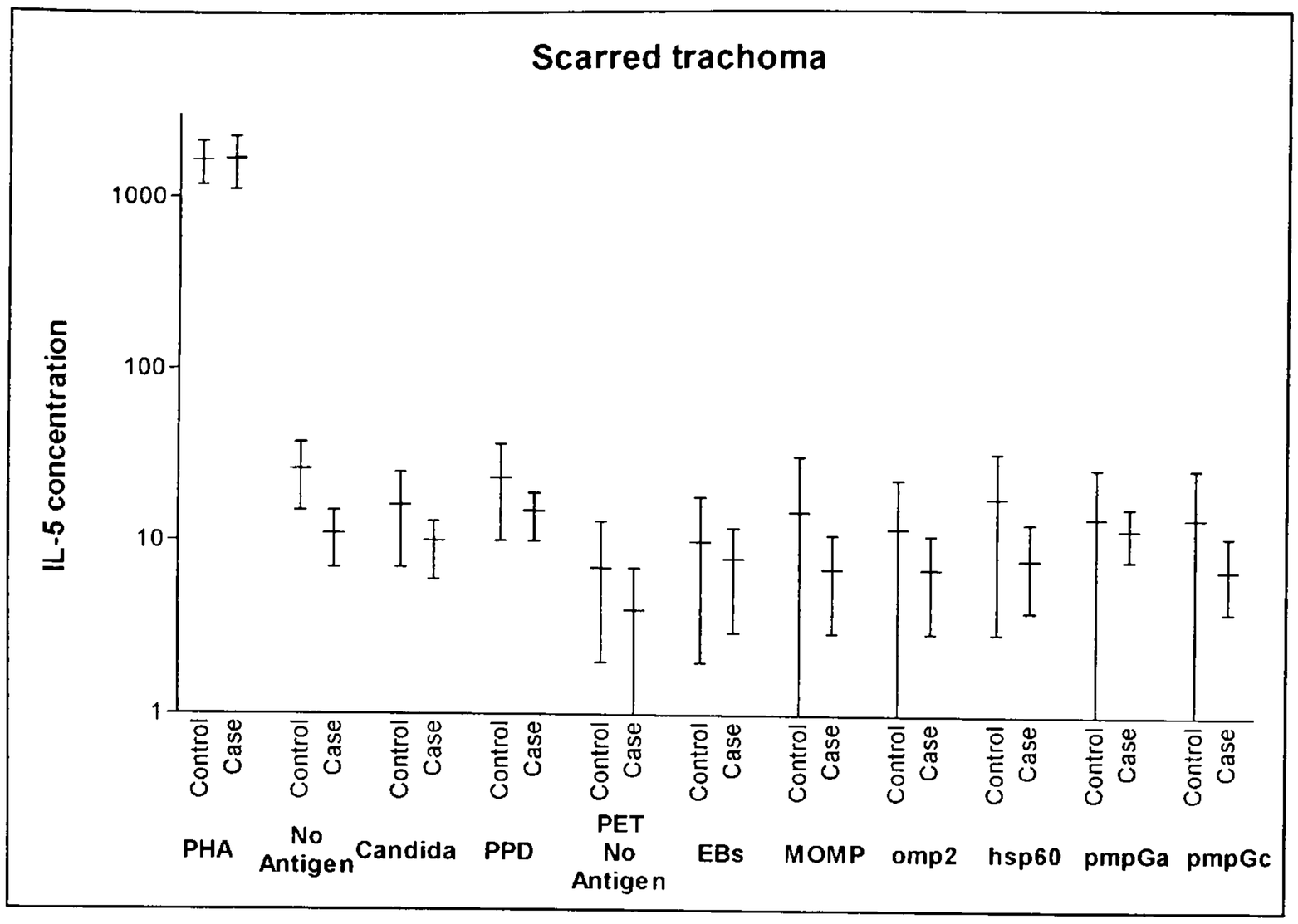


Figure 4-29: Mean IL-5 concentrations in individuals with trachomatous scarring and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-5	95% C.I.	p <
PHA	1.01	0.82 - 1.23	0.95
No antigen	0.82	0.66 - 1.01	0.06
Candida	0.91	0.77 - 1.07	0.24
PPD	0.92	0.79 - 1.06	0.24
PET - No antigen	0.96	0.89 - 1.05	0.38
EB's	0.98	0.91 - 1.06	0.67
MOMP	0.97	0.91 - 1.04	0.36
OMP2	0.97	0.91 - 1.04	0.42
HSP60	0.95	0.88 - 1.04	0.28
PMPGa	0.99	0.92 - 1.06	0.75
PMPGc	0.97	0.91 - 1.04	0.37

Table 4-48: Conditional logistic regression of IL-5 concentrations for individuals with trachomatous scarring and their controls

Conditional logistic regression analysis confirms no association between trachomatous scarring and IL-5 responses apart from that in the negative control wells.

4.3.2.2.5 IL-10

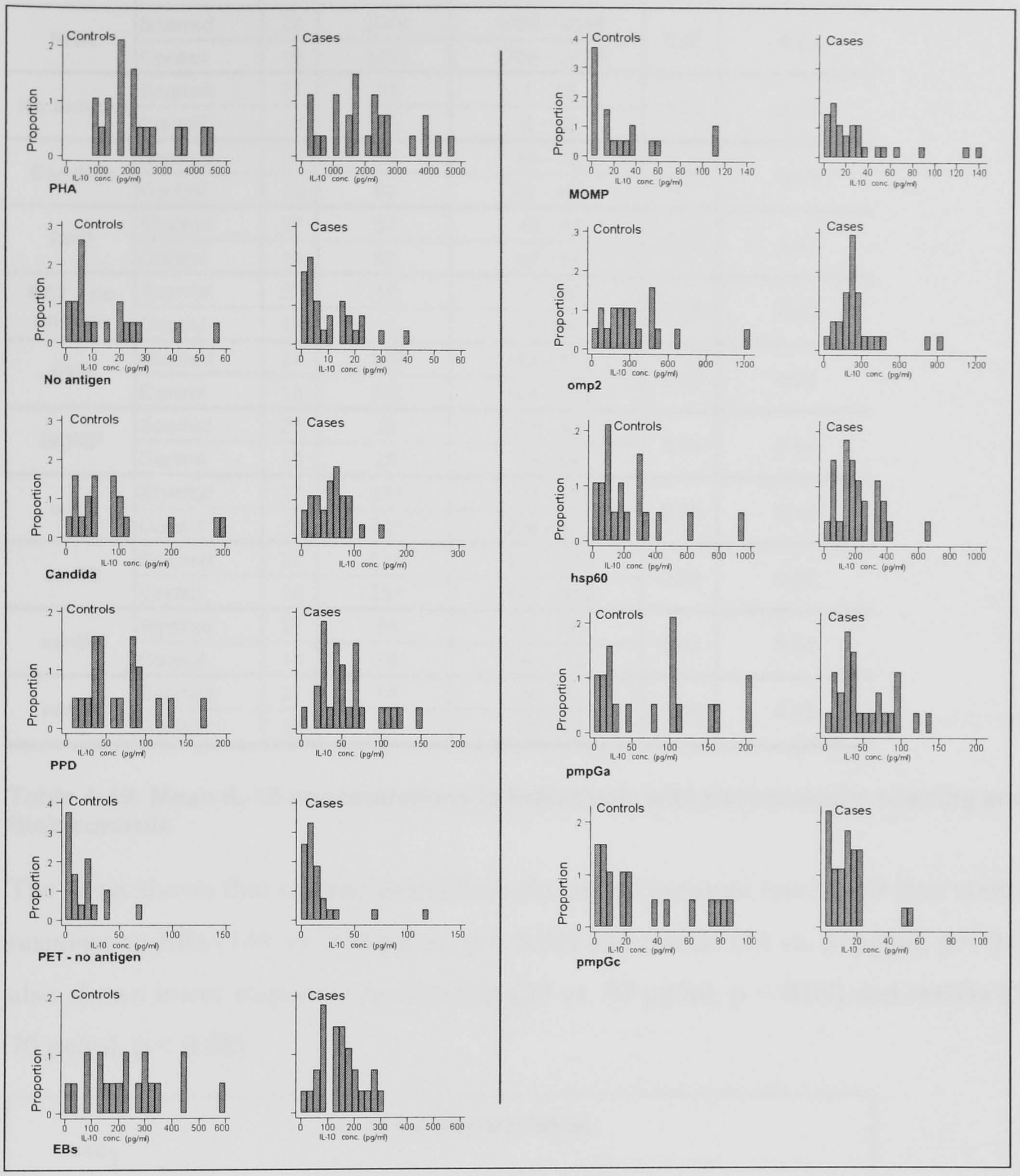


Figure 4-30: Frequency-distribution graphs of IL-10 responses in individuals with trachomatous scarring and their controls

As was seen with the active trachoma group, the IL-10 responses are much more normally distributed than those for the other cytokines. The graphs appear to show lower IL-10 responses to chlamydial EBs and to PMPGc in scarred individuals vs. controls.

Antigen	Group	n	Mean IL-10 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Scarred	27	2048	1559 - 2538	0.61	0.70
	Control	19	2230	1705 - 2755		
No antigen	Scarred	27	11	7 - 15	0.27	0.35
	Control	19	15	8 - 22		
Candida	Scarred	27	59	46 - 72	0.09	0.43
	Control	19	90	49 - 131		
PPD	Scarred	27	57	43 - 71	0.35	0.47
	Control	19	67	48 - 87		
PET - no antigen	Scarred	27	16	7 - 26	0.88	0.88
	Control	19	15	7 - 24		
EBs	Scarred	27	149	119 - 179	0.01	0.04
	Control	19	238	164 - 312		
MOMP	Scarred	27	32	18 - 46	0.81	0.60
	Control	19	29	13 - 46		
OMP2	Scarred	27	271	194 - 349	0.33	0.40
	Control	19	341	206 - 475		
HSP60	Scarred	27	214	159 - 270	0.68	0.80
	Control	19	237	128 - 345		
PMPGa	Scarred	27	51	37 - 66	0.08	0.33
	Control	19	79	46 - 112		
PMPGc	Scarred	27	14	9 - 19	0.02	0.13
	Control	19	31	16 - 45		

Table 4-49: Mean IL-10 concentrations in individuals with trachomatous scarring and their controls

The t-test shows that scarred individuals do indeed generate less IL-10 than controls in response to EBs (149 vs. 238 pg/ml, $p < 0.01$) and PMPGc (14 vs. 31pg/ml, $p < 0.02$). It also shows lower responses to *Candida* (59 vs. 90 pg/ml, $p < 0.09$) and PMPGa (51 vs. 70 pg/ml, $p < 0.08$).

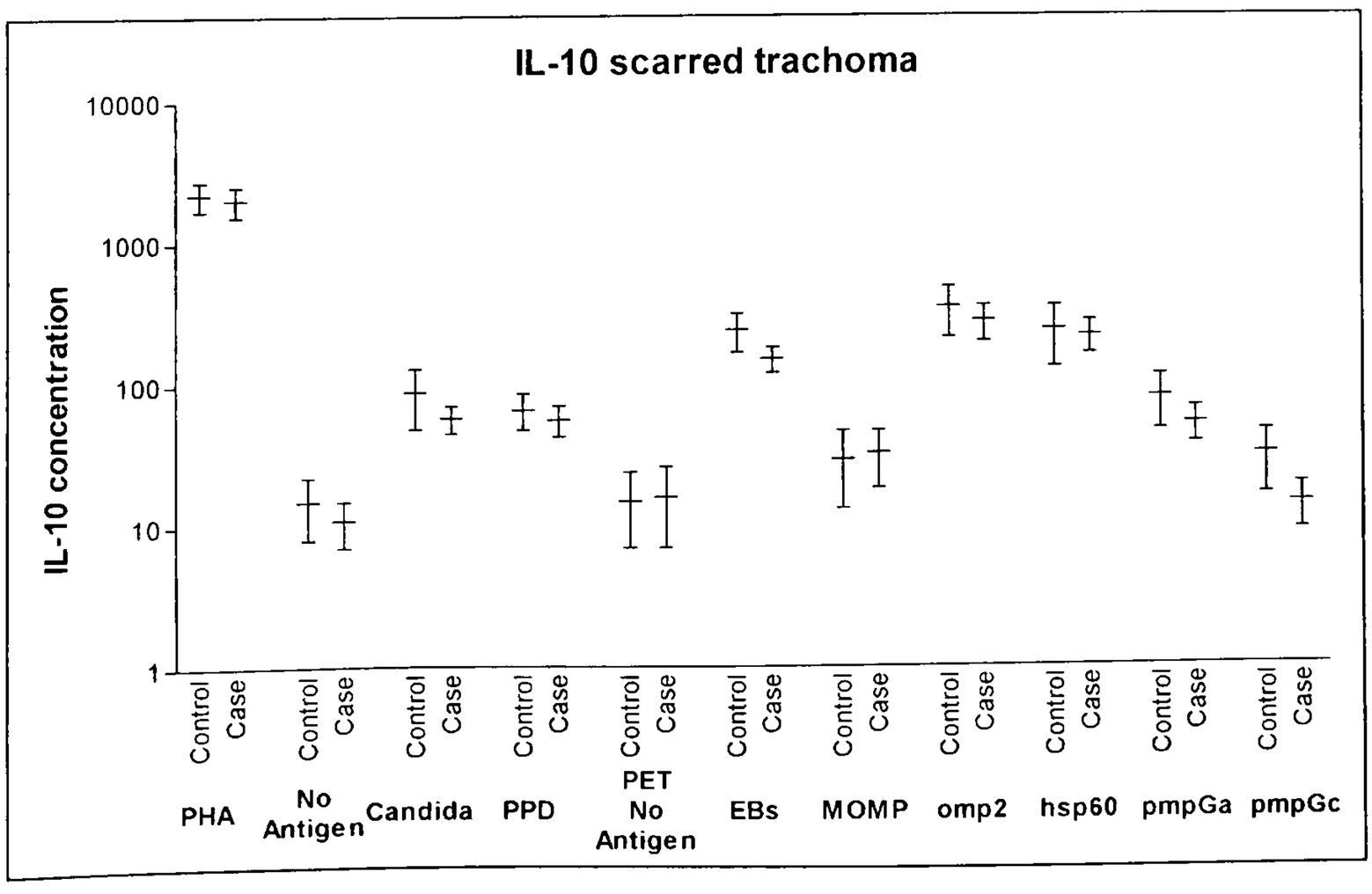


Figure 4-31: Mean IL-10 concentrations in individuals with trachomatous scarring and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-10	95% C.I.	p <
PHA	0.95	0.74 - 1.22	0.67
No antigen	0.93	0.79 - 1.11	0.42
Candida	0.88	0.72 - 1.08	0.22
PPD	0.86	0.64 - 1.16	0.32
PET - No antigen	1.02	0.91 - 1.15	0.74
EB's	0.80	0.62 - 1.03	0.08
MOMP	1.03	0.90 - 1.18	0.71
OMP2	0.94	0.79 - 1.11	0.46
HSP60	0.97	0.81 - 1.17	0.73
PMPGa	0.88	0.73 - 1.07	0.21
PMPGc	0.85	0.72 - 1.02	0.08

Table 4-50: Conditional logistic regression of IL-10 concentrations for individuals with trachomatous scarring and their controls

Conditional logistic regression provides further confirmation of lower IL-10 responses to EBs and PMPGc in scarred individuals but fails to confirm the association for *Candida* or PMPGa.

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Scarred	8	1.00	-	0.06
	Control	3			
IL-10 25 - 50%	Scarred	8	0.75	0.12 - 4.69	
	Control	4			
IL-10 50 - 75%	Scarred	7	0.66	0.10 - 4.21	
	Control	4			
IL-10 > 75th cent	Scarred	4	0.19	0.03 - 1.37	
	Control	8			

Table 4-51: χ^2 test for trend for IL-10 responses to chlamydial EBs in individuals with trachomatous scarring and their controls

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-10 < 25th cent	Scarred	8	1.00	-	0.11
	Control	3			
IL-10 25 - 50%	Scarred	7	0.53	0.09 - 3.22	
	Control	5			
IL-10 50 - 75%	Scarred	8	1.00	0.15 - 6.83	
	Control	3			
IL-10 > 75th cent	Scarred	4	0.19	0.03 - 1.37	
	Control	8			

Table 4-52: χ^2 test for trend for IL-10 responses to pmpGc in individuals with trachomatous scarring and their controls

The χ^2 test for trend shows individuals generating IL-10 responses in the highest quartile in response to EBs and to PMPGc are five times less likely to have scarring than those with responses in the lowest quartile.

4.3.2.2.6 TGFβ

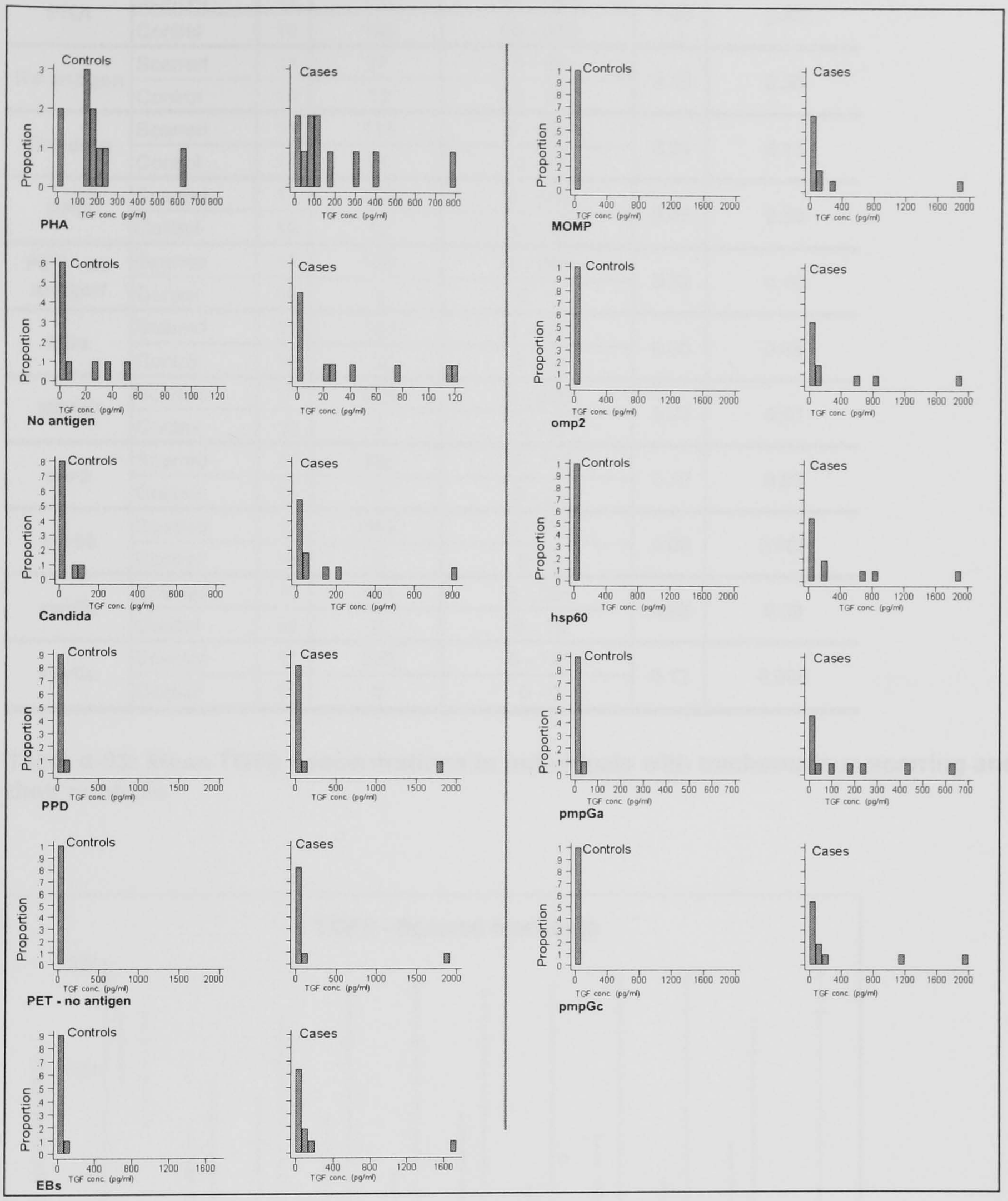


Figure 4-32: Frequency-distribution graphs of TGFβ responses in individuals with trachomatous scarring and their controls

As discussed earlier the numbers of TGFβ assays analysed is less than that for the other cytokines. Due to the presence of non-specific activation a small number of results included may still be spurious such as the values seen here at ~2000 pg/ml in response to PPD, PET and EBs amongst the cases.

Antigen	Group	n	Mean TGF level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Scarred	11	190	31 - 349	1.00	0.40
	Control	10	190	65 - 315		
No antigen	Scarred	11	37	5 - 68	0.15	0.30
	Control	10	12	0 - 27		
Candida	Scarred	11	116	0 - 281	0.24	0.13
	Control	10	21	0 - 54		
PPD	Scarred	11	175	0 - 538	0.35	0.36
	Control	10	12	0 - 38		
PET - no antigen	Scarred	11	186	0 - 569	0.32	0.16
	Control	10	3	0 - 11		
EBs	Scarred	11	189	0 - 538	0.30	0.09
	Control	10	12	0 - 38		
MOMP	Scarred	11	225	0 - 605	0.23	0.01
	Control	10	3	0 - 8		
OMP2	Scarred	11	333	0 - 735	0.10	0.03
	Control	10	7	0 - 24		
HSP60	Scarred	11	349	0 - 739	0.08	0.008
	Control	10	3	0 - 9		
PMPGa	Scarred	11	144	5 - 283	0.05	0.09
	Control	10	9	0 - 19		
PMPGc	Scarred	11	330	0 - 761	0.12	0.008
	Control	10	0	0 - 0		

Table 4-53: Mean TGFβ concentrations in individuals with trachomatous scarring and their controls

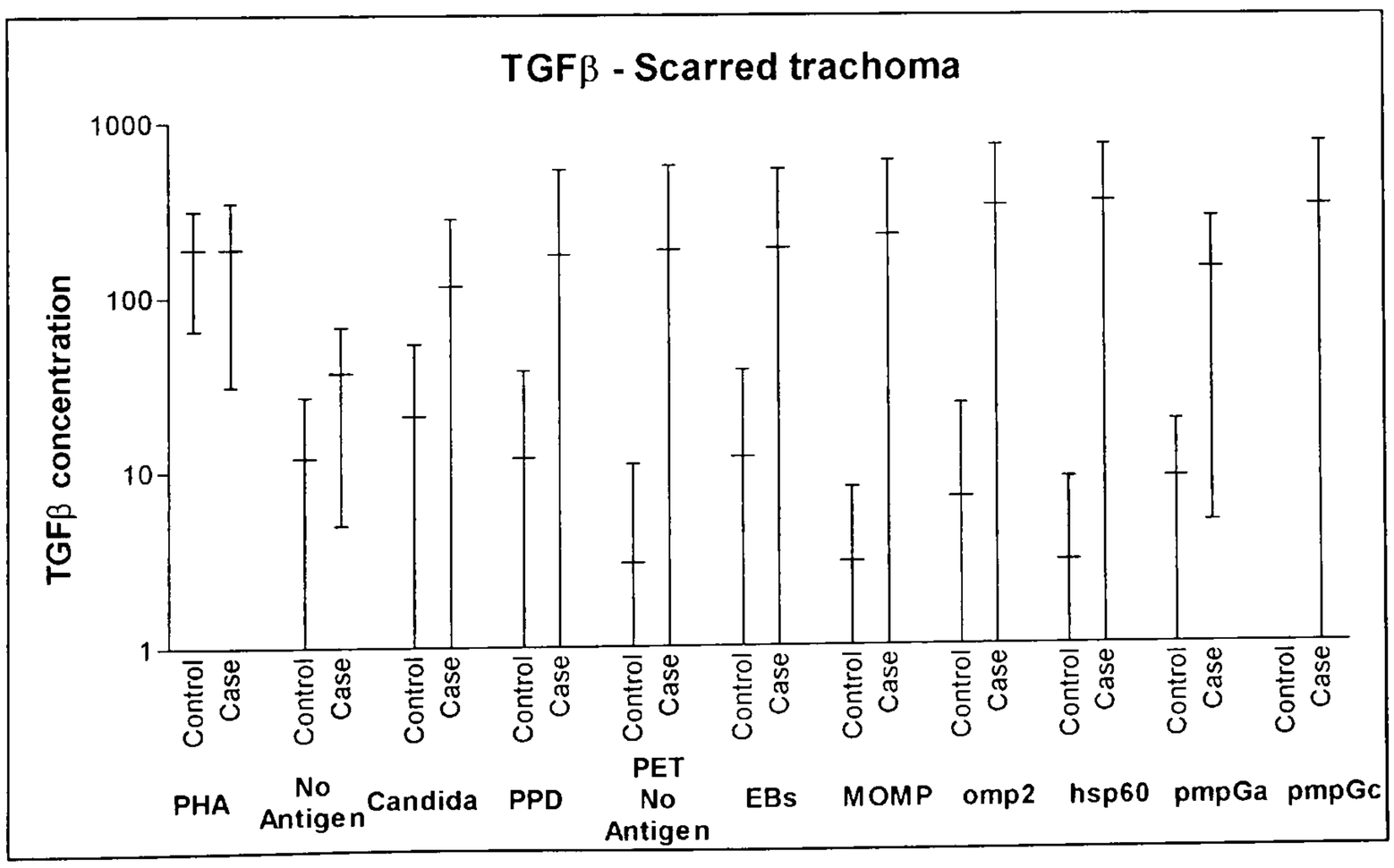


Figure 4-33: Mean TGFβ concentrations in individuals with trachomatous scarring and their controls

Antigen	logistic regression		
	O.R. for 25% rise in TGF	95% C.I.	p <
PHA	1.02	0.84 - 1.24	0.86
No antigen	1.34	0.93 - 1.38	0.21
Candida	1.13	0.84 - 1.53	0.41
PPD	1.06	0.80 - 1.40	0.69
PET - No antigen	2.00	0.53 - 7.57	0.31
EB's	1.29	0.76 - 2.19	0.35
MOMP	6.77	0.11 - 434	0.37
OMP2	2.83	0.47 - 17.00	0.26
HSP60	(3.45×10^7)	-	-
PMPGa	1.41	0.81 - 2.44	0.23
PMPGc	(5.03×10^6)	-	-

Table 4-54: Conditional logistic regression of TGFβ concentrations for individuals with trachomatous scarring and their controls

4.4 Results of studies in infertile women

4.4.1 Immunoblot results

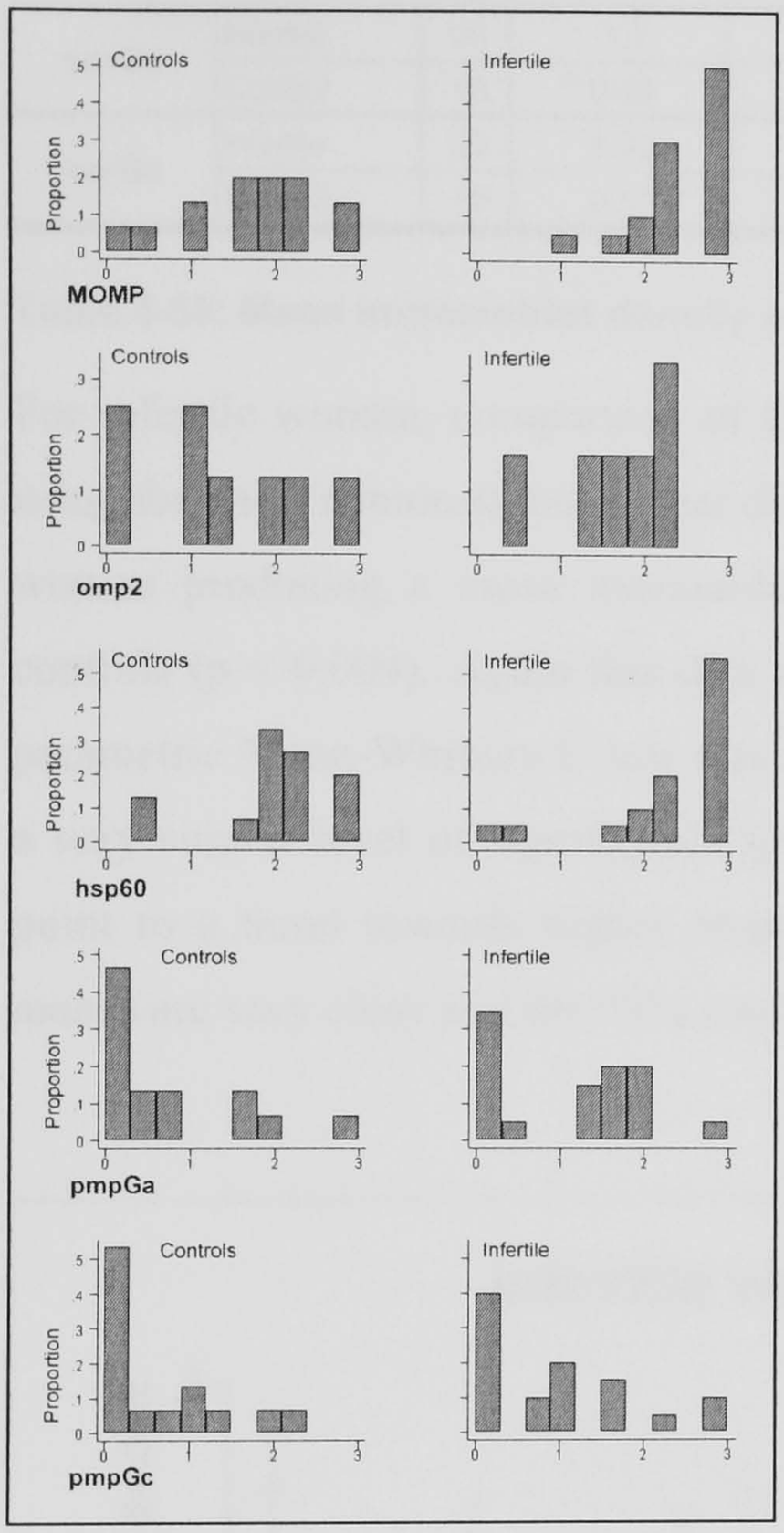


Figure 4-34: Frequency-distribution graphs of immunoblot density scores from infertile women and their controls

2/15 (13%) of controls and 0/22 infertile women had no detectable antibodies to MOMP ($p < 0.09$). It is clear from the frequency distribution graphs that infertile women had higher immunoblot density scores for MOMP than controls.

Again in contrast to previous studies, no association is seen with antibodies to HSP60 and infertility. 13/15 (87%) controls and 18/20 (90%) of infertile women have detectable antibodies to HSP60. Even at higher levels of response there is no difference between the cases and controls; 12/15 (80%) of controls and 17/20 (85%) of infertile women have immunoblot scores of 2 or greater.

As was seen in the two trachoma groups, a large number of individuals demonstrate detectable antibodies to the recombinant PMPG proteins. 4/15 (27%) of controls vs. 12/20 (60%) of the infertile women have antibodies to PMPGa – a significantly higher proportion in the infertile women ($p < 0.05$). 5/15 (33%) of the controls and 10/20 (50%) of the infertile women demonstrate antibodies to PMPGc ($p < 0.32$)

Antigen	Group	n	Mean immunoblot score	t-test		Mann-Whitney
				95% C.I.	p	p
MOMP	Infertile	20	2.50	2.24 - 2.75	0.004	0.005
	Control	15	1.75	1.27 - 2.23		
OMP2	Infertile	6	1.65	0.85 - 2.45	0.47	0.43
	Control	8	1.29	0.45 - 2.12		
HSP60	Infertile	20	2.40	1.98 - 2.08	0.22	0.07
	Control	15	2.03	1.58 - 2.48		
PMPGa	Infertile	20	1.1	0.65 - 1.55	0.22	0.27
	Control	15	0.69	0.17 - 1.22		
PMPGc	Infertile	20	0.93	0.47 - 1.38	0.26	0.32
	Control	15	0.57	0.14 - 1.01		

Table 4-55: Mean immunoblot density scores from infertile women and their controls

For infertile women, comparison of the population means between cases and controls using the t-test demonstrates a clear difference in the responses to MOMP with infertile women producing a mean immunoblot density score of 2.50 vs. 1.75 for matched controls ($p < 0.004$). Again this data is somewhat skewed to the right and so the non-parametric Mann-Whitney U test was also applied. This confirmed the association with a very similar level of significance ($p < 0.005$). The Mann-Whitney U test appears to point to a trend towards higher responses to HSP60 in infertile women however the means are very close and the 95% confidence intervals are overlapping.

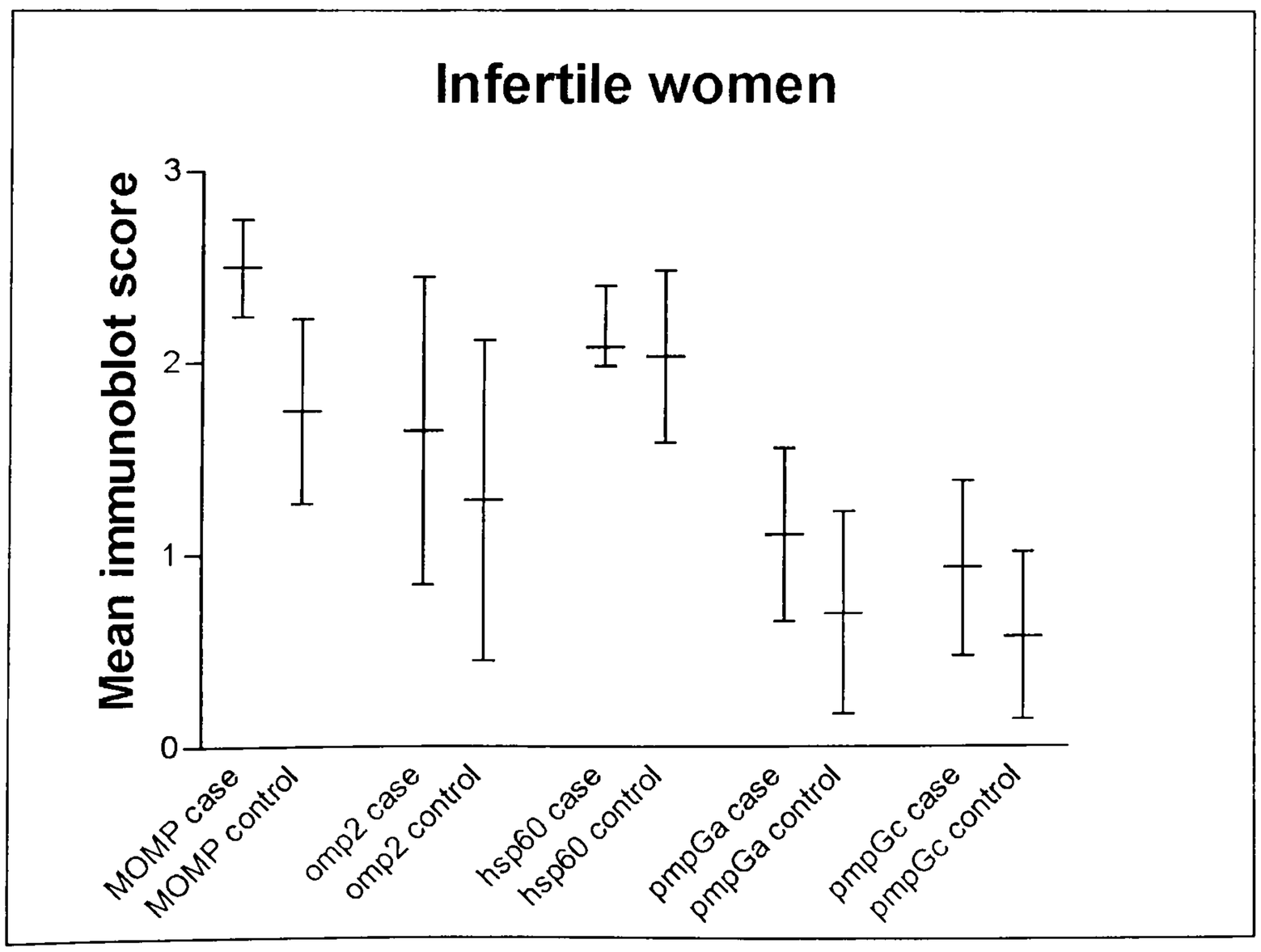


Figure 4-35: Mean immunoblot density scores from infertile women and their controls

Antigen	O.R.	logistic regression	
		95% C.I.	p <
MOMP	3.57	0.92 - 13.90	0.07
OMP2	1.31	0.41 - 4.20	0.65
HSP60	1.54	0.67 - 3.52	0.31
PMPGa	2.18	0.66 - 7.21	0.20
PMPGc	1.83	0.59 - 5.63	0.29

Table 4-56: Conditional logistic regression of immunoblot density scores for infertile women and their controls

Pair-wise conditional logistic analysis again suggests the association of antibody response to MOMP with the presence of infertility, each incremental increase of response to MOMP being associated with an odds ratio of 3.57 for being infertile, but the level of significance is reduced compared to that obtained using the t-test and Mann-Whitney U test (95% C.I. 0.92 – 13.90, $p < 0.07$). The response to HSP60 shows no association with infertility in this response. This conflict between the results of the Mann-Whitney U-test and conditional logistic regression may be explained by the fact that these tests use two different models and two different outcomes. The Mann-Whitney U-test tests if the immunoblot score is dependent on whether the individual is a case or control whilst the conditional logistic regression analysis estimates the likelihood of being a case for a given immunoblot score.

Immunoblot score	Group	n	Odds	95% C.I.	p <
MOMP < 0.4	Infertile	0	0	-	0.03
	Control	2			
MOMP 0.4 - 0.99	Infertile	1	0.50	0.05 - 5.51	
	Control	2			
MOMP 1.0 - 1.99	Infertile	3	0.50	0.13 - 2.00	
	Control	6			
MOMP 2.0 - 3.0	Infertile	18	1.64	0.77 - 3.46	
	Control	11			

Table 4-57: χ^2 test for trend for immunoblot density scores in response to MOMP in infertile women and their controls

χ^2 analysis for trend (Table 4-57) demonstrates an increase in the odds of being infertile with an increasing rise in antibody response as measured by the immunoblot density score for MOMP ($p < 0.03$). The confidence intervals however do include 1.

4.4.2 Cytokine ELISAs from whole blood assays

4.4.2.1 TNF-α

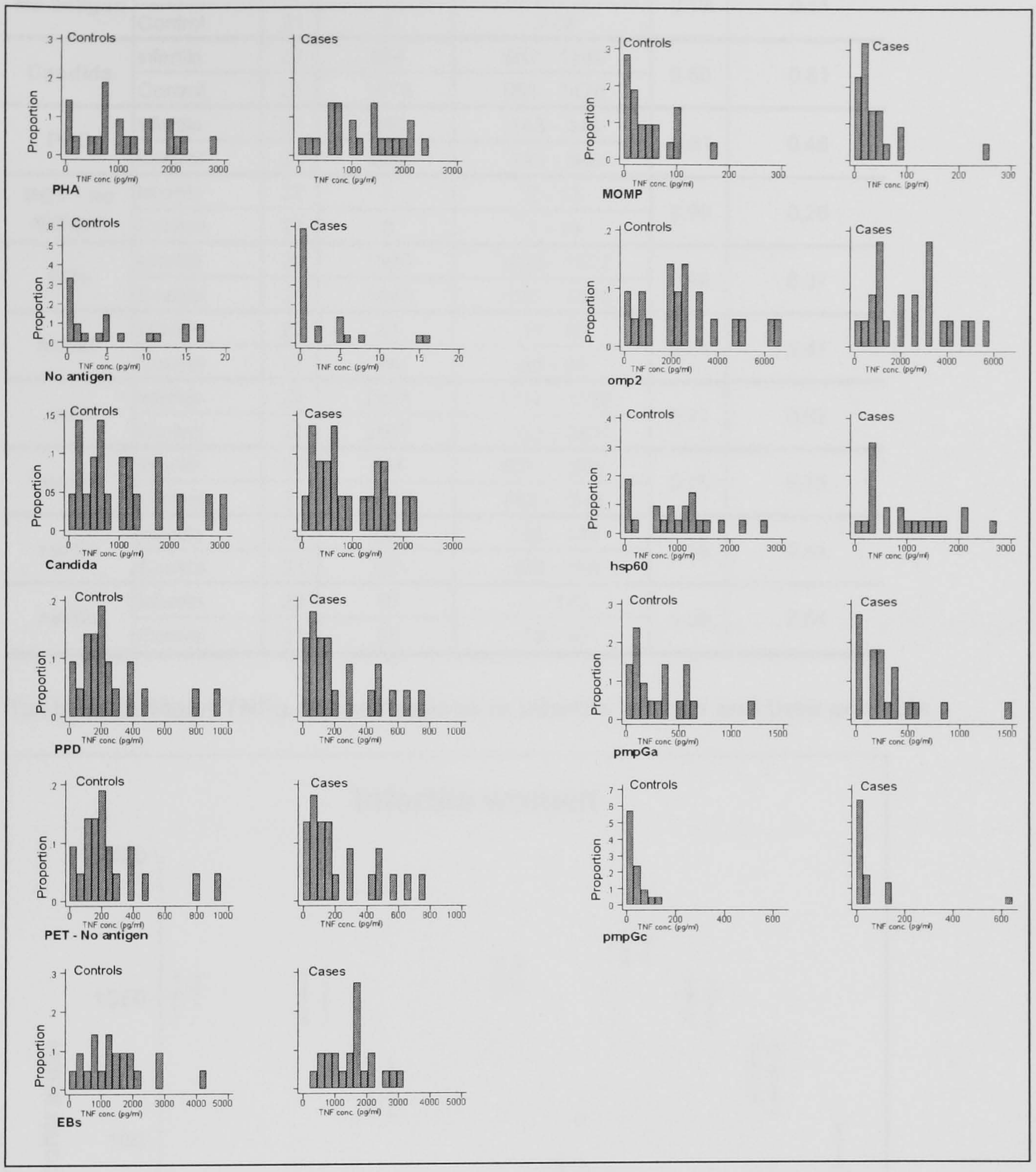


Figure 4-36: Frequency-distribution graphs of TNFα responses in infertile women and their controls

The whole blood assays and cytokine ELISAs failed to detect any difference between the infertile women and their controls in terms of the amount of TNFα generated in response to the 11 antigens and controls. This can be seen in the frequency-distribution plots (Figure 4-36) and in the subsequent formal statistical analysis by t-test and Mann-Whitney U test.

Antigen	Group	n	Mean TNF level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Infertile	22	1182	887 - 1477	0.70	0.66
	Control	21	1098	746 - 1449		
No antigen	Infertile	22	3	0.8 - 5	0.13	0.11
	Control	21	5	3 - 8		
Candida	Infertile	22	954	662 - 1245	0.60	0.81
	Control	21	1078	686 - 1470		
PPD	Infertile	22	243	143 - 343	0.81	0.46
	Control	21	260	153 - 366		
PET - no antigen	Infertile	22	7	2 - 13	0.90	0.26
	Control	21	8	1 - 14		
EBs	Infertile	22	1550	1223 - 1877	0.68	0.37
	Control	21	1441	1007 - 1875		
MOMP	Infertile	22	41	17 - 65	0.74	0.47
	Control	21	46	26 - 66		
OMP2	Infertile	22	2421	1712 - 3129	0.77	0.92
	Control	21	2577	1723 - 3431		
HSP60	Infertile	22	944	621 - 1266	0.75	0.76
	Control	21	1013	682 - 1344		
PMPGa	Infertile	22	296	152 - 440	0.78	0.64
	Control	21	323	188 - 458		
PMPGc	Infertile	22	62	1 - 123	0.39	0.64
	Control	21	35	19 - 50		

Table 4-58: Mean TNFα concentrations in infertile women and their controls

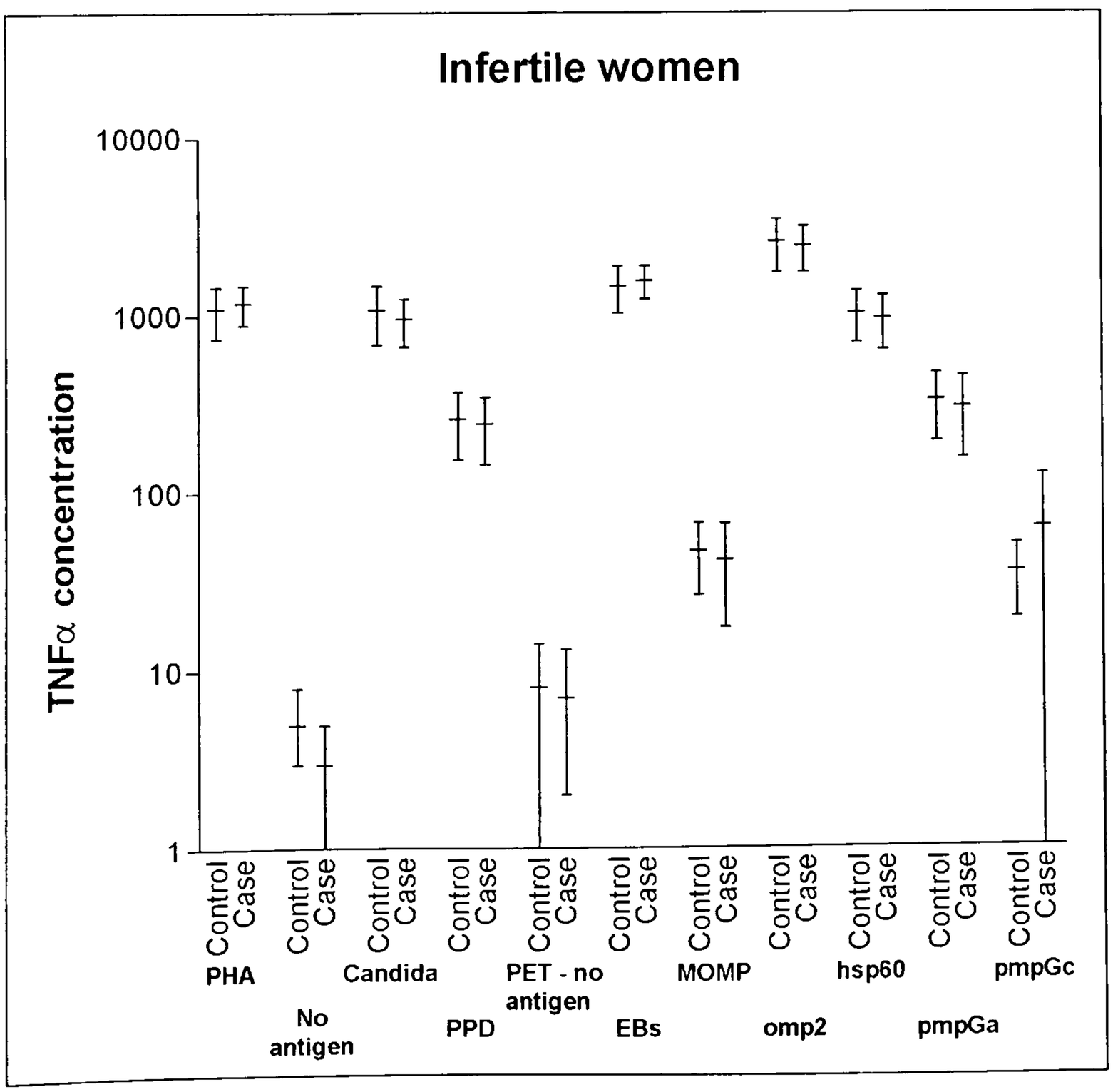


Figure 4-37: Mean TNFα concentrations in infertile women and their controls

Antigen	logistic regression		
	O.R. for 25% rise in TNF	95% C.I.	p <
PHA	1.08	0.83 - 1.41	0.55
No antigen	0.92	0.83 - 1.03	0.16
Candida	0.94	0.72 - 1.21	0.63
PPD	0.99	0.82 - 1.18	0.87
PET - No antigen	1.00	0.92 - 1.08	0.94
EB's	1.10	0.81 - 1.50	0.53
MOMP	0.98	0.86 - 1.12	0.78
OMP2	0.98	0.78 - 1.23	0.88
HSP60	0.98	0.78 - 1.23	0.86
PMPGa	0.98	0.83 - 1.16	0.85
PMPGc	1.05	0.93 - 1.20	0.41

Table 4-59: Conditional logistic regression of TNFα concentrations for infertile women and their controls

As expected from the frequency-distribution graphs and the t-test and Mann-Whitney U-test results, conditional logistic regression confirms that no detectable differences exist between the infertile women and their controls in respect of the levels of TNFα generated.

4.4.2.2 IFNγ

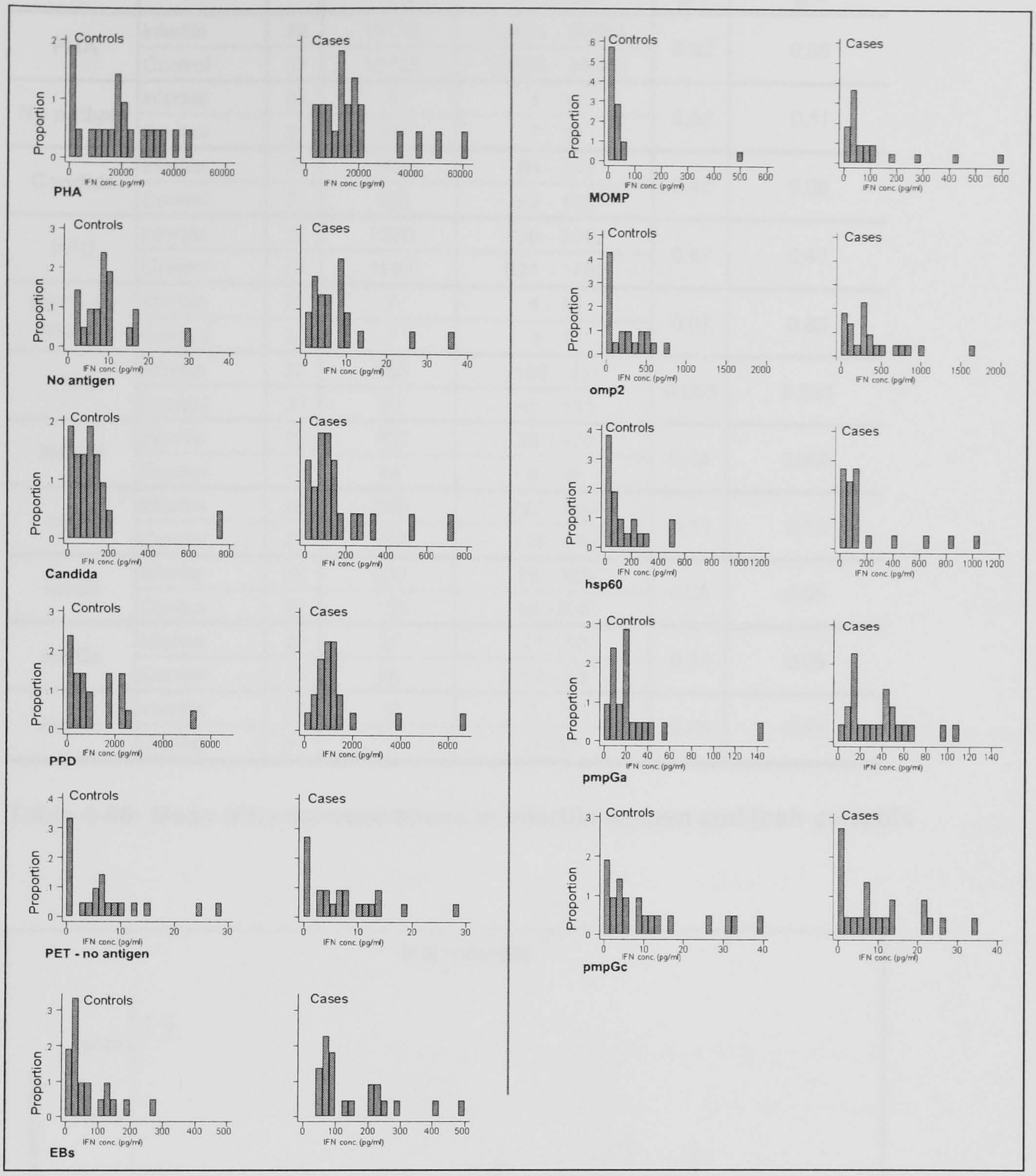


Figure 4-38: Frequency-distribution graphs of IFNγ responses in infertile women and their controls

Comparison of the IFNγ responses of infertile women and their controls using the t-test demonstrates significantly higher responses by the infertile women towards chlamydial elementary bodies (156 vs. 71, $p < 0.008$) and this difference is also confirmed by the Mann-Whitney U test. The latter test also suggests differences between the two groups in response to MOMP and PMPGa although the 95% confidence intervals show a wide overlap.

Antigen	Group	n	Mean IFN level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Infertile	22	19194	12431 - 25957	0.92	0.86
	Control	21	18725	12508 - 24943		
No antigen	Infertile	22	8	4 - 12	0.52	0.11
	Control	21	10	7 - 12		
Candida	Infertile	22	165	89 - 242	0.40	0.36
	Control	21	122	52 - 193		
PPD	Infertile	22	1370	738 - 2002	0.67	0.47
	Control	21	1197	631 - 1762		
PET - no antigen	Infertile	22	7	4 - 10	0.97	0.85
	Control	21	7	3 - 11		
EBs	Infertile	22	156	102 - 211	0.008	0.001
	Control	21	71	40 - 101		
MOMP	Infertile	22	104	38 - 170	0.14	0.006
	Control	21	44	0 - 93		
OMP2	Infertile	22	396	227 - 564	0.11	0.13
	Control	21	238	136 -339		
HSP60	Infertile	22	200	76 - 325	0.36	0.55
	Control	21	136	66 -206		
PMPGa	Infertile	22	37	25 - 50	0.21	0.09
	Control	21	26	12 - 40		
PMPGc	Infertile	22	10	5 - 14	0.79	0.88
	Control	21	11	5 - 16		

Table 4-60: Mean IFNγ concentrations in infertile women and their controls

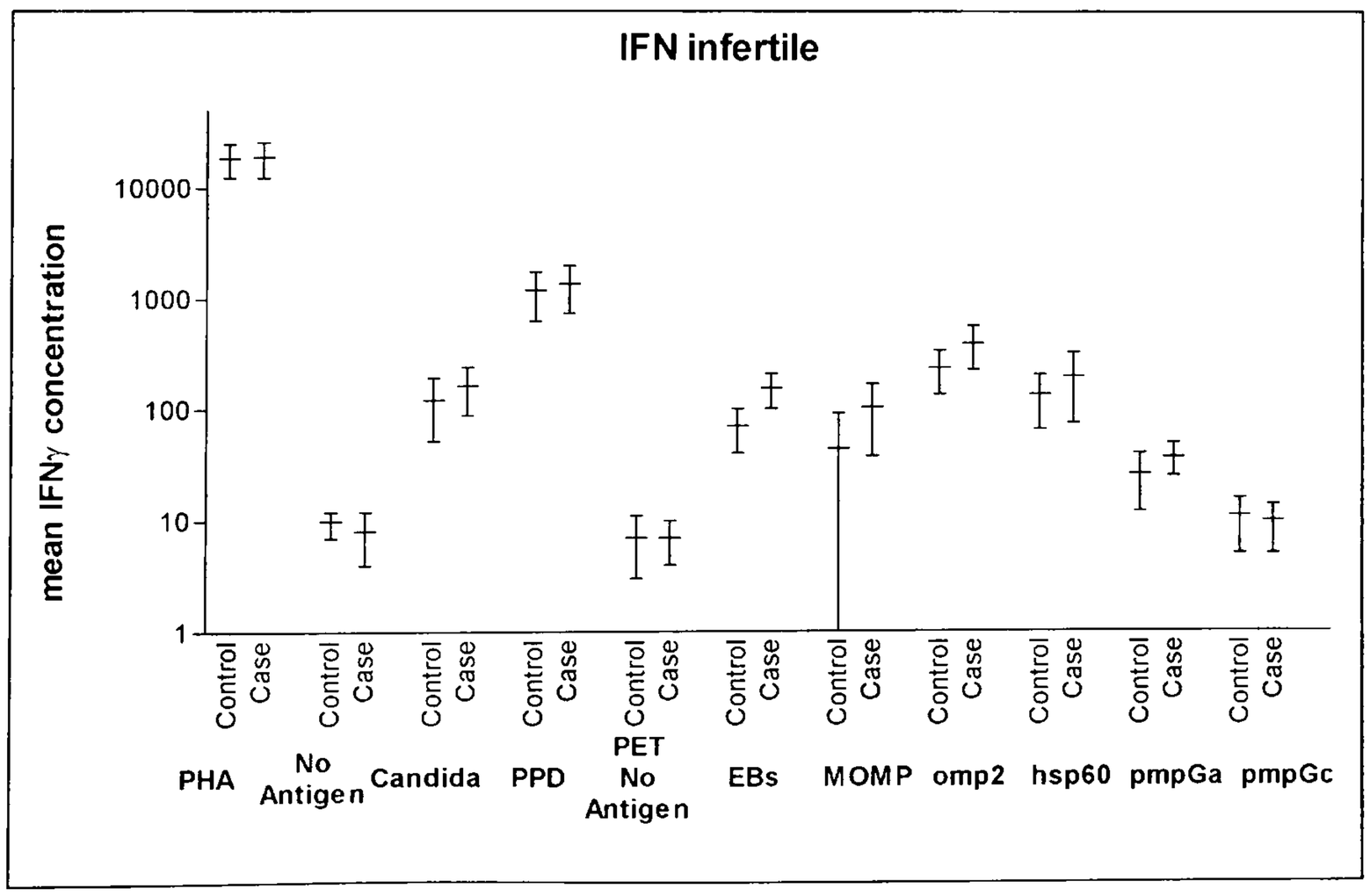


Figure 4-39: Mean IFNγ concentrations in infertile women and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IFN	95% C.I.	p <
PHA	1.03	0.82 - 1.31	0.80
No antigen	0.93	0.76 - 1.13	0.45
Candida	1.07	0.91 - 1.27	0.40
PPD	1.05	0.88 - 1.24	0.60
PET - No antigen	1.00	0.87 - 1.15	1.00
EB's	1.56	1.04 - 2.33	0.03
MOMP	1.08	0.95 - 1.23	0.23
OMP2	1.13	0.95 - 1.35	0.16
HSP60	1.08	0.93 - 1.26	0.31
PMPGa	1.16	0.92 - 1.46	0.20
PMPGc	0.95	0.77 - 1.18	0.66

Table 4-61: Conditional logistic regression of IFNγ concentrations for infertile women and their controls

Conditional logistic regression confirms the difference in responses to chlamydial elementary bodies with a 1.56 increase on odds ratio for each 25% rise in IFNγ but fails to confirm a difference for MOMP or PMPGa.

Quartile	Group	n	Odds	95% C.I.	p <
IFN < 25th cent	Infertile	0	0	-	0.001
	Control	10			
IFN 25 - 50%	Infertile	7	1.75	0.50 - 6.00	
	Control	4			
IFN 50 - 75%	Infertile	6	1.2	0.37 - 3.90	
	Control	5			
IFN > 75th cent	Infertile	8	4.0	0.85 - 18.84	
	Control	2			

Table 4-62: χ^2 test for trend for IFNγ responses to PHA in infertile women and their controls

The χ^2 test for trend (Table 4-62) demonstrates a much higher ratio for being a scarred for those generating IFNγ responses in the highest quartile than those in the lowest quartile (4.0 vs 0 $p < 0.001$). As the odds for the lowest quartile are 0 an odds ratio compared to this cannot be calculated.

4.4.2.3 IL-2

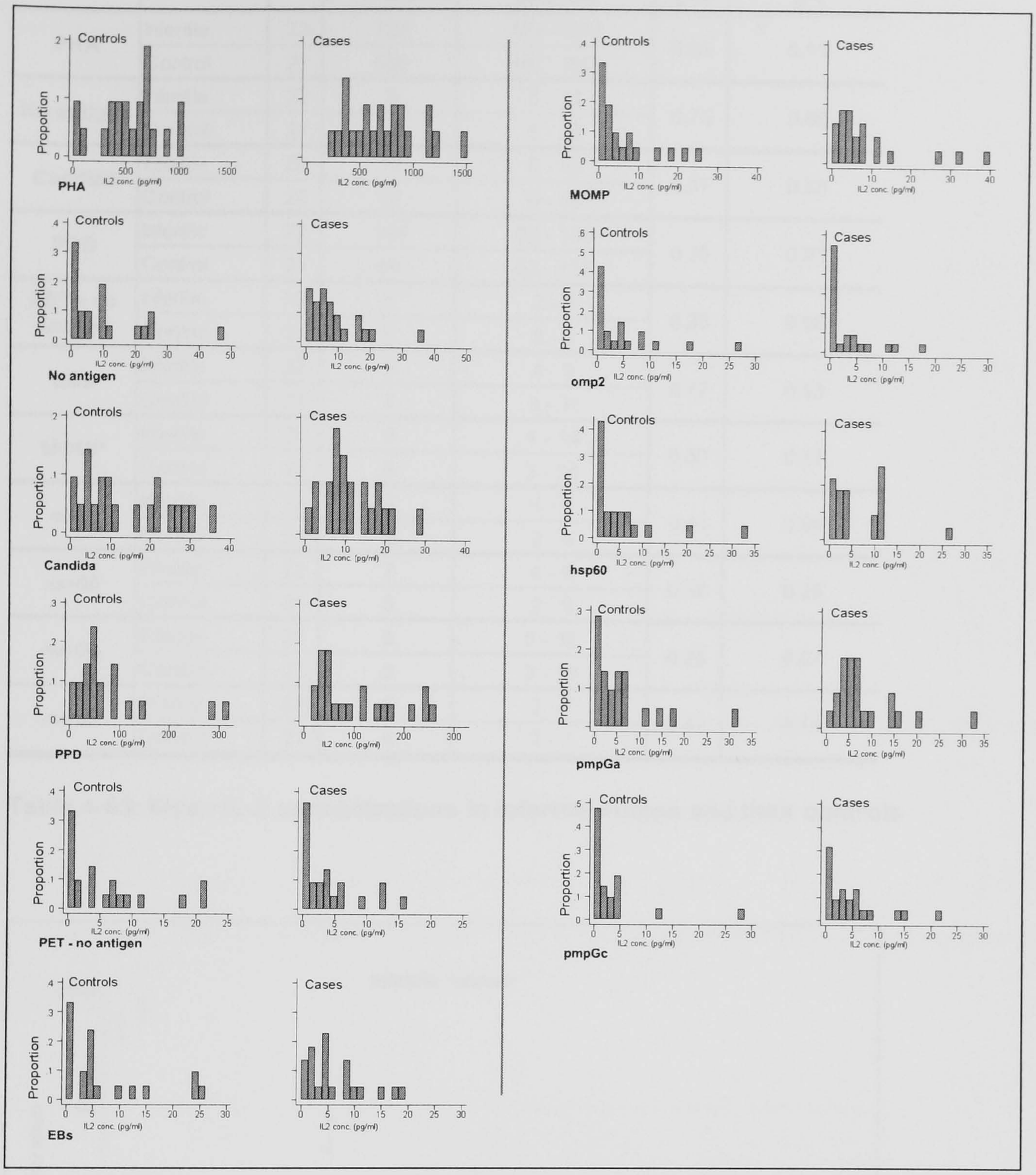


Figure 4-40: Frequency-distribution graphs of IL-2 responses in infertile women and their controls

The t-test demonstrates higher levels of IL-2 being produced by the infertile women in response to PHA compared to controls (724 vs. 535 pg/ml, $p < 0.05$). These data appear normally distributed thus the t-test is more appropriate than the Mann-Whitney U-test. There is a wide discrepancy between the significance levels ascribed to the IL-2 response to PMPGa and it is difficult to be certain if the data are normally distributed or not from the frequency-distribution graphs. However the 95% confidence intervals overlap considerably suggesting that the difference is probably not significant.

Antigen	Group	n	Mean IL2 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Infertile	22	724	573 - 874	0.05	0.13
	Control	21	535	407 - 663		
No antigen	Infertile	22	9	5 - 13	0.75	0.88
	Control	21	10	5 - 15		
Candida	Infertile	22	12	8 - 15	0.37	0.68
	Control	21	14	9 - 19		
PPD	Infertile	22	104	69 - 139	0.35	0.27
	Control	21	80	43 - 117		
PET - no antigen	Infertile	22	4	2 - 6	0.33	0.56
	Control	21	6	3 - 9		
EBs	Infertile	22	6	4 - 9	0.77	0.53
	Control	21	7	3 - 11		
MOMP	Infertile	22	9	4 - 14	0.30	0.17
	Control	21	6	3 - 10		
OMP2	Infertile	22	3	1 - 5	0.41	0.66
	Control	21	5	2 - 8		
HSP60	Infertile	22	7	4 - 9	0.58	0.26
	Control	21	5	2 - 9		
PMPGa	Infertile	22	9	5 - 12	0.28	0.07
	Control	21	6	3 - 10		
PMPGc	Infertile	22	5	2 - 7	0.47	0.18
	Control	21	4	1 - 7		

Table 4-63: Mean IL-2 concentrations in infertile women and their controls

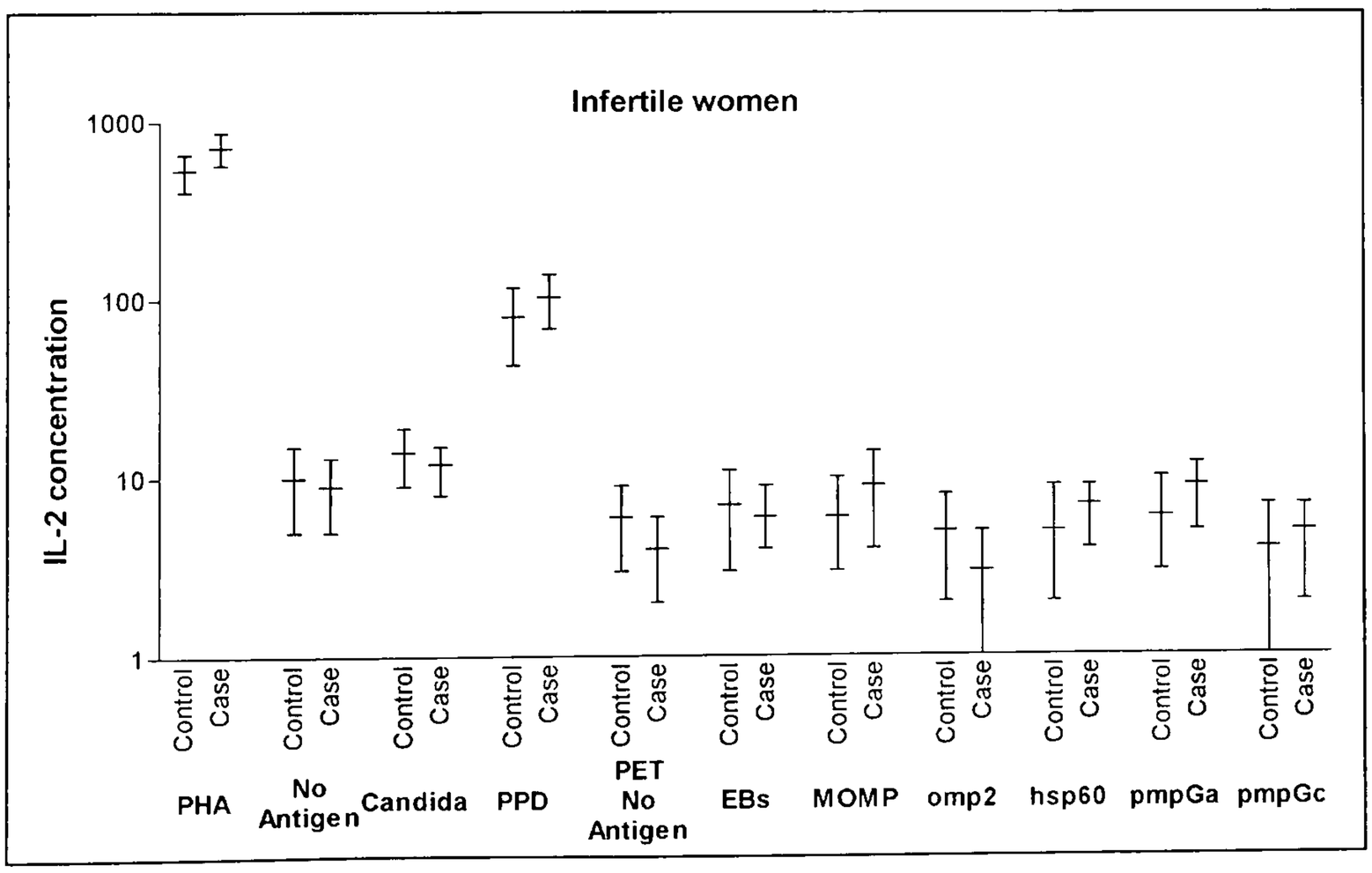


Figure 4-41: Mean IL-2 concentrations in infertile women and their controls

Figure: *IL2 infertile logistic.tbl*

Antigen	logistic regression		
	O.R. for 25% rise in IL-2	95% C.I.	p <
PHA	1.59	1.02 - 2.48	0.04
No antigen	0.98	0.84 - 1.13	0.80
Candida	0.91	0.73 - 1.13	0.34
PPD	1.10	0.91 - 1.33	0.32
PET - No antigen	0.93	0.82 - 1.07	0.31
EB's	0.97	0.83 - 1.13	0.69
MOMP	1.08	0.92 - 1.26	0.35
OMP2	0.95	0.86 - 1.06	0.40
HSP60	1.04	0.89 - 1.22	0.61
PMPGa	1.07	0.92 - 1.23	0.39
PMPGc	1.03	0.93 - 1.14	0.57

Table 4-64: Conditional logistic regression of IL-2 concentrations for infertile women and their controls

Conditional logistic regression confirms the association of higher levels of IL-2 in response to PHA for infertile women. The association with PMPGa is, as expected, not confirmed.

Quartile	Group	n	Odds ratio	95% C.I.	p <
IL-2 < 25th cent	Infertile	2	1.00	-	0.13
	Control	4			
IL-2 25 - 50%	Infertile	6	2.00	0.32 - 3.10	
	Control	6			
IL-2 50 - 75%	Infertile	5	1.25	0.15 - 10.11	
	Control	8			
IL-2 > 75th cent	Infertile	9	6.00	0.54 - 66.69	
	Control	3			

Table 4-65: χ^2 test for trend for IL-2 responses to PHA in infertile women and their controls

4.4.2.4 IL-5

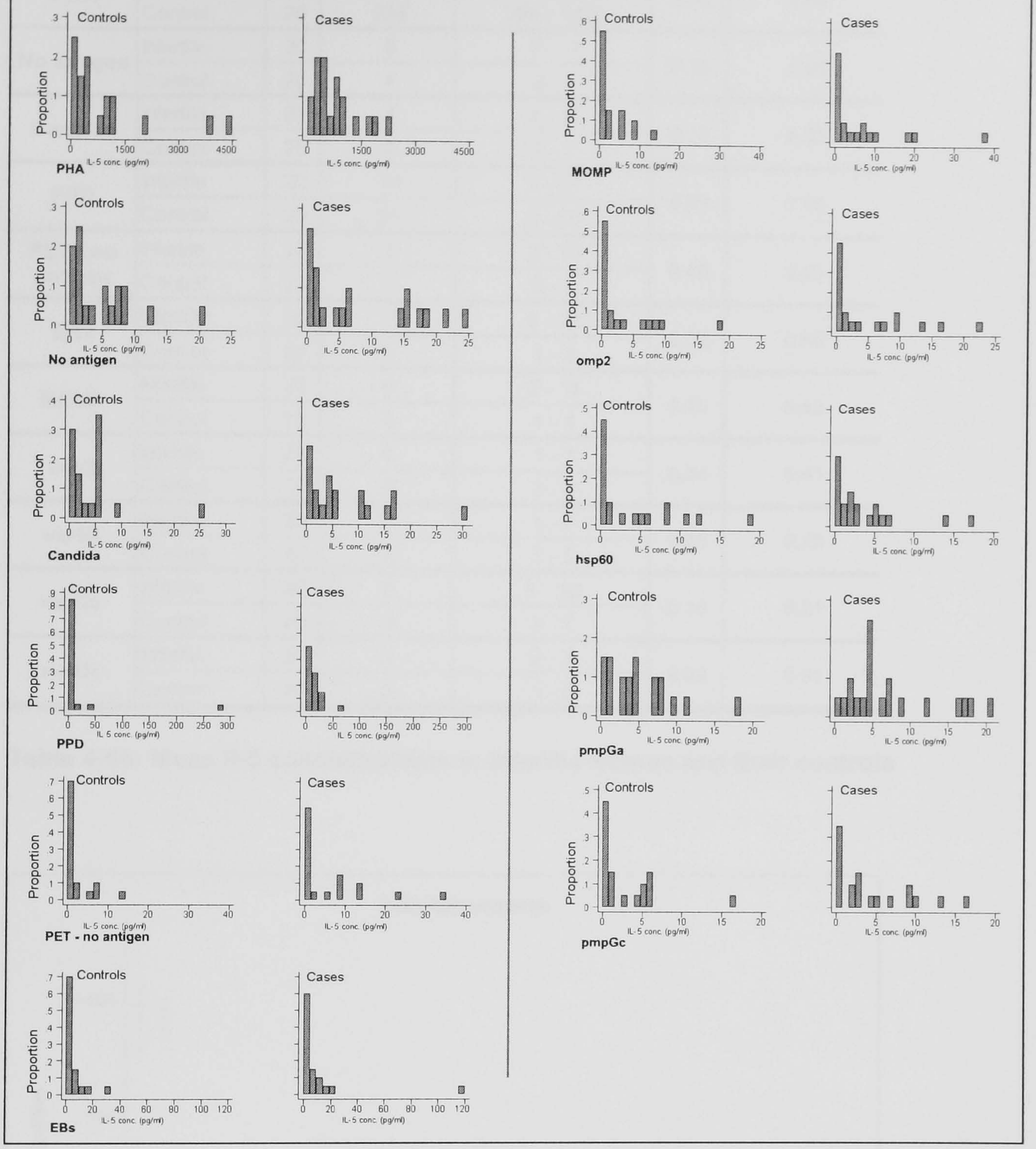


Figure 4-42: Frequency-distribution graphs of IL-5 responses in infertile women and their controls

No clear differences in IL-5 responses are apparent from the frequency-distribution graphs and this is confirmed by the lack of any statistically significant associations by t-test or Mann-Whitney U-test.

Antigen	Group	n	Mean IL-5 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Infertile	20	748	473 - 1024	0.54	0.64
	Control	20	933	368 - 1497		
No antigen	Infertile	20	8	4 - 11	0.16	0.51
	Control	20	4	2 - 7		
Candida	Infertile	20	7	4 - 11	0.18	0.32
	Control	20	4	2 - 7		
PPD	Infertile	20	14	7 - 21	0.64	0.10
	Control	20	21	0 - 50		
PET - no antigen	Infertile	20	6	2 - 10	0.08	0.23
	Control	20	2	0 - 4		
EBs	Infertile	20	11	0 - 23	0.32	0.56
	Control	20	5	1 - 8		
MOMP	Infertile	20	6	2 - 11	0.15	0.42
	Control	20	3	1 - 5		
OMP2	Infertile	20	4	1 - 7	0.34	0.41
	Control	20	3	1 - 5		
HSP60	Infertile	20	4	1 - 6	0.85	0.78
	Control	20	4	1 - 6		
PMPGa	Infertile	20	8	5 - 10	0.16	0.21
	Control	20	5	3 - 7		
PMPGc	Infertile	20	4	2 - 7	0.26	0.31
	Control	20	3	1 - 5		

Table 4-66: Mean Il-5 concentrations in infertile women and their controls

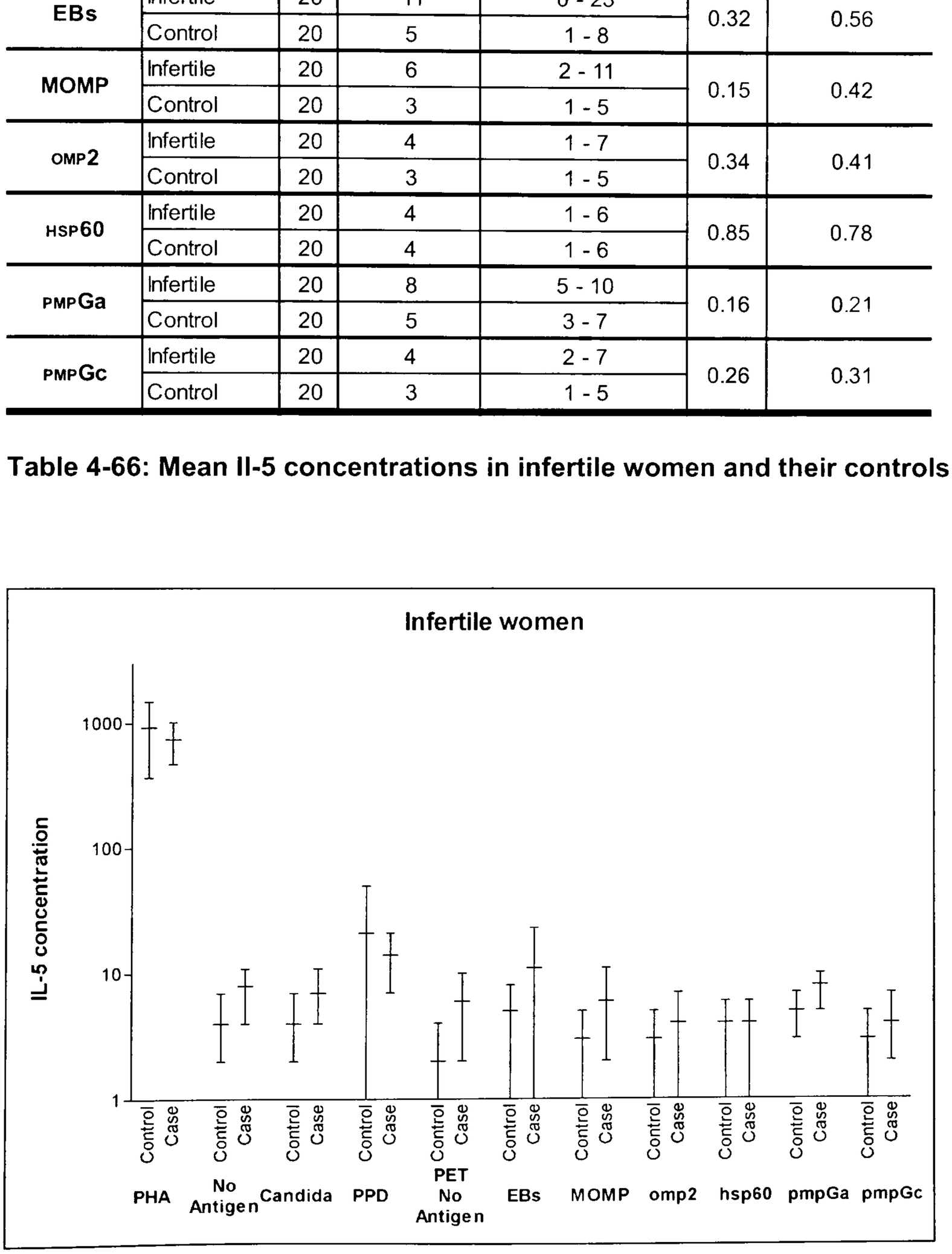


Figure 4-43: Mean IL-5 concentrations in infertile women and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-5	95% C.I.	p <
PHA	0.96	0.83 - 1.11	0.54
No antigen	1.11	0.95 - 1.30	0.18
Candida	1.10	0.94 - 1.29	0.22
PPD	0.97	0.89 - 1.07	0.58
PET - No antigen	1.11	0.97 - 1.28	0.14
EB's	1.05	0.94 - 1.17	0.42
MOMP	1.11	0.94 - 1.30	0.21
OMP2	1.06	0.94 - 1.20	0.34
HSP60	0.99	0.88 - 1.12	0.85
PMPGa	1.14	0.93 - 1.40	0.20
PMPGc	1.11	0.93 - 1.33	0.25

Table 4-67: Conditional logistic regression of IL-5 concentrations for infertile women and their controls

Conditional logistic regression provides further confirmation that there are no differences between infertile women and their controls with respect to IL-5 responses to these antigens.

4.4.2.5 IL-10

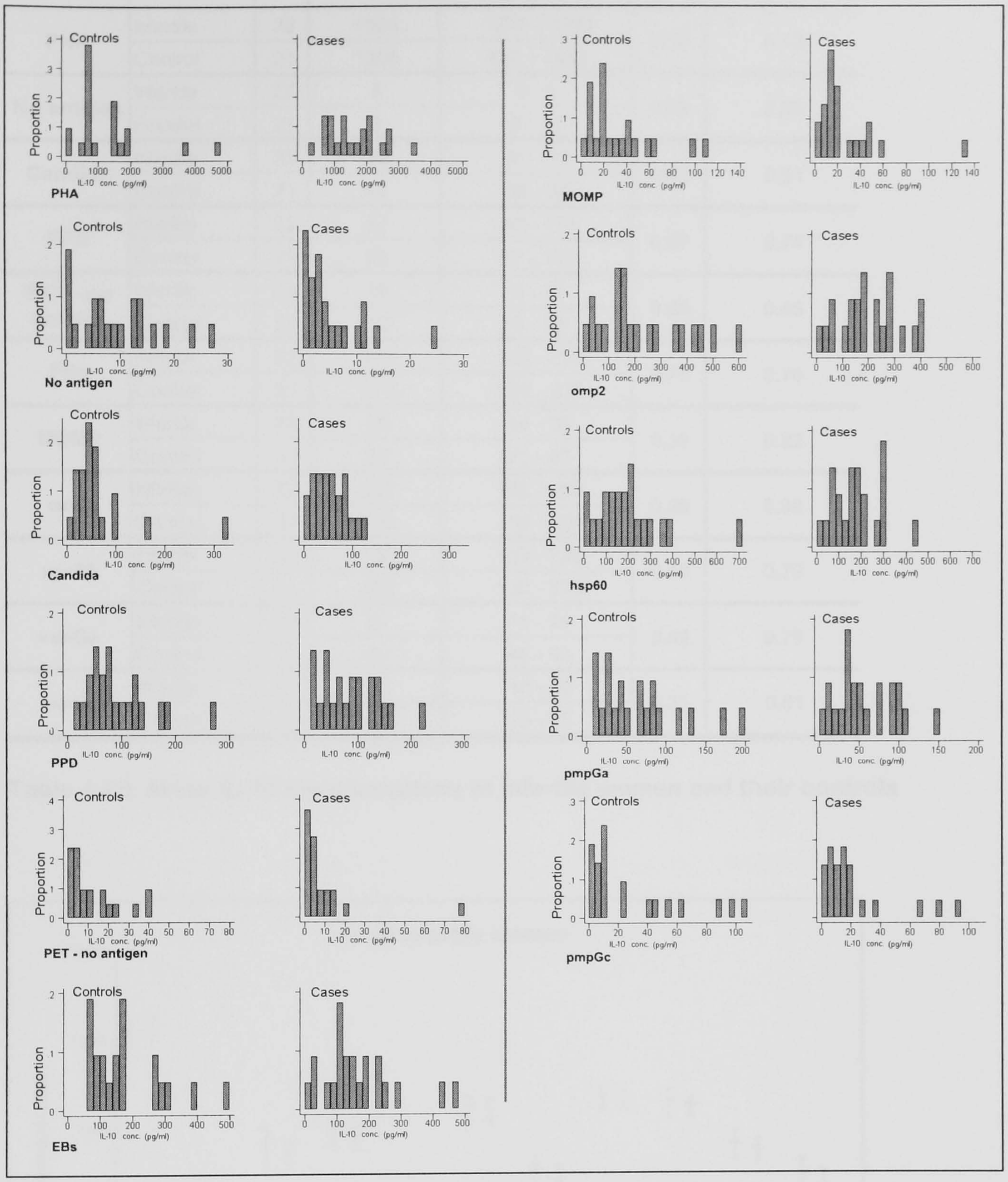


Figure 4-44: Frequency-distribution graphs of IL-10 responses in infertile women and their controls

The only association shown by the t-test and Mann-Whitney U-test is a lower IL-10 response in the negative control wells (5 vs. 9 pg/ml), of uncertain significance. No differences were noted with antigens.

Antigen	Group	n	Mean IL-10 level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Infertile	22	1565	1201 - 1929	0.39	0.10
	Control	21	1308	807 - 1808		
No antigen	Infertile	22	5	3 - 8	0.06	0.05
	Control	21	9	6 - 13		
Candida	Infertile	22	56	41 - 71	0.48	0.91
	Control	21	68	36 - 99		
PPD	Infertile	22	87	63 - 111	0.67	0.74
	Control	21	95	67 - 122		
PET - no antigen	Infertile	22	10	3 - 17	0.60	0.45
	Control	21	12	6 - 18		
EBs	Infertile	22	167	115 - 219	0.75	0.76
	Control	21	178	126 - 231		
MOMP	Infertile	22	26	14 - 39	0.38	0.22
	Control	21	34	21 - 47		
OMP2	Infertile	22	207	155 - 258	0.59	0.99
	Control	21	230	155 - 306		
HSP60	Infertile	22	180	133 - 226	0.59	0.79
	Control	21	202	130 - 273		
PMPGa	Infertile	22	57	41 - 74	0.48	0.78
	Control	21	67	44 - 90		
PMPGc	Infertile	22	22	10 - 33	0.35	0.61
	Control	21	30	15 - 45		

Table 4-68: Mean IL-10 concentrations in infertile women and their controls

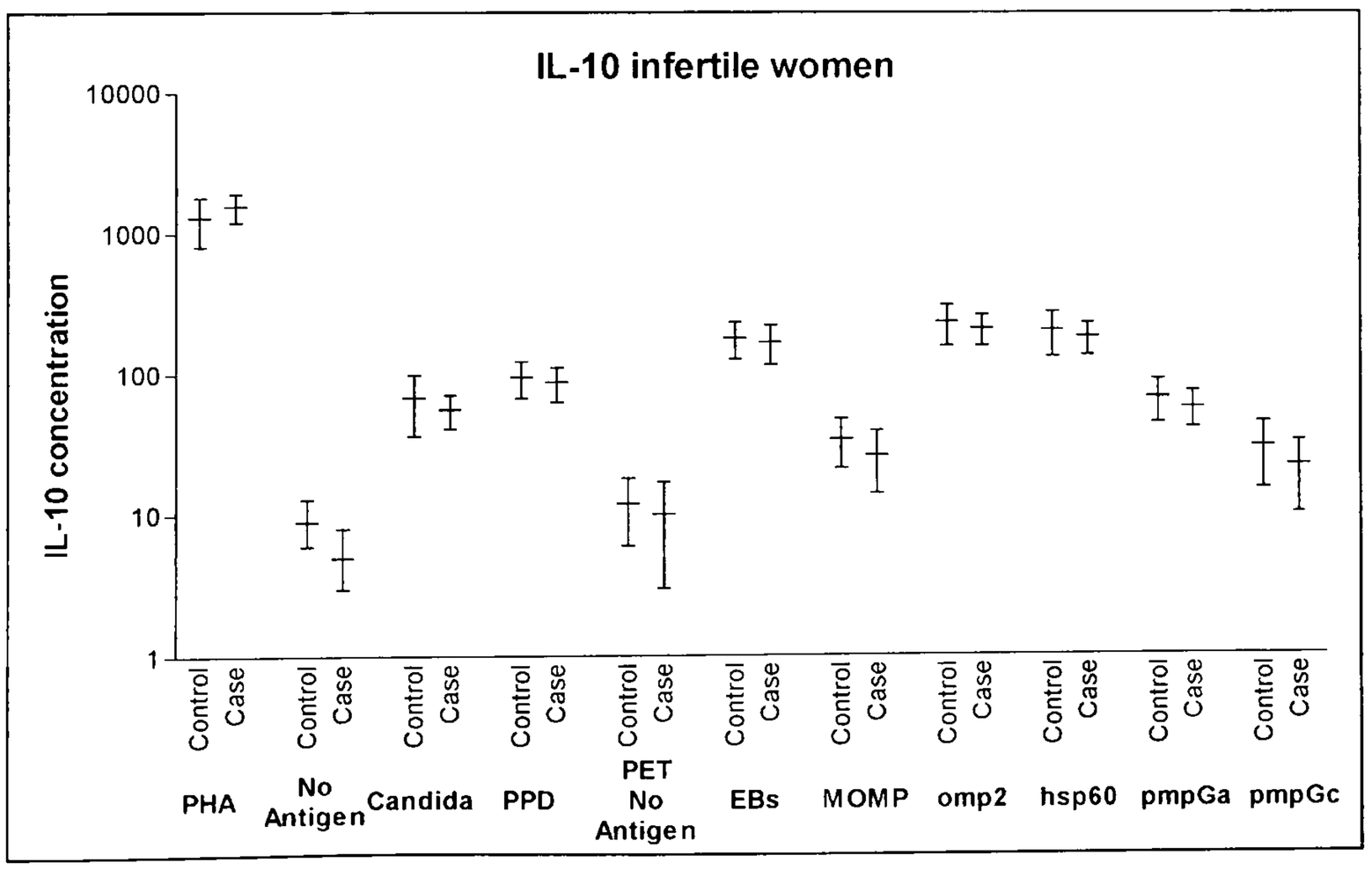


Figure 4-45: Mean IL-10 concentrations in infertile women and their controls

Antigen	logistic regression		
	O.R. for 25% rise in IL-10	95% C.I.	p <
PHA	1.16	0.88 - 1.52	0.29
No antigen	0.86	0.71 - 1.03	0.11
Candida	0.95	0.79 - 1.13	0.55
PPD	0.97	0.78 - 1.21	0.77
PET - No antigen	0.97	0.87 - 1.09	0.64
EB's	0.97	0.76 - 1.23	0.79
MOMP	0.94	0.81 - 1.10	0.46
OMP2	0.94	0.71 - 1.23	0.65
HSP60	0.95	0.75 - 1.20	0.66
PMPGa	0.95	0.78 - 1.15	0.60
PMPGc	0.94	0.83 - 1.07	0.37

Table 4-69: Conditional logistic regression of IL-10 concentrations for infertile women and their controls

Conditional logistic regression also shows a trend towards a reduced odds ratio of being infertile with increasing IL-10 responses in the negative control wells though this no longer reaches significance.

4.4.2.6 TGF-β

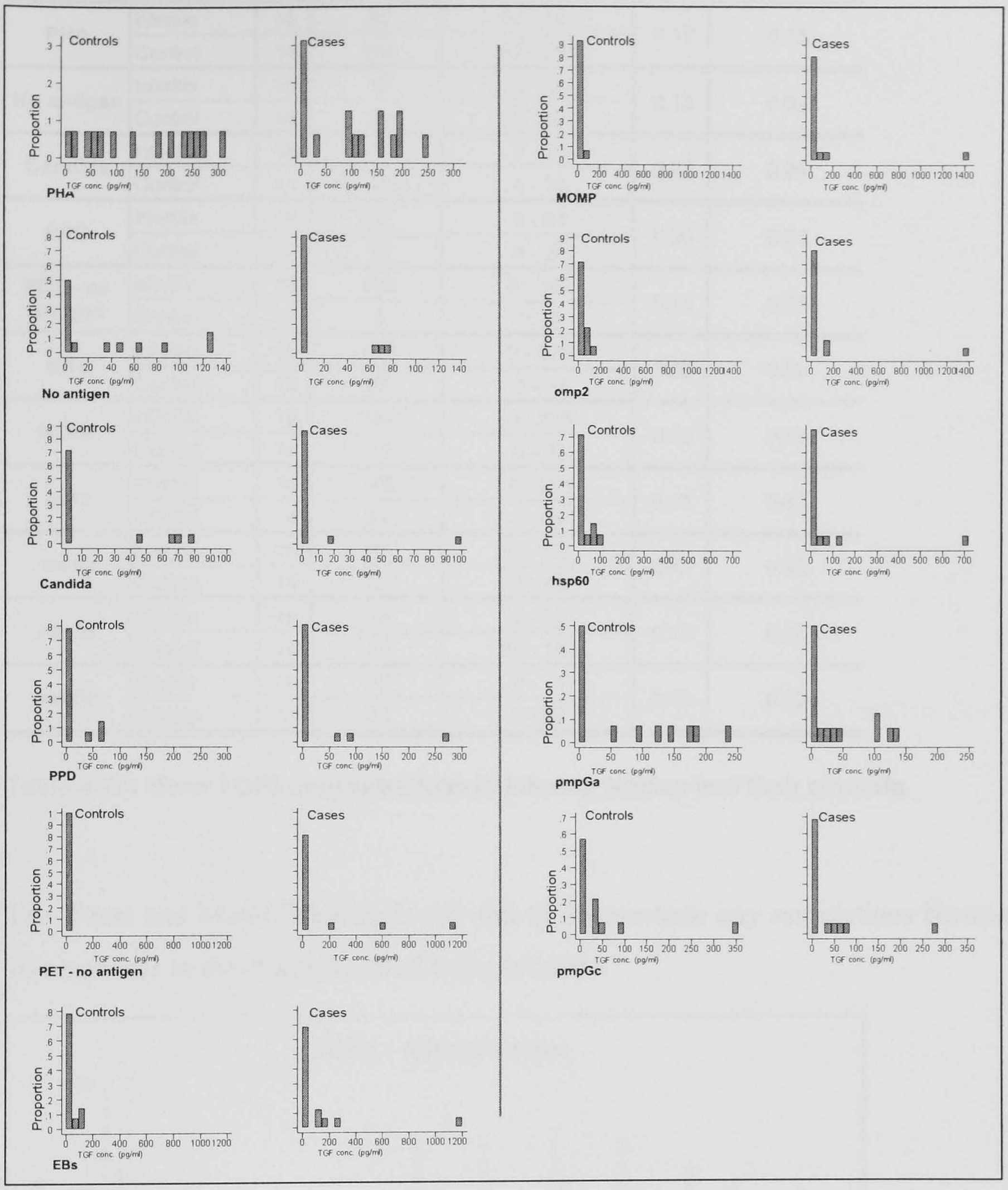


Figure 4-46: Frequency-distribution graphs of TGFβ responses in infertile women and their controls

As for the two trachoma groups, there are a reduced number of data for the infertile women and their controls and again perhaps a few spuriously high results due to non-specific activation.

Antigen	Group	n	Mean TGF level (pg/ml)	t-test		Mann-Whitney
				95% C.I.	p <	p <
PHA	Infertile	16	99	54 - 144	0.13	0.13
	Control	14	153	92 - 214		
No antigen	Infertile	16	13	0 - 28	0.13	0.09
	Control	14	35	8 - 62		
Candida	Infertile	16	7	0 - 20	0.27	0.28
	Control	14	18	1 - 36		
PPD	Infertile	16	26	0 - 63	0.50	0.74
	Control	14	13	0 - 28		
PET - no antigen	Infertile	16	123	0 - 289	0.16	0.61
	Control	14	3	0 - 8		
EBs	Infertile	16	117	0 - 273	0.26	0.57
	Control	14	25	0 - 53		
MOMP	Infertile	16	104	0 - 295	0.36	0.74
	Control	14	15	0 - 33		
OMP2	Infertile	16	109	0 - 291	0.38	0.66
	Control	14	27	0 - 54		
HSP60	Infertile	16	61	0 - 157	0.43	0.92
	Control	14	22	1 - 42		
PMPGa	Infertile	16	36	9 - 63	0.15	0.42
	Control	14	73	24 - 123		
PMPGc	Infertile	16	32	0 - 71	0.70	0.72
	Control	14	44	0 - 98		

Table 4-70: Mean TGFβ concentrations in infertile women and their controls

The T-test and Mann-Whitney U-test fail to demonstrate any associations between IL-10 responses to these antigens and being infertile.

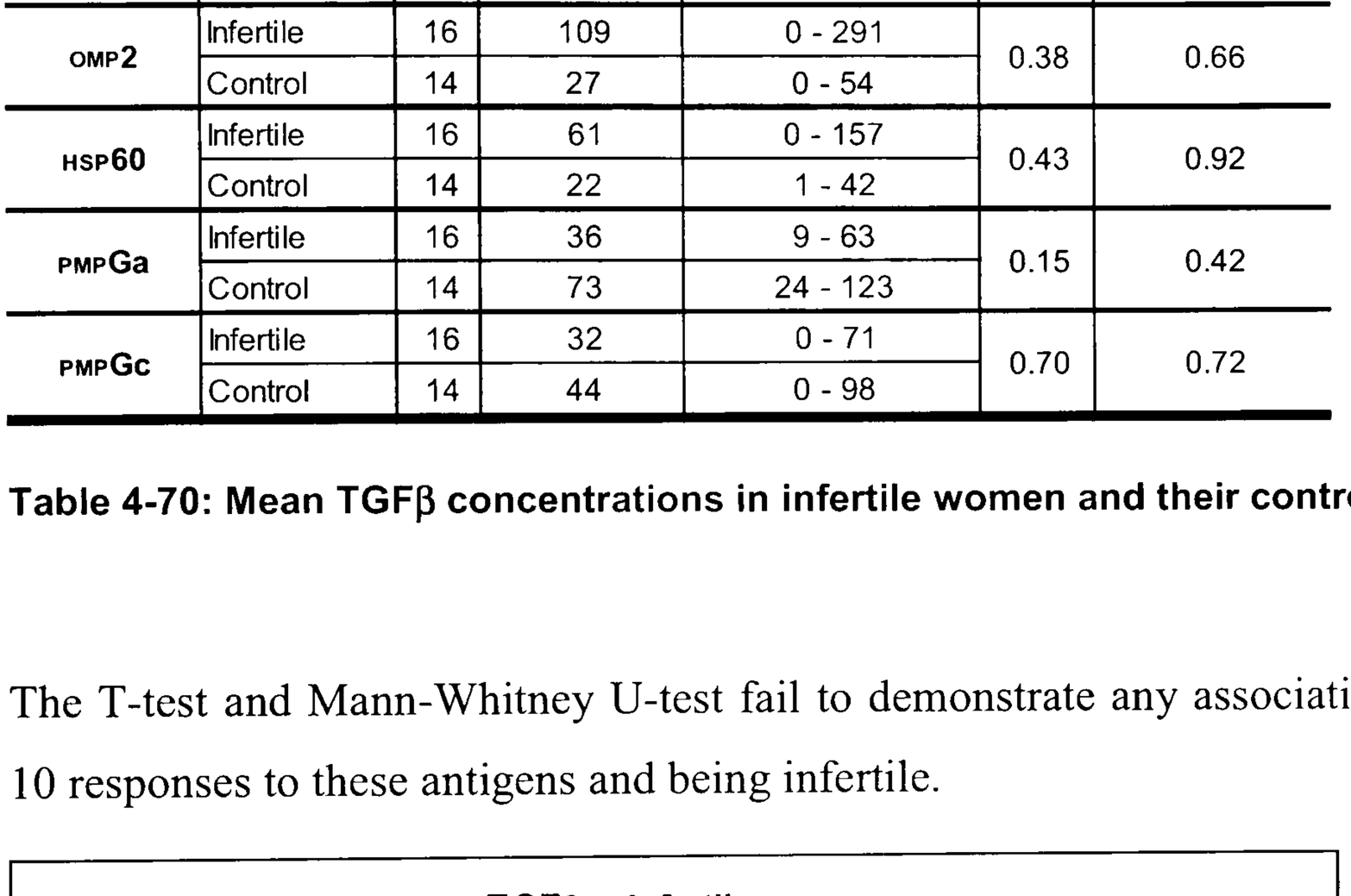


Figure 4-47: Mean TGFβ concentrations in infertile women and their controls

Antigen	logistic regression		
	O.R. for 25% rise in TGF	95% C.I.	p <
PHA	0.81	0.61 - 1.07	0.14
No antigen	0.86	0.71 - 1.04	0.12
Candida	0.91	0.80 - 1.05	0.19
PPD	1.03	0.95 - 1.11	0.53
PET - No antigen	1.19	0.74 - 1.92	0.47
EB's	1.09	0.86 - 1.37	0.48
MOMP	1.04	0.92 - 1.18	0.55
OMP2	1.04	0.93 - 1.15	0.49
HSP60	1.03	0.94 - 1.13	0.48
PMPGa	0.87	0.73 - 1.04	0.14
PMPGc	0.99	0.91 - 1.07	0.76

Table 4-71: Conditional logistic regression of TGFβ concentrations for infertile women and their controls

Conditional logistic regression also shows no differences between infertile women and their controls with respect to TGFβ production in response to these antigens.

4.5 Miscellaneous results

As described in section 3.12.2, the following tables show the results for a number of other variables; gender, ethnicity, *Chlamydia* PCR results from conjunctival swabs, effects of prior BCG vaccination, and the presence of faecal helminth ova and protozoan cysts. As the numbers are small in some instances, cases and controls have been pooled. Only the statistically significant results are presented.

The results are presented first for univariate analysis using a t-test and Mann-Whitney U-test, and then for multivariate analysis controlling for age, sex, ethnicity, prior BCG vaccination, presence of faecal helminth ova and protozoan cysts and whether the individual is a case or control.

4.5.1 Effect of gender

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
Immunoblot MOMP	Male	28	1.65	1.32 - 1.97	0.05	0.08	3.09	0.09
	Female	34	2.05	1.80 - 2.30				
TNF PHA	Male	36	1037	853 - 1221	0.02	0.02	6.23	0.02
	Female	39	756	607 - 905				
TNF EB	Male	36	1396	1074 - 1718	0.01	0.003	5.80	0.02
	Female	39	900	663 - 1137				
IL-2 PHA	Male	36	621	485 - 757	0.01	0.02	5.42	0.02
	Female	39	417	334 - 499				
IL-2 No antigen	Male	36	5	2 - 8	0.09	0.07	3.52	0.07
	Female	39	9	5 - 12				
IL-5 PHA	Male	37	1464	1100 - 1827	0.003	0.002	8.04	0.006
	Female	35	762	490 - 1034				
IL-10 EB	Male	35	327	277 - 378	0.06	0.09	3.20	0.08
	Female	35	267	229 - 304				
Scarred trachoma								
TNF PPD	Male	21	128	78 - 178	0.06	0.05	2.78	0.10
	Female	33	76	46 - 107				
TNF HSP60	Male	21	957	755 - 1159	0.006	0.002	3.21	0.08
	Female	33	579	408 - 750				
TGF No antigen	Male	10	18	10 - 25	0.002	0.004	28.40	0.0005
	Female	9	4	1 - 6				
TGF PPD	Male	10	68	46 - 90	0.006	0.004	3.87	0.08
	Female	9	34	24 - 43				
TGF HSP60	Male	10	243	192 - 294	0.02	0.009	3.44	0.10
	Female	9	127	33 - 222				
TGF PMPGc	Male	10	20	10 - 29	0.02	0.006	25.5	0.0007
	Female	9	6	1 - 11				

Table 4-72: Effects of gender

Where a difference was noted between males and females, males had a higher response with the exceptions of antibody responses to MOMP and the IL-2 response in the negative controls for children with active trachoma and their controls.

4.5.2 Effect of ethnicity

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
Immunoblot MOMP	Mandinka	24	1.57	1.21 - 1.92	0.02	0.03	11.93	0.001
	Not Mandinka	38	2.06	1.82 - 2.29				
Immunoblot OMP2	Mandinka	23	1.66	1.29 - 2.02	0.03	0.02	7.70	0.008
	Not Mandinka	29	2.22	1.86 - 2.59				
Immunoblot HSP60	Mandinka	24	1.93	1.62 - 2.23	0.008	0.01	9.05	0.004
	Not Mandinka	38	2.38	2.19 - 2.56				
IFN Candida	Mandinka	30	155	59 - 251	0.04	0.04	3.75	0.06
	Not Mandinka	42	65	37 - 93				
IFN PPD	Mandinka	30	834	358 - 1310	0.09	0.06	4.15	0.06
	Not Mandinka	42	454	280 - 627				
IL-2 Candida	Mandinka	33	21	12 - 29	0.0001	0.00001	14.3	0.0004
	Not Mandinka	42	5	4 - 7				
IL-2 PPD	Mandinka	33	119	69 - 170	0.001	0.00001	9.62	0.003
	Not Mandinka	42	40	25 - 55				
IL-10 MOMP	Mandinka	28	47	31 - 62	0.09	0.06	2.46	0.12
	Not Mandinka	42	65	50 - 80				
TGF MOMP	Mandinka	3	13	0 - 42	0.05	0.01	12.64	0.02
	Not Mandinka	10	56	34 - 79				
Scarred trachoma								
IFN PPD	Mandinka	44	544	327 - 761	0.001	0.04	5.26	0.03
	Not Mandinka	10	2076	208 - 3945				
IL-5 No antigen	Mandinka	44	21	14 - 29	0.06	0.02	6.09	0.02
	Not Mandinka	10	7	2 - 11				
Infertile women								
TNF OMP2	Mandinka	25	3063	2375 - 3751	0.009	0.006	3.46	0.07
	Not Mandinka	18	1710	964 - 2458				
TNF HSP60	Mandinka	25	1259	971 - 1547	0.002	0.002	8.21	0.008
	Not Mandinka	18	587	310 - 864				

Table 4-73: Effects of ethnicity - Mandinka

In the active trachoma and infertile women groups, where there was a difference between Mandinka and other individuals, the Mandinka showed what might be described as a more pro-inflammatory or T_H1-type response, i.e. higher IFNγ, higher IL-2, lower IL-5 and lower IL-10. In the scarred trachoma group the lower IFNγ and higher IL-5 responses are more T_H2 type responses however it would be wrong to reach any conclusions from only two such results.

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
Immunoblot PMPGa	Fula	7	0.19	0 - 0.53	0.008	0.009	7.98	0.007
	Not Fula	55	1.16	0.91 - 1.41				
Immunoblot PMPGc	Fula	7	0.04	0 - 0.15	0.07	0.05	4.60	0.04
	Not Fula	55	0.71	0.45 - 0.96				
IL-2 PHA	Fula	9	194	130 - 258	0.003	0.0005	6.47	0.01
	Not Fula	66	558	473 - 643				
IL-2 EB	Fula	9	14	2 - 26	0.006	0.0003	7.94	0.007
	Not Fula	66	3	1 - 6				
IL-2 MOMP	Fula	9	19	2 - 35	0.003	0.002	9.89	0.003
	Not Fula	66	5	2 - 8				
IL-2 OMP2	Fula	9	14	1 - 26	0.003	0.0001	10.04	0.002
	Not Fula	66	3	1 - 5				
IL-2 HSP60	Fula	9	5	3 - 8	0.003	0.01	4.38	0.04
	Not Fula	66	3	2 - 3				
IL-5 PHA	Fula	9	270	145 - 395	0.006	0.0007	4.83	0.03
	Not Fula	63	1244	986 - 1503				
IL-10 Candida	Fula	9	190	138 - 243	0.01	0.01	6.20	0.02
	Not Fula	61	126	107 - 144				
IL-10 PPD	Fula	9	154	116 - 193	0.09	0.05	4.62	0.04
	Not Fula	61	118	102 - 133				
IL-10 MOMP	Fula	9	85	26 - 144	0.05	0.36	4.13	0.05
	Not Fula	61	54	44 - 63				

Table 4-74: Effects of ethnicity – Fula

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
Immunoblot MOMP	Wolof	6	2.67	2.22 - 3.12	0.008	0.006	4.47	0.04
	Not Wolof	56	1.78	1.57 - 1.99				
Immunoblot HSP60	Wolof	6	2.68	2.30 - 3.07	0.06	0.06	4.78	0.03
	Not Wolof	56	2.15	1.97 - 2.32				
TNF Candida	Wolof	6	2249	949 - 3550	0.02	0.02	3.21	0.08
	Not Wolof	69	1165	922 - 1409				
IL-2 No antigen	Wolof	6	19	0 - 39	0.002	0.02	13.69	0.0005
	Not Wolof	69	6	4 - 8				
IL-5 PHA	Wolof	6	305	0 - 686	0.04	0.004	3.63	0.06
	Not Wolof	66	1197	946 - 1448				
IL-10 Candida	Wolof	6	226	136 - 315	0.001	0.006	9.70	0.003
	Not Wolof	64	125	109 - 142				
IL-10 EB	Wolof	6	423	277 - 568	0.01	0.02	5.15	0.03
	Not Wolof	64	285	254 - 317				
IL-10 MOMP	Wolof	6	107	89 - 124	0.005	0.001	7.20	0.01
	Not Wolof	64	53	42 - 64				
IL-10 OMP2	Wolof	5	802	413 - 1191	0.02	0.04	3.45	0.07
	Not Wolof	60	507	439 - 575				
IL-10 HSP60	Wolof	6	656	381 - 931	0.0009	0.007	8.12	0.006
	Not Wolof	64	386	342 - 430				
IL-10 PMPGa	Wolof	6	278	170 - 386	0.0002	0.002	12.64	0.0008
	Not Wolof	64	124	101 - 147				

Table 4-75: Effects of ethnicity - Wolof

The most marked difference between the Wolof and those of other ethnic groups is the higher levels of IL-10 generated in response to all the chlamydial antigens except PMPGc. Higher antibody responses were also noted which, like high IL-10 responses are

usually taken to be T_H2 type responses, however the Wolof also demonstrated some higher TNFα and IL-2 results more typical of an T_H1 type response.

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
Immunoblot OMP2	Jola	18	2.54	2.15 - 2.92	0.001	0.0009	10.61	0.002
	Not Jola	34	1.67	1.36 - 1.99				
Immunoblot PMPGa	Jola	18	1.66	1.28 - 2.03	0.0007	0.001	9.77	0.003
	Not Jola	44	0.80	0.53 - 1.07				
IL-2 PHA	Jola	19	680	470 - 890	0.01	0.05	4.96	0.03
	Not Jola	56	458	380 - 537				
IL-2 Candida	Jola	19	3	1 - 5	0.009	0.00001	4.05	0.05
	Not Jola	56	15	10 - 20				
IL-2 PPD	Jola	19	42	20 - 64	0.12	0.09	3.63	0.06
	Not Jola	56	86	54 - 118				
IL-5 PHA	Jola	19	1660	1182 - 2138	0.006	0.002	8.69	0.005
	Not Jola	53	930	666 - 1194				
IL-5 No antigen	Jola	19	15	8 - 23	0.08	0.05	2.74	0.10
	Not Jola	53	9	6 - 13				
IL-5 Candida	Jola	19	15	9 - 22	0.003	0.01	11.00	0.002
	Not Jola	53	8	6 - 10				
IL-10 PHA	Jola	19	2450	1760 - 3139	0.0002	0.007	9.97	0.003
	Not Jola	51	1418	1202 - 1634				
IL-10 Candida	Jola	19	101	81 - 121	0.02	0.03	4.26	0.04
	Not Jola	51	146	124 - 168				
IL-10 PMPGa	Jola	19	91	64 - 118	0.02	0.05	4.52	0.04
	Not Jola	51	155	124 - 186				
IL-10 PMPGc	Jola	19	25	12 - 38	0.06	0.0005	3.25	0.08
	Not Jola	51	58	38 - 78				
Scarred trachoma								
IFN PPD	Jola	10	2076	208 - 3945	0.001	0.04	5.26	0.03
	Not Jola	44	544	327 - 761				
IL-5 No antigen	Jola	10	7	2 - 11	0.06	0.02	6.09	0.02
	Not Jola	44	21	14 - 29				

Table 4-76: Effects of ethnicity - Jola

Where differences were noted between Jola and other ethnic groups, they tend to show more of a T_H2-like response, e.g. higher antibody responses, higher IL-5 and lower IFNγ results. However they also tend to have lower IL-10 responses so no clear pattern emerges.

4.5.3 Chlamydial PCR results

	Active		Scarred	
	Case	Control	Case	Control
PCR negative				
No	18	20	21	24
%	*36%*	*80%*	*78%*	*92%*
PCR positive				
No	32	5	6	2
%	*64%*	*20%*	*22%*	*8%*

Table 4-77: Chlamydial PCR results

As expected, the prevalence of chlamydial organisms being detected in the eye was highest from those with active trachoma where 32/50 cases had positive PCRs vs. 5/25 controls ($p < 0.0005$). The prevalence was also higher in those with trachomatous scarring vs. controls but this did not reach significance ($p < 0.14$).

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
TNF Candida	PCR positive	37	1606	1179 - 2034	0.005	0.02	2.74	0.10
	PCR negative	37	928	712 - 1145				
IL-2 PPD	PCR positive	37	50	33 - 67	0.06	0.21	3.19	0.08
	PCR negative	37	98	51 - 146				
Scarred trachoma								
Immunoblot HSP60	PCR positive	7	2.81	2.57 - 3.06	0.06	0.05	7.29	0.01
	PCR negative	39	2.32	2.10 - 2.53				
Immunoblot PMPGa	PCR positive	7	2.31	1.56 - 3.07	0.01	0.007	6.18	0.02
	PCR negative	39	1.38	1.11 - 1.65				
IFN Candida	PCR positive	8	61	12 - 109	0.12	0.06	6.71	0.01
	PCR negative	45	75	47 - 102				
IFN OMP2	PCR positive	8	61	12 - 109	0.21	0.24	3.67	0.06
	PCR negative	45	160	94 - 225				
IFN hsp60	PCR positive	8	27	0 - 56	0.22	0.14	3.18	0.08
	PCR negative	45	96	49 - 142				
TGF PMPGa	PCR positive	3	0	0 - 0	0.37	0.13	6.86	0.03
	PCR negative	18	93	7 - 178				
TGF PMPGc	PCR positive	3	380	0 - 2015	0.44	0.91	10.57	0.01
	PCR negative	18	138	0 - 369				

Table 4-78: Effects of positive chlamydial PCR

Where a difference in response was noted between those with positive and negative chlamydial PCR results amongst the scarred individuals and their controls, those with a positive chlamydial PCR tended to show more T_H2-type responses, e.g. lower IFNγ and higher antibody levels. There was no such pattern amongst children with active trachoma and their controls.

4.5.4 Effect of BCG vaccination

Of all study participants 58.3% had a BCG scar. Females were more likely to have been vaccinated than males, 66.7% vs. 41.4% ($p < 0.001$). Rates of BCG vaccination appeared to be declining as the younger children were far less likely to have been vaccinated than the adults and the proportion vaccinated increased with age.

Age	Group	n	Odds ratio	95% C.I.	p <
0 - 9	BCG	31	1.0	-	0.003
	No BCG	35			
10 - 19	BCG	15	1.21	0.50 - 2.92	
	No BCG	14			
20 - 29	BCG	8	1.81	0.53 - 6.20	
	No BCG	5			
30 - 39	BCG	24	2.71	1.09 - 6.73	
	No BCG	10			
> 40	BCG	24	3.01	1.18 - 7.70	
	No BCG	9			

Table 4-79: Likelihood of having received BCG vaccination by age group

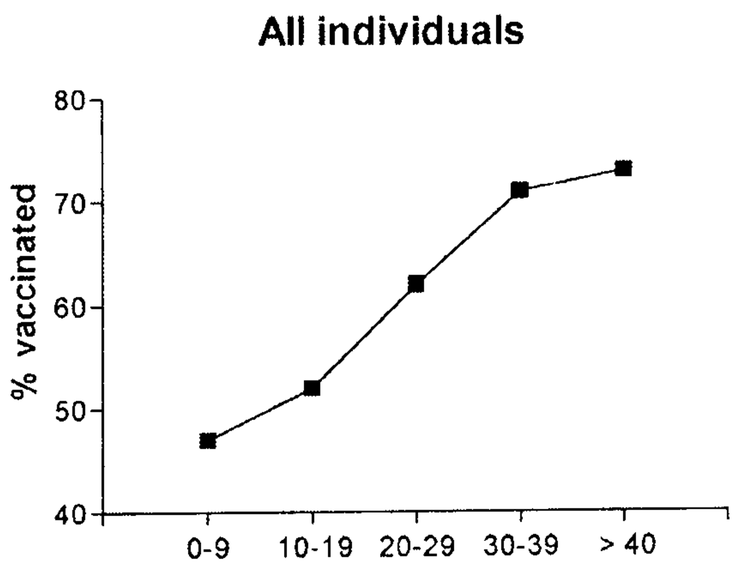


Figure 4-48: Likelihood of having received BCG vaccination by age group

The presence of a BCG scar was not associated with the presence or absence of active trachoma, trachomatous scarring or infertility ($p < 0.75$, $p < 0.10$ and $p < 0.17$ respectively by χ^2 analysis).

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
Immunoblot OMP2	BCG	27	2.32	2.02 - 2.62	0.005	0.009	9.47	0.004
	No BCG	25	1.60	1.18 - 2.01				
IL-5 No antigen	BCG	36	9	5 - 12	0.10	0.19	3.06	0.09
	No BCG	36	14	9 - 19				
IL-5 Candida	BCG	36	8	4 - 11	0.12	0.01	4.94	0.03
	No BCG	36	12	8 - 15				
IL-5 PPD	BCG	36	9	5 - 13	0.04	0.03	3.34	0.07
	No BCG	36	16	10 - 22				
IL-5 PMPGa	BCG	36	9	6 - 11	0.04	0.15	3.80	0.06
	No BCG	36	19	9 - 30				
IL-10 Candida	BCG	36	143	120 - 165	0.31	0.16	3.51	0.07
	No BCG	34	125	97 - 153				
IL-10 PPD	BCG	36	135	112 - 157	0.08	0.12	4.04	0.05
	No BCG	36	109	92 - 127				
Scarred trachoma								
Immunoblot HSP60	BCG	26	2.31	2.01 - 2.62	0.28	0.56	3.56	0.07
	No BCG	21	2.52	2.29 - 2.74				
TNF PPD	BCG	28	119	78 - 160	0.07	0.08	8.06	0.007
	No BCG	26	72	38 - 106				
IFN OMP2	BCG	28	206	105 - 306	0.02	0.09	5.85	0.02
	No BCG	26	75	45 - 105				
IFN HSP60	BCG	28	99	37 - 160	0.45	0.20	3.19	0.08
	No BCG	26	69	17 - 120				
IL-2 PHA	BCG	28	699	530 - 867	0.12	0.21	5.12	0.03
	No BCG	26	538	425 - 651				
IL-10 PHA	BCG	25	2474	1928 - 3019	0.03	0.07	3.51	0.01
	No BCG	21	1706	1340 - 2073				
Infertile women								
TNF PMPGa	BCG	27	322	171 - 473	0.52	0.52	6.83	0.01
	No BCG	27	254	94 - 414				

Table 4-80: Effects of prior BCG vaccination

Amongst the scarred individuals, where there was a difference in response between those with prior BCG vaccination and those without, those with BCG vaccination tended to have a more T_H1-type response as might be expected, e.g. lower antibody levels and higher TNFα, IFNγ and IL-2 levels. The pattern was however much more mixed for the children with active trachoma and their controls. Interestingly there was much less difference than might have been expected between responses to PPD with only a lower IL-5 response in the active trachoma group though this is only significant to the 7% level.

4.5.5 Effects of helminth and parasitic infestation

	No	%	Knight 1981	Bundy 1995
Helminths	**24**	**16.0**		
Hookworm	14	9.3	80-94%	30%
Hymenolepis nana	5	3.3	0.7%	0
Ascaris	5	3.3	0.7%	25%
Enterobius	1	0.7	0	0
Strongyloides	1	0.7	0	0
Trichuris	0	0	3.7%	2.4%
Schistosoma mansoni	0	0	0.3%	1.5%
Taenia saginata	0	0	0.3%	0
Wucheraria bancrofti	not tested	-	8-21%	not tested
Mansonella perstans	not tested	-	25-30%	not tested
Protozoa	**35**	**23.3**		
Entamoeba coli	33	21.6	not tested	not tested
Entamoeba histolytica/dispar	1	0.7	not tested	not tested
Giardia lamblia	1	0.7	not tested	not tested

Table 4-81: Prevalence of helminth and protozoan infection, and comparison with previous studies in The Gambia

24 (16%) individuals had helminth ova in their stools. Of these, 2 had dual infections. 35 (23.3%) had protozoan cysts detected in their stool. Three individuals had both helminth ova and protozoan cysts. The species involved are detailed in the table above. As expected helminths were found more commonly in children; the mean age of those with helminths was 12.3 years vs. 23.7 years for those without helminths ($p < 0.001$). Corresponding figures are given from the two most recently published surveys of helminth infection in rural Gambia.[243,244] The prevalence of hookworm infection is very much lower than in previous studies although the prevalence had fallen from 94% in 1981 to 30% in 1995 and so may well have continued to fall. All samples were checked by a very experienced parasitologist. It has been noted previously that there are large variations in the prevalence of intestinal helminths both between and within villages.[244]

The presence of helminth ova was unrelated to gender or previous BCG vaccination and there was no relationship to having active trachoma or to being infertile. There was however an association with scarring; 4/24 of those with trachomatous scarring (16.7%) had helminth ova whilst 0/23 controls had ova detected ($p < 0.04$).

There was no relationship between the presence of protozoan cysts and gender, age, previous BCG vaccination or to having active trachoma, trachomatous scarring or to being infertile.

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
IFN OMP2	Worms	16	110	13 - 207	0.03	0.48	3.17	0.08
	No worms	52	47	33 - 62				
IFN PMPGa	Worms	16	26	8 - 44	0.03	0.31	4.88	0.03
	No worms	52	14	10 - 17				
IL-10 PMPGc	Worms	16	74	12 - 136	0.09	0.77	3.91	0.05
	No worms	50	42	31 - 53				
TGF EB	Worms	2	114	0 - 673	0.06	0.05	14.64	0.03
	No worms	10	277	204 - 349				
Scarred trachoma								
IFN Candida	Worms	4	5	0 - 11	0.14	0.003	3.19	0.08
	No worms	43	75	47 - 104				
IL-2 PHA	Worms	4	400	106 - 693	0.19	0.14	5.59	0.02
	No worms	43	664	543 - 786				
IL-10 PHA	Worms	4	1295	0 - 2651	0.12	0.14	6.87	0.01
	No worms	37	2287	1881 - 2693				

Table 4-82: Effects of intestinal helminth infection

The presence of gut helminths did not induce a particularly T_H2 bias in immune responses, where differences were noted they presented a mixed picture.

Assay	Group	n	Mean response	t-test		Mann - Whitney	Multivariate analysis	
				95% C.I.	p <	p <	F	p <
Active trachoma								
IL-5 No antigen	Protozoa	14	16	6 - 26	0.10	0.13	4.53	0.04
	No protozoa	54	10	7 - 13				
TGF PHA	Protozoa	2	1703	0 - 7827	0.12	0.09	5.47	0.10
	No protozoa	10	2947	2255 - 3639				
TGF EB	Protozoa	2	178	0 - 1118	0.35	0.39	9.43	0.05
	No protozoa	10	264	183 - 345				
TGF HSP60	Protozoa	2	240	0 - 1384	0.43	0.39	5.77	0.10
	No protozoa	10	314	232 - 396				
Scarred trachoma								
TNF Candida	Protozoa	12	953	600 - 1307	0.06	0.10	3.34	0.08
	No protozoa	35	658	512 - 805				
IL-5 PMPGa	Protozoa	12	25	0 - 55	0.04	0.11	2.87	0.10
	No protozoa	35	8	5 - 11				
IL-5 PHA	Protozoa	7	3170	2046 - 4294	0.02	0.03	6.82	0.01
	No protozoa	34	1989	1595 - 2383				
Infertile women								
TNF PMPGc	Protozoa	9	55	6 - 104	0.11	0.46	6.70	0.02
	No protozoa	23	27	14 - 41				
IFN PMPGa	Protozoa	9	34	10 - 58	0.27	0.67	8.50	0.008
	No protozoa	23	25	17 - 32				
IL-5 PPD	Protozoa	9	47	0 - 116	0.05	0.57	8.58	0.008
	No protozoa	21	8	5 - 10				
TGF PHA	Protozoa	5	2540	567 - 4515	0.03	0.07	6.79	0.02
	No protozoa	18	1311	903 - 1720				

Table 4-83: Effects of intestinal protozoan infection

4.6 Summary of results

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots							↑			↓	
TNF	↑		↑			↑		↑	↑	↑	
IFN	↓					↓					
IL2											
IL5											
IL10			↑			↑		↑	↑	↑	↑
TGF											
Scarred trachoma											
Immunoblots											↑
TNF											
IFN											
IL2											
IL5		↓									
IL10						↓					↓
TGF											
Infertile women											
Immunoblots							↑				
TNF											
IFN						↑					
IL2	↑										
IL5											
IL10											
TGF											

Figure 4-49: Summary of results - cases vs. controls for all groups

KEY

- Not investigated
- No difference
- ↑ Increase $p < 0.05$
- ↑ Increase $p < 0.1$
- ↓ Decrease $p < 0.05$
- ↓ Decrease $p < 0.1$

This key is used for the figures in this and the following pages. For example in Figure 4-49, the red arrow in the box corresponding to immunoblot responses to MOMP indicates that children with active trachoma generated stronger responses to MOMP than their controls ($p < 0.05$), whilst the arrow in the box corresponding to immunoblot responses to PMPGa indicates that children with active trachoma showed a trend towards weaker responses to PMPGa ($p < 0.1$).

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots							↓	↓	↓		
TNF											
IFN			↑	↑							
IL2			↑	↑							
IL5											
IL10											
TGF							↓				
Scarred trachoma											
Immunoblots											
TNF											
IFN				↓							
IL2											
IL5		↑									
IL10											
TGF											
Infertile women											
Immunoblots											
TNF								↑	↑		
IFN											
IL2											
IL5											
IL10											
TGF											

Figure 4-51: Summary of results - Mandinka vs. other ethnicity

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots								↑		↑	
TNF											
IFN											
IL2	↑		↓	↓							
IL5	↑	↑	↑								
IL10	↑		↓							↓	↓
TGF											
Scarred trachoma											
Immunoblots											
TNF											
IFN				↑							
IL2											
IL5		↓									
IL10											
TGF											
Infertile women											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											

Figure 4-50: Summary of results - Jola vs. other ethnicity

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots										↓	↓
TNF											
IFN											
IL2	↓					↑	↑	↑	↑		
IL5	↓										
IL10			↑	↑			↑				
TGF											
Scarred trachoma											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											
Infertile women											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											

Figure 4-53: Summary of results - Fula vs. other ethnicity

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots							↑		↑		
TNF			↑								
IFN											
IL2		↑									
IL5	↓										
IL10			↑			↑	↑	↑	↑	↑	
TGF											
Scarred trachoma											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											
Infertile women											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											

Figure 4-52: Summary of results - Wolof vs. other ethnicity

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots											
TNF											
IFN								↑		↑	
IL2											
IL5											
IL10											↑
TGF						↓					
Scarred trachoma											
Immunoblots											
TNF											
IFN			↓								
IL2	↓										
IL5											
IL10	↓										
TGF											
Infertile women											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											

Figure 4-55: Summary of results – intestinal helminths vs. no intestinal helminths

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots											
TNF											
IFN											
IL2											
IL5		↑									
IL10											
TGF	↓					↓			↓		
Scarred trachoma											
Immunoblots											
TNF			↑								
IFN											
IL2											
IL5	↑									↑	
IL10											
TGF											
Infertile women											
Immunoblots											
TNF											↑
IFN										↑	
IL2											
IL5				↑							
IL10											
TGF	↑										

Figure 4-54: Summary of results - intestinal protozoa vs. no intestinal protozoa

	PHA	Neg Ctrl	Candida	PPD	PET	EBs	MOMP	omp2	hsp60	pmpGa	pmpGc
Active trachoma											
Immunoblots							↓				
TNF	↑					↑					
IFN											
IL2	↑										
IL5	↑										
IL10						↑					
TGF											
Scarred trachoma											
Immunoblots											
TNF				↑					↑		
IFN											
IL2											
IL5											
IL10											
TGF		↑		↑					↑		↑
Infertile women											
Immunoblots											
TNF											
IFN											
IL2											
IL5											
IL10											
TGF											

Figure 4-56: Summary of results - male vs. female

Chapter Five

Discussion

5 Discussion

This discussion first presents the principal findings of the study and discusses these findings in relation to the current literature. Potential weaknesses of the study are then presented together with possible solutions. Finally unanswered questions and potential future research directions are discussed.

5.1 Principal findings

5.1.1 PMP-G IgG antibodies are commonly detected in individuals from a trachoma-endemic population. Higher levels of antibody to the amino-terminus half are associated with a reduced risk of trachoma but higher levels of antibody to the carboxyl-terminus half are associated with scarring

The immunoblot analysis demonstrated that 22/44 (50%) of children with active trachoma and 12/18 (67%) of controls had detectable IgG antibodies to the amino-terminal half of PMPG, PMPGa. In this group it was also noted that higher levels of antibody to PMPGa were associated with a reduced likelihood of having acute trachoma (O.R 0.32, $p < 0.06$ for an immunoblot density score of > 2) which raises the possibility that these antibodies might give some protection against re-infection. This contrasts sharply with the situation for MOMP where children with the highest immunoblot density scores were 13.5 times more likely to have active trachoma than those without detectable antibody.

In the trachomatous scarring group 19/22 (86%) of those with scarring and 17/25 (68%) of controls had antibodies to PMPGa. A similar pattern was seen with the infertile women where 12/20 (60%) of the infertile women but only 4/15 (27%) of the controls had anti-PMPGa antibodies, a significant difference between the infertile women and the controls ($p < 0.05$).

Antibodies to the recombinant half of PMPG which includes the carboxyl terminus, PMPGc, were also found in natural infection though at a lower prevalence and a lower level than PMPGa. In the active trachoma group 12/44 (27%) of children with active trachoma and 7/18 (39%) of controls had antibodies to PMPGc (p = N.S.). Again the prevalence of antibodies is higher in those without active disease but there was no direct

relationship between the immunoblot density and likelihood of acute trachoma as was seen for PMPGa. Antibodies to PMPGc were more prevalent in individuals with trachomatous scarring than in controls, 14/22 (64%) vs. 9/25 (36%) ($p < 0.06$) and individuals with trachomatous scarring demonstrated higher immunoblot density scores to PMPGc than the controls, 1.24 vs. 0.64 ($p < 0.05$). Increasing immunoblot density scores were associated with a corresponding increase in the likelihood of being scarred. Those with responses in the highest quartile were 4.5 times more likely to be scarred than those with undetectable antibody ($p < 0.02$)

Working with a PMP protein from *Chlamydia psittaci*, Longbottom et al demonstrated that although the carboxyl fragment was capable of generating antibody responses, such antibodies were not generated during natural infection in sheep.[159] Immuno-staining also suggests that the carboxyl terminus is not surface exposed.[163] The carboxyl-terminal recombinant PMPG fragment used for this study also included a significant portion of the central region of the PMPG protein which, on examination of predicted trans-membrane regions, is likely to be surface exposed and hence in contact with the immune system effectors, and this may be responsible for its recognition by immune responses in this study. Additionally both the prevalence and the immunoblot density scores were lower for PMPGc than for PMPGa.

5.1.2 *PMPG stimulates cell-mediated responses and PMPGa stimulates higher levels of TNFα and IL-10 in children with active trachoma than controls, and lower levels of IL-10 in scarred individuals.*

Both PMPGa and PMPGc are potent stimulators of TNFα and IL-10 production in the whole blood assay. PMPGa, but not PMPGc, also stimulates the production of IFNγ and TGFβ but to a lesser degree (Table 5-1).

PMPGa is a particularly potent stimulator of TNFα production. Wells stimulated with PMPGa generated between 24 and 127 times more TNFα than the negative control wells. Children with active trachoma also appear to generate higher TNFα levels in response to PMPGa than their controls (463 vs. 235 pg/ml, $p < 0.06$). PMPGc also stimulates TNFα production but at a very much lower level, approximately two to seven times that of the negative control wells.

		No antigen	pmpGa	p <	pmpGc	p <
TNF						
Active	**Case**	7	463	0.00005	23	0.005
	Control	10	235	0.0002	-	-
Scarred	**Case**	5	322	0.0002	10	0.02
	Control	2	254	0.003	10	0.003
Infertile	**Case**	3	296	0.0004	62	0.06
	Control	5	323	0.0001	35	0.0004
IL-10						
Active	**Case**	19	161	0.00005	58	0.0007
	Control	16	92	0.0001	30	0.001
Scarred	**Case**	11	51	0.00005	14	0.02
	Control	15	79	0.0001	31	0.004
Infertile	**Case**	5	57	0.00005	22	0.003
	Control	9	67	0.00005	30	0.004
IFN						
Active	**Case**	8	15	0.0002	-	-
	Control	10	18	0.01	-	-
Scarred	**Case**	12	24	0.006	17	0.01
	Control	15	33	0.01	-	-
Infertile	**Case**	8	37	0.00005	-	-
	Control	10	26	0.02	-	-
TGF						
Active	**Case**	21	102	0.04	-	-
	Control	39	88	0.07	-	-
Scarred	**Case**	37	144	0.11	-	-
	Control	-	-	-	-	-
Infertile	**Case**	13	36	0.04	-	-
	Control	35	73	0.03	-	-

Table 5-1: Stimulation of TNFα, IL-10, IFNγ and TGFβ by PMPG recombinant proteins

The generation of IL-10 is also stimulated by both PMPGa and PMPGc and here again IL-10 levels are significantly higher after stimulation with PMPGa (5 - 11 times higher than the negative controls) than they are with PMPGc (1.4 - 4.4 times higher). Blood from children with active trachoma is again more responsive to stimulation with PMPGa than their controls (161 vs. 92 pg/ml, $p < 0.02$) and a similar trend is seen for PMPGc (58 vs. 30 pg/ml, $p < 0.09$). In scarred individuals there was a trend towards less IL-10 production in response to PMPGc (14 vs. 31 pg/ml, $p < 0.08$), and a similar pattern was seen for PMPGa though this was not significant (51 vs. 79 pg/ml, $p < 0.21$).

PMPGa also stimulated the generation of IFNγ at levels between 1.8 and 4.6 times that of the negative control wells. Scarred individuals produced slightly elevated levels of IFNγ in response to PMPGc but this was not seen in their controls or in any other groups. PMPGa also stimulated TGFβ production in all groups at levels approximately two to five times that of the negative controls and stimulated IL-2 production at low levels in the scarred individuals and their controls.

Confidence that these results are due to the recombinant proteins themselves and not due to contaminants is provided by the differences between the responses to PMPGa and PMPGc and also by the fact that for all the assays there was no significant difference between the responses to the recombinant protein preparation using the PET vector without an insert and the negative control.

The only published work in relation to cell-mediated responses to the PMP proteins demonstrated that CD_4 T-cells, from individuals with *Chlamydia*-associated reactive arthritis, proliferated in response to recombinant PMPD. The results of this current study clearly indicate that both the amino and carboxyl-terminal halves of PMPG provoke cell-mediated responses, particularly TNFα and IL-10 production, that children with active trachoma produce more IL-10 in response to PMPGa than controls and that scarred individuals appear to produce less IL-10 than controls in response to PMPGc and possibly PMPGa. The IL-10 responses are discussed further in section 6.1.10

As discussed in section 1.3.4 TNFα is known to be produced in the conjunctivae and the reproductive tract during chlamydial infection.[100,103] Blocking TNFα can lead to an increase in organism load in murine pneumonia,[104] but has little or no effect in reproductive tract infection of mice or guinea pigs.[107] This study demonstrates that PMPG stimulates TNFα production and moreover, that children with active trachoma produce more TNFα than their controls in response to the amino-terminal half of the protein. Although certain TNFα polymorphisms have been shown to be associated with scarring in trachoma,[102] this study did not demonstrate any differences between those with scarring and their controls with respect to the levels of TNFα produced in response to PMPG or any other antigen.

5.1.3 Anti-MOMP IgG antibodies are associated with active trachoma and with tubal infertility

The children with active trachoma demonstrated a higher prevalence of detectable anti-MOMP IgG than their controls, 43/44 (98%) vs. 14/19 (74%) ($p < 0.003$). The mean immunoblot score was higher for children with active trachoma than for controls 2.08 vs. 1.33 ($p < 0.0003$), and there was a clear relationship between the immunoblot density score and the likelihood of having active disease; children with the immunoblot density scores in the highest quartile were 13.5 times more likely to have active disease than those without detectable antibody ($p < 0.01$).

A similar pattern was seen in women with tubal infertility where 20/20 infertile women and 13/15 (87%) of controls had detectable antibodies to MOMP ($p < 0.09$). The infertile women also demonstrated stronger antibody responses than their controls, 2.5 vs. 1.75 ($p < 0.005$).

In a study of Nepalese children with trachoma, 22/40 (55%) of children with active disease had anti-MOMP IgG, a figure not significantly different from that seen in controls.[141] Similar figures were seen in patients with either urethral or cervical *Chlamydia trachomatis* infection where 26/45 (58%) of the cases and 10/31 (32%) of the controls had antibody to MOMP.[245] In the current study the prevalence of antibodies to MOMP is higher in both cases and controls and this may reflect the higher levels of trachoma transmission in this community.

Although neutralising antibodies to MOMP can be clearly demonstrated in vitro[117-119], serum antibodies very often do not provide protection in vivo[127] as appears to be the case here.

5.1.4 MOMP stimulates cell-mediated production of IFNγ and TNFα but this is not associated with pathology

MOMP was shown to act as a stimulant to IFNγ production in the whole blood assay with approximately four-fold increases in IFNγ concentration (39 vs. 10 pg/ml overall, $p < 0.00005$). Significant production of IFNγ in response to MOMP was seen for all the children with active infection and their controls and for the scarred adults and infertile women but not for the controls in either group.

T-cells isolated from cord-blood are known to proliferate and produce IFNγ in response to MOMP[246] and IFNγ is produced by PBMCs from women 'at risk of chlamydial PID'

on incubation with MOMP.[247] Working with patients with trachomatous scarring, Holland et al demonstrated higher IFNγ production by PBMCs of controls on incubation with MOMP than those from cases.[248] In the present study MOMP is shown to be capable of stimulating IFNγ production but there are no significant differences in response between those with long-term sequelae of chlamydial infection and controls.

TNFα production in response to MOMP was higher than that seen in the negative control wells in all groups with mean concentrations of 48 vs. 6 pg/ml ($p < 0.00005$). No published work has addressed the question of whether MOMP stimulates the production of TNFα, this study demonstrates that it does but the level of response does not correlate with the presence of acute infection or long-term sequelae.

5.1.5 Anti-OMP2 IgG antibodies are detectable in the majority of individuals but are not associated with pathology

IgG antibodies to OMP2 were highly prevalent in all groups. Ninety-one percent (31/34) of children with active trachoma had detectable anti-OMP2 antibodies vs. 16/19 (84%) of controls. A slightly higher prevalence of anti-OMP2 antibodies was also noted amongst the infertile women, 5/6 (83%) vs. 6/8 (75%), but in neither case was the difference significant, perhaps due to the low numbers. All of the individuals with trachomatous scarring and their controls had anti-OMP2 antibodies.

These findings are in keeping with other studies which have demonstrated that anti-OMP2 antibodies are almost always present after chlamydial infection. All of 17 patients with *Chlamydia trachomatis* urethritis,[133] 89% of individuals with urethritis or cervicitis[116] and almost all women with either PID or ectopic pregnancy[132] have been demonstrated to have anti-OMP2 antibodies, as have 43% of 'healthy' Swiss blood donors.[116] It has even been suggested as a marker for *Chlamydia pneumoniae* infection in patients with coronary heart disease.[249]

Despite the high prevalence, there have been no suggestions that immune responses to OMP2 are associated with pathology other than the observation of a higher intensity of binding to OMP2 of IgG from the synovial fluid of patients with *Chlamydia trachomatis*-associated reactive arthritis.[250] This study confirms the high prevalence of antibodies to OMP2 and shows no association with pathology.

5.1.6 OMP2 stimulates the production of TNFα, IFNγ and IL-10. TNFα responses are higher in those with active trachoma than controls

This study demonstrates that OMP2 is a potent stimulant of TNFα production. This was true for each group and for all individuals the mean TNFα concentration after incubation with OMP2 was 2152 pg/ml compared to 6 pg/ml in the negative control wells. Children with active trachoma generated higher levels of TNFα in response to OMP2 than their controls (2726 vs. 1455, $p < 0.01$). There was no difference in response in those with long-term sequelae.

IFNγ was also produced in response to OMP2. For the study population as a whole the mean IFNγ concentration was 169 pg/ml after incubation with OMP2 compared to 10 pg/ml for the negative controls ($p < 0.00005$). This was true of all study groups but there were no significant differences between cases and controls in any group. It should be remembered that most if not all individuals, both cases and controls, will have been exposed to *Chlamydia* though ocular infection. The only exceptions to this are likely to be a few of the younger controls in the active infection study arm.

OMP2 also stimulated the production of IL-10 in all groups, with a mean concentration of 374 pg/ml compared to 13 pg/ml for the negative controls ($p < 0.00005$). Again there were no differences between cases and controls.

T-cells responsive to OMP2 have been isolated from individuals with *Chlamydia*-associated reactive arthritis and with chlamydial urethritis[137] and also from atheromatous plaques,[251] but results of cellular cytokine responses to OMP2 have not been published.

Both OMP2 and HSP60 were generously donated by another laboratory and therefore control antigens, similar to the 'PET-no antigen' control used in this study, were not available. The possibility of contaminants, notably lipopolysaccharide (LPS), affecting the results must therefore be considered. The supplier of these recombinant proteins has addressed this issue and estimates the LPS contamination to be at very low levels (< 0.2 endotoxin units/μg protein).[251] Correlations between responses to OMP2 and HSP60 are quite high in several cases; 0.38 for IFNγ, 0.89 for IL-10 and 0.69 for IL-2 and this raises concerns that the responses may be due to contaminants common to both recombinant proteins. However, these levels of correlation are not significantly different from the correlation between responses to these antigens and to EBs. For the examples quoted above corresponding correlation coefficients between responses to HSP60 and

EB's are 0.41 for IFNγ, 0.81 for IL-10 and 0.74 for IL-2 and for responses to OMP2 and EB's the corresponding coefficients are 0.59 for IFNγ, 0.82 for IL-10 and 0.89 for IL-2. Similar figures are seen in correlations with responses to MOMP. It seems likely therefore that the responses seen to OMP2 and HSP60 are made to the recombinant proteins themselves and not to any contaminants.

5.1.7 Antibodies to HSP60 are detectable in most individuals but are not associated with pathology

Almost all individuals studied had detectable IgG antibodies to chlamydial HSP60. Of the children with active trachoma 43/44 (98%) had anti-HSP60 antibodies as did 17/19 (89%) of controls. Similarly high levels were seen in adults with scarring (22/22) and their controls (24/25 [96%]), and in the infertile women (18/20 [90%]) and their controls (13/15 [87%]).

The mean immunoblot density scores for the infertile women are slightly higher than for their controls, 2.40 vs. 2.03 ($p < 0.07$) and this trend is supported by the multivariate analysis ($p < 0.06$) although a straight case-control conditional logistic regression analysis finds the difference not to be significant.

These findings are at odds with other studies which show a clear relationship between antibodies to HSP60 and pathology. Previous work by Peeling et al in The Gambia showed that 47/148 (32%) individuals with trachomatous scarring had antibodies to HSP60 compared to 24/148 (16%) of controls ($p < 0.001$). Similar associations have been shown for infertility; two separate studies demonstrated anti-chlamydial HSP60 antibodies in 79% and 59% of women with tubal infertility compared to 23% and 16% of controls respectively.[209,210] The levels of anti-HSP60 antibodies in controls in this study are higher than in most other published work and such a high background level would make the detection of a difference between cases and control difficult. The Peeling study used an ELISA method to determine anti-HSP60 antibodies and thus the sensitivity may have been less than this current study depending on how the cut-offs were determined. Another important difference between the current study and the early study by Peeling et al is in the number of individuals investigated. The current study only investigated 25 patients with scarring and an equal number of controls compared to 148 in each group in the Peeling study. Sample size calculations, taking into account the mean values for cases and controls reveals that 83 individuals in each group would have been enough to demonstrate a statistically significant difference in the current study.

Thus, if more individuals had been investigated this study may have confirmed the association between HSP60 and scarring and/or infertility.

5.1.8 *HSP60 stimulates the production of TNFα, IFNγ and IL-10. TNFα and IL-10 responses are higher in children with active trachoma than in controls*

Production of TNFα, IFNγ and IL-10 were all seen to be increased after incubation with HSP60. TNFα production was the most marked with mean levels in HSP60-stimulated wells being 912 pg/ml compared to 6 pg/ml in the negative control wells ($p < 0.00005$). Children with active trachoma also produced higher levels of TNFα in response to HSP60 than did their controls (1188 vs. 731 pg/ml, $p < 0.02$).

IFNγ production was also increased after incubation with HSP60. Mean IFNγ concentration after incubation with HSP60 was 96 pg/ml compared to 10 pg/ml in the negative control wells ($p < 0.00005$). There were no differences between cases and controls in any of the groups.

IL-10 concentrations in response to HSP60 were also increased, 296 vs. 14 ($p < 0.00005$) and were also higher in children with active trachoma than in controls (469 vs. 295, $p < 0.002$).

A variety of bacterial heat shock proteins have been shown to induce the production of TNFα, IFNγ, IL-1 and IL-10.[145,252,253] Debattista has previously demonstrated reduced IFNγ responses to chlamydial HSP60 in women with PID or multiple previous chlamydial infections.[254] In their study there was no difference in IL-10 responses to HSP60 in any of the groups. In another study however, T-cells isolated from salpingeal tissues of women with tubal factor infertility responded to chlamydial HSP60 by producing predominantly IL-10.[255] Taken together these might suggest a switch from a T_H1-type to a T_H2-type response to chlamydial HSP60 in women with chronic chlamydial infection of the reproductive tract. However, in mice T-cells isolated from animals immunized with chlamydial HSP60 produce less IL-10 and more IFNγ than those immunized with mouse HSP60.[256] In a study of a small number of patients with *Chlamydia*-associated reactive arthritis, T-cells isolated from the synovium secreted primarily IL-10 in response to MOMP and chlamydial HSP60.[257]

In the present study no differences were noted in IFNγ or IL-10 responses in the infertile women although the IFNγ:IL-10 ratio was higher (i.e. more T_H1-like) in infertile

women than in their controls (1.34 vs. 0.75, $p < 0.07$). Higher IL-10 as well as TNFα responses were seen in children with active trachoma. This has not been documented previously.

5.1.9 Secretion of TNFα in response to both common recall and *Chlamydia*-specific antigens is increased during active trachoma

Children with active trachoma demonstrated higher TNFα production in the whole blood assay in response to PHA, *Candida*, EBs, OMP2, HSP60 and PMPGa with cases generating between 1.5 and 2 times the amount of TNFα as controls. Higher TNFα concentrations in the assay were much more strongly associated with the presence of trachoma; children generating TNFα levels in the top quartile in response to OMP2 were 14.4 times more likely to have active trachoma than those in the lowest quartile ($p < 0.004$). Similar associations were seen for HSP60 (3.75, $p < 0.03$), PMPGa (5.33, $p < 0.05$) and for the common recall antigen *Candida* (5.33, $p < 0.03$).

TNFα has been clearly demonstrated to be important in the successful response to chlamydial infections in general (reviewed in 1.3.4). In trachoma, increased levels of TNFα have been found in the tears[102] and conjunctivae[103] of individuals with acute trachoma and certain polymorphisms of the TNFα-promoter are also associated with scarring.[102] In this study no significant differences were noted between those with trachomatous scarring or infertile women and their controls. This study does however demonstrate an increase in either the number, or the TNFα producing ability or both, of circulating immune cells in response to *Chlamydia*-specific and common recall antigens and mitogen in children with active trachoma.

5.1.10 Secretion of IL-10 in response to both common recall and *Chlamydia*-specific antigens is increased in active trachoma

Children with active trachoma produced higher levels of IL-10 in the whole blood assay than their controls for a variety of common recall as well as *Chlamydia*-specific antigens: *Candida* 153 vs. 98 pg/ml ($p < 0.007$); PPD 133 vs. 101 pg/ml ($p < 0.04$); EBs 321 vs. 251 pg/ml ($p < 0.05$); OMP2 588 vs. 124 pg/ml (0.02); HSP60 469 vs. 295 pg/ml ($p < 0.002$); PMPGa 161 vs. 92 pg/ml (0.02) and PMPGc 58 vs. 30 pg/ml ($p < 0.09$).

IL-10 is known to be secreted in response to chlamydial infection. IL-10 mRNA was detected in primary[258] and secondary[57] reproductive tract infection in macaques and is

more common in endocervical samples from women with chlamydial PID than in controls.[259] High levels of IL-10 are also produced by synovial fluid mononuclear cells from patients with *Chlamydia*-associated reactive arthritis in response to chlamydial antigens,[257] and peripheral blood mononuclear cells from anonymous blood donors also produce high levels of IL-10 in response to *Chlamydia pneumoniae* EBs.[260] Interestingly in this latter study low doses of EBs generated predominantly IL-12 responses whilst high levels of EB's produced predominantly IL-10 responses.

IL-10 is a T_H2-type cytokine which down-regulates T_H1-type responses.[261] In contrast to the situation in mice, where it is produced only by T_H2 cells, in humans it is produced by both T_H1 and T_H2 cells and appears to down-regulate both types of response.[262] In keeping with this, an immunohistochemical study of genital chlamydial infection in mice showed that maximal IL-10 expression was seen at three weeks post-infection when the infection was nearing resolution, whilst pro-inflammatory cytokines such as IL-12 and TNFα were seen in the earlier stages.[87]

Experimental evidence suggests that strong IL-10 responses to chlamydial infection benefit the organism but not the host. BALB/c mice demonstrate higher IL-10 and lower IFNγ responses to chlamydial infection and this is associated with reduced clearance of *Chlamydia* and a worse prognosis. Treatment of the animals with anti-IL-10 monoclonal antibodies results in increased bacterial clearance and improved survival.[89] IL-10 knockout mice clear chlamydial infection much more rapidly, have higher IFNγ responses and are more resistant to re-infection,[263] but these improvements can be abrogated by the administration of exogenous IL-10.[264] In humans, PBMCs from women with tubal factor infertility demonstrated higher levels of IL-10 secretion in response to chlamydial EBs and chlamydial HSP60 than did controls, and increased responsiveness to chlamydial HSP60 was associated with the IL-10-1082 gene promoter polymorphism[265] which is associated with increased IL-10 production.[266] Associations between this polymorphism and scarring trachoma were looked for in The Gambia and homozygosity for IL-10-1082G was found more commonly in cases than controls amongst the Mandinka but not amongst other ethnic groups.[223] In the current study IL-10 responses to chlamydial antigens amongst Mandinkas were only significantly different between scarred cases and controls with respect to PMPGa (15 pg/ml for cases vs. 31 pg/ml for controls, $p < 0.04$).

It may be that the ability to stimulate IL-10 production is an evolutionary adaptation by *Chlamydia* to enable them to survive within the host. Human mononuclear cells infected

with *Chlamydia pneumoniae* showed high levels of IL-10 secretion and an associated reduction in MHC Class I expression.[77] Since chlamydial antigens are presented to CD_8 cytotoxic T-cells through MHC Class I, this down-regulation would render the infected cells less likely to be killed. In addition to protecting against CTL-mediated killing, stimulation of IL-10 production may also prevent apoptosis. Host cells frequently respond to intracellular bacteria with apoptosis. This has been clearly demonstrated for a number of organisms including *Shigella*,[267] *Listeria*,[268] *Mycobacterium tuberculosis*,[269] and *Salmonella*.[270] Infection with *Chlamydia trachomatis* and with *Chlamydia psittaci* has also been shown to induce apoptosis in macrophages, epithelial cells and activated T-cells.[271-273] Human PBMCs, when infected by *Chlamydia pneumoniae*, respond by secreting IL-10 and become resistant to apoptosis.[274] Inhibition of apoptosis through IL-10 has also been seen in *Mycobacterium tuberculosis* infection.[275] *Chlamydia trachomatis* infection has also been shown to render HeLa cells resistant to apoptosis although the mechanism was not defined.[276] At least in some cases this inhibition appears to be mediated through NF-κB and caspase-3 dependent mechanisms,[277] however the picture remains unclear as IL-10 can also promote apoptosis through a CD95 receptor/ligand pathway.[278] The importance of apoptosis in chlamydial infections remains unclear but it is an area receiving much more attention recently. One recent study suggested that acute infection of alveolar carcinoma cells with *Chlamydia pneumoniae* provoked apoptosis whilst chronic infection rendered the cells resistant to apoptotic stimuli.[279] It may be that *Chlamydia* facilitate the release of EBs from cells early in the course of infection by provoking apoptosis but then maintain chronic infection later by inhibiting the same process. Stimulating the production of high levels of IL-10 would be an effective way to prevent apoptosis and maybe also to provoke it.

5.1.11 Intestinal helminth infection is associated with trachomatous scarring

Examination of faecal samples revealed an association between intestinal helminth infection and conjunctival scarring; 4/24 of those with trachomatous scarring (16.7%) had helminth ova whilst 0/23 controls had ova detected ($p < 0.04$).

The effects of helminth infection on down-regulating T_H1-type responses and up-regulating T_H2-type responses have been clearly demonstrated. Sabin et al demonstrated that individuals previously vaccinated with tetanus toxoid produced relatively large

amounts of IFNγ in response to re-stimulation with tetanus toxoid but that individuals infected with *Schistosoma mansoni* produced low or immeasurable levels of IFNγ on re-stimulation.[280] *Schistosoma mansoni* infection has also been shown to markedly reduce IL-2 and IFNγ responses to sperm whale myoglobin in naïve animals whilst, at the same time, increasing IL-4 responses[281] and in a similar study PPD was shown to induce IFNγ responses in healthy mice but IL-4 and IL-5 responses in mice infected with *Brugia malayi*.[282] In a clinical study in Ethiopia individuals with intestinal helminths were randomised to receive either albendazole (an anti-helminthic) or placebo and were subsequently vaccinated with BCG. Those who had received placebo subsequently had significantly lower levels of T-cell proliferation and IFNγ responses to PPD.[283] One implication of these experiments is that a helminth-infected individual may have impaired cell-mediated responses to other microbial agents.

Since T_H1-type responses are much more effective in clearing chlamydial infection and T_H2-type responses have been shown to be associated with scarring in trachoma, it is plausible that ongoing helminth infection might give rise to a T_H2-type environment and hence predispose to scarring. In this study only the presence of intestinal helminths was investigated. The serous cavity-inhabiting filarial nematode *Mansonella perstans* is present at high prevalence in The Gambia (25-30% in 1981)[243] and there is also focal infection with *Wucheraria bancrofti* (8-21% in 1981 but probably less prevalent now)[243]. These would be expected to provide a higher level of immune stimulation than intestinal helminths as they migrate through soft tissue and their microfilaria live in the bloodstream. Serum is stored on all patients and can be tested for filarial antigens.

5.2 Limitations of this study

5.2.1 'Intense' trachoma is under-represented in this study

In this study 51/52 (98%) of the children with active trachoma or were classified as having follicular trachoma (TF). Only a single case of intense trachoma (TI) was identified. These figures are somewhat lower than for other studies. In Tanzania 100/137 (73%) of children with active trachoma had TF vs. 37/137 (27%) with TI[284] whilst in Nepal 195/210 (93%) had TF vs. 15/210 (7%) with TI.[285]

Grading of trachoma in this study was confirmed by a very experienced field-worker who has been working on trachoma for many years. There is an excellent national eye care programme in The Gambia and most of the villages have taken part in studies of trachoma for many years. Research staff and workers from the eye care programme are frequent visitors to the villages. It is likely therefore that individuals with more severe trachoma would seek treatment thus resulting in a reduction in the prevalence of intense trachoma. A reduction in the proportion of cases with TI is commonly seen as trachoma prevalence falls.

It is thought that children with TI are more likely to go on to develop scarring than children with TF and thus it would have been interesting to compare immune responses between these two groups. Clearly this was not possible with only one child having TI.

5.2.2 Controls for the children with active trachoma may themselves be recovering from or about to develop active trachoma

The natural history of trachoma in an endemic area such as The Gambia is that children will have many episodes of acute trachoma in the first decade or so of their lives then, as they develop immunity, the frequency and duration of acute episodes declines and acute trachoma is uncommon in adulthood. One implication of this is that the children without active trachoma at the time of the study may well have had active trachoma in recent weeks or may be incubating infection, indeed 5/26 (19%) of the controls had *Chlamydia trachomatis* DNA detected in conjunctival swabs by PCR compared to 32/50 (64%) of cases. In recent comparable studies, 110/378 (29%) of Egyptian children with clinically normal eyes had *Chlamydia trachomatis* detected in conjunctival swabs vs. 245/353 (69%) of those with clinically apparent active

trachoma,[286] whilst in Tanzania 31/696 (4%) of children with 'normal' eyes had PCR evidence of *Chlamydia trachomatis* infection vs. 17/63 (27%) of children with TF on examination.[287]

As a result of this somewhat blurred distinction between 'cases' and 'controls' it is necessary to be cautious when drawing conclusions about differences between the two groups. It is reasonable however to assume that those with clinical signs of active trachoma are at the peak of their inflammatory response to infection and this study has demonstrated clear differences in responses to both chlamydial and common recall antigens between the two groups. Perhaps of more significance is the relationship between the responses during acute infection and the subsequent development of scarring as discussed below.

5.2.3 The women with tubal infertility form a heterogenous group and not all infertility will be due to Chlamydia trachomatis infection

The infertile women selected for inclusion in this study had all been investigated for secondary infertility and found to have tubal occlusion on hysterosalpingography. At the current time the aetiology of their infertility is not known. In many instances their tubal infertility is likely to be due to *Chlamydia trachomatis* infection, however other infections and pathologies will be the cause in some. In one group of women with tubal infertility in Egypt, 51.8% had active *Chlamydia trachomatis* infection of the cervix at the time of investigation for their infertility,[288] whilst in a similar group in South Africa 14/40 (35%) had active cervical chlamydial infection.[289] The incidence of previous infection is likely to be very much higher. Brunham et al found that 13/18 (72%) of American women with tubal infertility had serological evidence of previous chlamydial infection[211], a similar study in Turkey found evidence of prior chlamydial infection in 103/185 (56%),[290] and 65% of Australian women with tubal infertility had positive serological tests for *Chlamydia*.[291] Elsewhere in Africa 104/135 (77%) of women with tubal infertility in Zimbabwe had antibodies to *Chlamydia trachomatis*, 52/135 (39%) at high level[292] and even higher levels were found in South Africa where 87% of women with tubal pathology and 38% of pregnant women had antibodies to *Chlamydia trachomatis*.[293] It seems reasonable therefore to speculate that at least 50% of the infertile women in our study would have had previous reproductive tract chlamydial infection, indeed a previous serological study of women with secondary tubal infertility in The Gambia demonstrated that 17/24 (71%) had antibodies to *Chlamydia*

trachomatis.[294]. The infertile women in this study have been part of a larger study of sexual health in The Gambia.[295] In this study the women as a whole (not just the infertile women), were shown to have *Chlamydia trachomatis* prevalence of 1.2% by PCR of DNA extracted from cervical swabs. However the sensitivity of the PCR technique used was subsequently questioned (West, B. Personal communication) and therefore it is difficult to know how much reliance can be placed upon this figure.

Additionally, these women are all living in an area endemic for trachoma. It is likely therefore that most of the women will have been exposed to trachoma during their lives and some of the responses may be due to this exposure to *Chlamydia* rather than to urogenital exposure. However, both the infertile women and their controls were selected from the same villages and it is therefore likely that they will have had the same exposure to non-urogenital *Chlamydia*.

5.2.4 Are antibodies in the serum representative of antibodies in the mucosae?

The lacrimal glands, conjunctivae and accessory glands secrete immunoglobulins which are predominantly of the s-IgA type but also include IgG and IgM.[296] The pattern of locally produced antibodies found in tears might be expected to differ from that of serum immunoglobulins. This certainly appears to be the case for s-IgA; *Chlamydia*-specific s-IgA was detected in the tears of 8/8 patients with chlamydial conjunctivitis but not in the serum of any.[297] Interestingly anti-chlamydial tear IgA was also found in 20/50 patients with chlamydial urethritis and no exposure to ocular trachoma,[298] though tear IgG is found only in patients with ocular chlamydial infection.[299] Serum IgG does however appear to be more representative of the local mucosal situation. In the non-human primate IgG directed against specific chlamydial antibodies was found in both tears and serum, and serum IgG antibodies were directed at the same antigens as tear IgA.[300] In men with chlamydial urethritis *Chlamydia*-specific IgG and IgA were detected in urethral swabs[39] and *Chlamydia*-specific IgG is also found in the sera of men with urethritis.[116] The detection antibody used for immunoblotting in this study detected all IgG subtypes but not IgM or IgA.

5.2.5 Is cytokine production in blood a reliable indicator of cytokine production in mucosal surfaces?

One of the important questions in studies of immunity to mucosal pathogens is whether antigen-specific T-cells found in the peripheral blood are reliable indicators of the presence of such T-cells at infected mucosal sites and vice versa. Most of the work undertaken using the whole blood assay method has been with more systemic infections such as tuberculosis and HIV and the validity of the method is well established under these conditions. A review of the literature did not reveal any studies using this methodology to investigate more localized or mucosal infections and this study is the first to apply the technique to chlamydial infection.

Studies of lymphocyte proliferation and the production of cytokines by separated peripheral blood mononuclear cells (PBMCs) have been undertaken previously in the context of chlamydial infection. These have demonstrated: reduced lymphocyte proliferation in response to chlamydial antigens in individuals with trachomatous scarring;[301] proliferation in response to chlamydial HSP60 in women with chlamydial cervicitis;[302] and proliferation in response to MOMP in patients with various chlamydial infections.[303] *Chlamydia*-reactive T-cells have been isolated from atheromatous plaques[251] and the synovium of patients with *Chlamydia*-associated arthritis[304] and in animal studies genital tract T-cells have been shown to produce high levels of TNFα[100] and IFNγ,[90] but the responses of both PBMCs and local mononuclear cells have never been directly compared. It would be expected that investigation of the infected mucosa would provide the most accurate information however this may not always be the case, one immuno-histological study of conjunctival biopsies in individuals with trachoma demonstrated the expression of IL-1, TNFα and platelet-derived growth factor by conjunctival mononuclear cells but no expression of IFNγ[305] despite the fact that IFNγ has been shown to be of major importance in the immune response to *Chlamydia* (reviewed in 2.3.4).

In the absence of any definitive study comparing responses in mucosal sites with those of PBMCs or in the whole blood assay it is necessary to compare the results of such assays with experimental data from all available studies in order to determine the likely validity of the new data.

5.2.6 The TGFβ assay is unsuited to a whole blood assay method

The TGFβ ELISA results showed high levels of non-specific activation unrelated to the antigen being used for stimulation and including the negative control wells. These high levels of TGFβ production were unrelated to clinical status and independent of all the other variables measured. As a consequence the results of 41/106 individuals had to be excluded from analysis, and of those that were used for analysis quite a number of suspiciously high values remained rendering any conclusions unreliable. TGFβ is produced by many cells including activated T-cells and macrophages, platelets, tumour cells, osteoblasts and osteoclasts[306,307] and high levels of non-specific activation have been seen by other workers using the whole blood assay, leading them to conclude that these high levels probably result from platelet activation and that the whole blood assay is an inappropriate methodology for investigating TGFβ responses (Omer, F.M. personal communication).[308]

5.2.7 The cytokine ELISA used may not have been sensitive enough for the IL-2 and IL-5 concentrations generated

Statistical comparison of the IL-5 responses to the common recall and *Chlamydia*-specific antigens with those of the negative control wells demonstrates that none of these antigens stimulated an IL-5 response significantly higher than that seen in the negative control. The results were better for IL-2, for example mean IL-2 concentration after incubation with PPD was 87 pg/ml vs. 7 pgμl for the negative control ($p < 0.00005$), corresponding figures for *Candida* were 13 vs. 7 pg/ml ($p < 0.00005$).

Inspection of the raw results shows that for the IL-5 assay a very significant proportion of the results were below the lowest reliably accurate optical standard (8 pg/ml). This proportion varied from 71/166 (43%) for PPD to 127/166 (77%) for MOMP. In all 734/1162 (63%) were below the minimum reliable level of accurate measurement. All of the responses to the mitogen PHA were well above this level.

The IL-2 results were better. Results below the minimum level for accurate measurement (also 8 pg/ml) varied from 3/172 (2%) for PPD to 115/172 (67%) for OMP2. Overall 530/1204 (44%) were below this lowest accurate standard.

Whilst it is perfectly plausible that many of the samples may generate low levels of cytokine in response to stimulation, or even not respond at all, it is slightly worrying that only PHA stimulated a detectable response in the IL-5 assays and it may be that this

assay simply was not sensitive enough. Clearly no differences detected with this assay can be relied upon. The IL-2 ELISA is clearly also lacking in sensitivity for the levels of IL-2 generated in many of the assays, however the significant levels of IL-2 measured in response to the common recall antigens *Candida* and PPD suggest that the results generated are likely to be reliable.

5.2.8 The number of individuals studied did not always provide adequate power to detect differences between cases and controls?

At the initial planning stage a number of decisions were made in order to decide the number of individuals to be investigated in each group. The numbers chosen provided 80% power to detect a 50% difference in measurement between cases and controls for the infertile women, a 40% increase for the scarred individuals and a 35% increase for children with active trachoma. The studies subsequently demonstrated a variety of differences between the children with active trachoma and their controls and rather less for the scarred individuals and the infertile women. Some of that may be explained by the higher power provided by the larger numbers in the active trachoma group. Using sample size calculations, the figures (Table 5-2) show the number of cases required to demonstrate a significant difference between cases and controls using the means and standard deviations from this study. An equal number of controls would also be required. Boxes highlighted in red are those for which significant differences between cases and controls ($p < 0.05$) were demonstrated in this study and those highlighted in yellow are those for which a trend ($p < 0.1$) was demonstrated.

In most instances for the scarred individuals and infertile women, the number of cases and controls required to demonstrate differences are too large to make such a study feasible, for example in a resource-poor country such as The Gambia it would be difficult to recruit a hundred or more women with secondary infertility and tubal blockage confirmed by hysterosalpingography. Such a study may be feasible in a developed country setting however.

	Immuno-blot	TNF	IFN	IL-2	IL-10
Active trachoma					
EB	-	62	38	122,827	50
MOMP	16	494	130	276	236
omp2	4030	21	547	5820	40
hsp60	1123	38	147	233	16
pmpGa	81	51	1142	105,343	30
pmpGc	398	1563	124	46	65
Scarred trachoma					
EB	-	223	673	75	29
MOMP	273	11,517	716	63	2139
omp2	604	146	2313	143	186
hsp60	666	489	880	93	1042
pmpGa	144	524	375	418	60
pmpGc	46	52,895	1920	85	32
Infertile women					
EB	-	959	22	1925	1676
MOMP	16	1490	74	150	215
omp2	96	1958	62	248	576
hsp60	83	1741	195	560	569
pmpGa	86	2080	106	141	342
pmpGc	96	219	2319	321	191

Table 5-2: Number of cases required to give adequate power to determine significant differences between cases and controls

5.3 Conclusions and implications

The molecular biology methods and tools used in this work enabled the production of two recombinant fragments of PMPG enabling separate study of immune responses to the amino and carboxyl halves. The recombinant proteins were produced in ample quantity and high purity and there was no evidence of unwanted effects from contaminants in the assays used for the field work. Restriction fragment length polymorphism analysis of the *pmp* genes demonstrated only minor differences between the *pmp* genes from a clinical isolate of *Chlamydia trachomatis* serovar A from the Gambia used to generate the recombinant PMPG, and the reference strain (Serovar D (D/UW-3/Cx), trachoma biovar) used for sequencing in the *Chlamydia* Genome Project.

Field studies in The Gambia confirmed that antibodies to PMPG are generated during natural trachoma infection and that these are more commonly directed towards the amino-terminal fragment (PMPGa) than to the carboxyl-terminal half (PMPGc) supporting animal studies and *in vitro* work which suggests that the amino-terminal is exposed on the surface of the organism whilst the carboxyl-terminal is located within the membrane or cytosol. Antibodies to PMPGa were found more commonly, and at a higher titre, in children without active trachoma suggesting that they may confer some protection. This clearly requires further investigation as, if confirmed, this may be a useful component of a future vaccine. Conversely antibodies to PMPGc were more prevalent and found at a higher titre in those with trachomatous scarring.

PMPG was also able to stimulate the production of TNFα, IFNγ, IL-10 and TGFβ in the whole blood assay and again the amino-terminal fragment was much more active than the carboxyl-terminal fragment. Children with active trachoma produced significantly higher levels of TNFα and IL-10 than their controls in response to PMPGa whilst scarred individuals produced less IL-10 than controls.

Antibodies to MOMP were seen at a higher prevalence and titre in children with active trachoma suggesting that serum anti-MOMP antibodies do not provide good protection, perhaps due to strain variation in MOMP. A higher prevalence and titre of antibodies to MOMP was also seen in infertile women and may reflect recurrent or prolonged infection. In the whole blood assay MOMP stimulated the production of both IFNγ and TNFα but there was no association with pathology.

OMP2 was shown to be highly immunogenic, confirming the findings of other published studies. Antibodies to OMP2 where highly prevalent in all groups but were not associated with pathology. OMP2 also stimulated the production of TNFα, IFNγ and IL-10 in the whole blood assay and TNFα responses were higher in children with active trachoma than in their controls, but no differences were seen in those with long-term sequelae.

Similarly antibodies to HSP60 were highly prevalent in all groups but this study failed to demonstrate any association with pathology, a finding contrary to much previously published work. This is probably due to the sample size being inadequate to detect a significant difference and the higher sensitivity of this assay compared to the previously used ELISA methods causing a high background level rather than due to a lack of real difference. HSP60 was also shown to be capable of stimulating TNFα, IFNγ and IL-10 in the whole blood assay. Both TNFα and IL-10 concentrations were higher in children with active trachoma than in their controls but no differences were found in the groups with long-term sequelae of infection.

High levels of IL-10 were noted in response to both common recall antigens and all chlamydial antigens except MOMP, in children with active trachoma. Though normally associated with resolving inflammation, high levels of IL-10 secretion are also promoted by a variety of intracellular pathogens in order to subvert host responses by down-regulating MHC expression and inhibiting apoptosis. Increased production of IL-10 on infection with *Chlamydia* has been noted previously, as has the ability of *Chlamydia* to inhibit apoptosis. Clearly further work is required to tease out the details of this relationship.

One further fascinating, and previously undescribed, finding was that of an association between intestinal helminth infection and scarring trachoma. Ongoing helminth infection is known to predispose to a T_H2-type response to other microbial pathogens and trachomatous scarring is itself known to be associated with T_H2-type responses to chlamydial antigens. Only intestinal helminth infection was investigated in this study and it is likely that many of the individuals would have also been infected with *Mansonella perstans* which may also have an effect. One implication of this observation is that regular de-worming of children in a trachoma-endemic area might reduce the incidence of scarring as well as that of anaemia and malnutrition. As evidence increases of the detrimental effects of ongoing helminth infection on other diseases such as HIV,

TB and leishmaniasis it is important that these associations are determined and anti-helminthic medication given where appropriate.

Fewer statistically significant differences were noted in cytokine responses between cases and controls in the trachomatous scarring and infertile women groups than in the active trachoma group. This may reflect an expansion of circulating, *Chlamydia*-reactive, mononuclear cells in children with acute infection whilst those with trachomatous scarring or tubal damage have much lower numbers of circulating memory cells. As a consequence of this the differences between cases and controls are not sufficiently large to be detected using the whole blood assay unless unfeasibly large numbers of individuals are studied. The implication of this finding is that whilst the whole blood assay has been shown to be an effective tool to detect differences in the responses of children with active trachoma, even though the infection is mucosally located and affects only a relatively small amount of tissue, it may not be an appropriate methodology for studying individuals after the acute phase when few *Chlamydia*-specific cells are likely to be in circulation.

One of the most important fundamental questions regarding chlamydial infections is why some individuals develop long-term sequelae whilst others resolve the infection with no long-term consequences. Due to limitations in the sensitivity of the assays and the number of individuals studied, this study has not shed a great deal of light on this question. It has however provided new information on the acute responses to trachoma. Given the blurred distinctions between cases and controls in this population as described above, it is difficult to draw any important conclusions from these findings. However, a review of these individuals in five to ten years time may allow conclusions to be drawn as to the association between acute responses to trachoma and the subsequent development of scarring.

This work has perhaps raised more questions than it has answered and certainly further work is indicated. Potential avenues for future research are described below.

5.4 Unanswered questions and future research

5.4.1 Do antibody or cytokine responses during an episode of active trachoma predict subsequent development of scarring?

Children with active trachoma were shown to demonstrate differences in a variety of humoral and cytokine responses compared to controls. The importance of these is difficult to determine due to the blurring of the division between cases and controls in this population as discussed earlier. The fundamentally important question in chlamydial infection is why some individuals develop scarring of the conjunctivae or fallopian tubes whilst others resolve infection without any long-term sequelae. Most studies attempt to answer that question by looking at the differing responses between those who have developed scarring and those who have not, long after the acute illness has resolved. This study provides an opportunity to revisit these children in five to ten years time to determine which children have developed scarring. It may then be possible to relate their responses during active infection with the subsequent development of long-term sequelae.

5.4.2 Do in vivo cytokine responses correlate with those predicted from cytokine gene and gene-promoter polymorphism analysis?

Previous studies in The Gambia have looked at associations between scarred trachoma and polymorphisms of the TNFα and IL-10 gene promoters.[102,223] Some of the individuals investigated during the course of those studies were also included in this current study. It would be interesting therefore to compare the results of cytokine production in response to common recall and chlamydial antigens with the results of those polymorphism analyses to determine if the phenotypic responses are in keeping with the genotype. As DNA has been isolated and stored from all individuals in this study it will be possible to genotype for other cytokine genes and gene-promoters and correlate these with responses seen in the whole blood assay.

5.4.3 Do antibodies to the amino-terminal fragment of PMPG protect against re-infection?

Antibodies to the amino-terminal fragment of PMPG were found at higher prevalence and higher titre in controls than in children with active trachoma. Those with the highest titres had an odds ratio for having active trachoma of 0.32 when compared to those with

undetectable antibody. These differences were found to be significant at a level of $p < 0.04$ using non-parametric tests and $p < 0.06$ using a t-test and χ^2 for trend, but the significance was reduced to $p < 0.13$ using conditional logistic regression. Hence this finding needs to be confirmed using larger numbers of individuals. Sample size calculations suggest that 81 cases and 81 controls would provide 80% power to detect a significant difference using the means determined from this pilot study. If this finding is confirmed then further studies using *in vitro* neutralisation of infectivity and passive immunisation in animal models would be indicated.

5.4.4 Are antibodies to the carboxyl-terminal of PMPG associated with scarring?

Antibodies to the carboxyl-terminal fragment of PMPG were shown to be associated with the presence of scarring. Those with conjunctival scarring had a higher prevalence of antibodies and these were present at higher titres. Those with the highest antibody titres were 4.5 times more likely to be scarred than those with undetectable antibodies ($p < 0.02$). In general, evidence from the current study supports the conclusions of previous work which suggest that the carboxyl-terminal of the molecule is not surface exposed and thus less antigenic. The recombinant protein used in this study however includes a significant portion of the protein's central region which is predicted to be surface exposed and this may account for it having some antigenicity. Clearly this association with scarring needs further investigation and in the first instance this should be by the production of further, smaller recombinant fragments of PMPG to allow the region responsible for this association to be determined. It would also be interesting to determine if the PMPG protein includes predicted HLA-binding motifs and indeed if PMPG epitopes are presented through MHC.

5.4.5 Does TGFβ have a role in the development of scarring?

TGFβ is produced by a wide variety of cells and has widespread effects as most cells have TGFβ receptors. It plays an important role in the control of immune responses and wound healing. At low levels it stimulates fibroblast proliferation and the deposition of extracellular matrix whilst inhibiting proliferation at higher levels.[306] TGFβ suppresses macrophage function and this is utilised by *Leishmania*, *Trypanosoma cruzi* and *Mycobacterium avium* to suppress macrophage killing and allow intracellular proliferation of the organisms.[309,310] In the context of chlamydial infection, pulmonary

infection of mice is associated with increases in the local expression of TGFβ[311] and human synovial cells infected with *Chlamydia trachomatis* also produce TGFβ,[312] thus *Chlamydiae* may also make use of the immunosuppressive effects of TGFβ. In addition high levels of TGFβ are though to be associated with the development of keloid scarring[313] and therefore TGFβ, produced in response to chlamydial infection, may be important in the development of scarring in trachoma.

As a result of the difficulties associated with the TGFβ assays used in this study it was not possible to make any meaningful comments on the TGFβ responses in relation to active trachoma, trachomatous scarring or infertility but these remain an important area for investigation using alternative methods, perhaps by measuring local mRNA transcripts.

5.4.6 How much does helminth infection contribute to scarring in trachoma?

Intestinal helminth infection was shown to be associated with trachomatous scarring and this may be due to the induction of T_H2-type responses to chlamydial antigens. Although the higher prevalence of intestinal helminth infection was statistically significant, the numbers were small and it would be important to confirm this finding with a larger study. Additionally the effects of *Mansonella perstans* and *Wucheraria bancrofti* were not assessed in this study and may also be important. Sera from all patients are in storage and filarial serology could easily be undertaken on these samples. Any larger scale studies of this association should also include filarial serology. When these children are reviewed in 5 to 10 years time it will be interesting to see if helminth infection detected during this study is associated with subsequent scarring although clearly all individuals found to have helminth infection were given appropriate treatment. The intestinal helminths isolated in this study are all easily treated and may themselves be associated with anaemia and reduced nutrition. It would be useful to know if treatment may also reduce the incidence of the long term sequelae of trachoma.

5.4.7 What role does IL-10 play in chlamydial infection?

High levels of IL-10 production were seen in children with active trachoma. As discussed earlier IL-10 tends to be produced in the later stages of an immune response to infection and is an effective mechanism through which the host terminates the inflammatory response. However IL-10 production seems to occur earlier in the course

of chlamydial infection and may be induced by *Chlamydia* itself in order to evade host responses, perhaps through the inhibition of apoptosis. Further studies of this phenomenon are indicated, initially perhaps by studying temporal changes in mRNA transcripts during infection of cell cultures or through *in vivo* work in animal models.

Bibliography

Bibliography

1. Szeredi L, Schiller I, Sydler T, Guscetti F, Heinen E, Corboz L *et al*. **Intestinal Chlamydia in finishing pigs**. *Vet.Pathol.* 1996;33:369-74.

2. Berger L, Volp K, Mathews S, Speare R, Timms P. **Chlamydia pneumoniae in a free-ranging giant barred frog (Mixophyes iteratus) from Australia**. *J.Clin.Microbiol.* 1999;37:2378-80.

3. Everett KD, Bush RM, Andersen AA. **Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms**. *Int.J.Syst.Bacteriol.* 1999;49 Pt 2:415-40.

4. Schachter J, Stephens RS, Timms P, Kuo C, Bavoil PM, Birkelund S *et al*. **Radical changes to chlamydial taxonomy are not necessary just yet (Letter)**. *Int.J.Syst.Evol.Microbiol.* 2001;51:249.

5. Thylefors B, Negrel AD, Pararajasegaram R, Dadzie KY. **Global data on blindness**. *Bull.World Health Organ* 1995;73:115-21.

6. Dawson, C. R., Jones, B. R., and Tarizzo, M. L. **Guide to trachoma control in programmes for the prevention of blindness.** 1981. Geneva, World Health Organization.

7. Dolin PJ, Faal H, Johnson GJ, Ajewole J, Mohamed AA, Lee PS. **Trachoma in The Gambia**. *Br.J.Ophthalmol.* 1998;82:930-3.

8. Somani J, Bhullar VB, Workowski KA, Farshy CE, Black CM. **Multiple drug-resistant Chlamydia trachomatis associated with clinical treatment failure**. *J.Infect.Dis.* 2000;181:1421-7.

9. Schachter J. **Chlamydial infections** *N.Engl.J.Med.* 1978;298:540-9.

10. Washington AE, Johnson RE, Sanders LL, Jr. **Chlamydia trachomatis infections in the United States. What are they costing us?** *JAMA* 1987;257:2070-2.

11. The NIMH Collaborative HIV/STD Prevention Trial Group, Stamper, P. D, and Quinn, T. C. **International multi-centre evaluation for Chlamydia trachomatis infections in diverse cultural settings**. 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 6-6-2002. p 149-152.

12. Fenton KA, Korovessis C, Johnson AM, McCadden A, McManus S, Wellings K *et al.* **Sexual behaviour in Britain: reported sexually transmitted infections and prevalent genital Chlamydia trachomatis infection**. *Lancet* 2001;358:1851-4.

13. Paavonen J,.Lehtinen M. **Chlamydial pelvic inflammatory disease**. *Hum.Reprod.Update.* 1996;2:519-29.

14. Nikkanen V, Terho P, Punnonen R, Meurman O. **The significance of chlamydial genital infection in male infertility**. *Arch.Androl.* 1980;4:57-61.

15. Gdoura R, Keskes-Ammar L, Bouzid F, Eb F, Hammami A, Orfila J. **Chlamydia trachomatis and male infertility in Tunisia**. *Eur.J.Contracept.Reprod.Health Care* 2001;6:102-7.

16. Saikku P, Wang SP, Kleemola M, Brander E, Rusanen E, Grayston JT. **An epidemic of mild pneumonia due to an unusual strain of Chlamydia psittaci**. *J.Infect.Dis.* 1985;151:832-9.

17. Saikku P. **Epidemiology of Chlamydia pneumoniae in atherosclerosis**. *Am.Heart J.* 1999;138:S500-S503.

18. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH *et al.* **Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction**. *Lancet* 1988;2:983-6.

19. Kuo CC, Grayston JT, Campbell LA, Goo YA, Wissler RW, Benditt EP. **Chlamydia pneumoniae (TWAR) in coronary arteries of young adults (15-34 years old)**. *Proc.Natl.Acad.Sci.U.S.A* 1995;92:6911-4.

20. Campbell LA, O'Brien ER, Cappuccio AL, Kuo CC, Wang SP, Stewart D *et al.* **Detection of Chlamydia pneumoniae TWAR in human coronary atherectomy tissues**. *J.Infect.Dis.* 1995;172:585-8.

21. Moazed TC, Kuo C, Patton DL, Grayston JT, Campbell LA. **Experimental rabbit models of Chlamydia pneumoniae infection**. *Am.J.Pathol.* 1996;148:667-76.

22. Campbell LA,.Kuo C. **Mouse models of Chlamydia pneumoniae infection and atherosclerosis**. *Am.Heart J.* 1999;138:S516-S518.

23. Muhlestein JB, Anderson JL, Hammond EH, Zhao L, Trehan S, Schwobe EP *et al.* **Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model**. *Circulation* 1998;97:633-6.

24. Anderson JL,.Muhlestein JB. **The ACADEMIC study in perspective (Azithromycin in coronary artery disease: elimination of myocardial infection with Chlamydia)**. *J.Infect.Dis.* 2000;181 Suppl 3:S569-S571.

25. Brunham RC, Kimani J, Bwayo J, Maitha G, Maclean I, Yang C *et al.* **The epidemiology of Chlamydia trachomatis within a sexually transmitted diseases core group**. *J.Infect.Dis.* 1996;173:950-6.

26. Mabey DC, Robertson JN, Ward ME. **Detection of Chlamydia trachomatis by enzyme immunoassay in patients with trachoma**. *Lancet* 1987;2:1491-2.

27. Piot, P., van den Hoek, J. A. R., Van Damme, L., and Laga, M. Orfila, J., Byrne, G. I., and Chernesky, M. A. 7-16. 1994. Bologna, Societa Editrice Esculapio. Chlamydial Infections.

28. Buendia AJ, de Oca RM, Navarro JA, Sanchez J, Cuello F, Salinas J. **Role of polymorphonuclear neutrophils in a murine model of Chlamydia psittaci-induced abortion**. *Infect.Immun.* 1999;67:2110-6.

29. de Oca RM, Buendia AJ, Del Rio L, Sanchez J, Salinas J, Navarro JA. **Polymorphonuclear neutrophils are necessary for the recruitment of CD8(+) T cells in the liver in a pregnant mouse model of Chlamydophila abortus (Chlamydia psittaci serotype 1) infection**. *Infect.Immun.* 2000;68:1746-51.

30. Montes dO, Buendia AJ, Sanchez J, Del Rio L, Seva J, Navarro JA *et al.* **Limited role of polymorphonuclear neutrophils in a pregnant mouse model of secondary infection by Chlamydophila abortus (Chlamydia psittaci serotype 1)**. *Microb.Pathog.* 2000;29:319-27.

31. Manor E,.Sarov I. **Fate of Chlamydia trachomatis in human monocytes and monocyte- derived macrophages**. *Infect.Immun.* 1986;54:90-5.

32. Yamaguchi H, Haranaga S, Widen R, Friedman H, Yamamoto Y. **Chlamydia pneumoniae infection induces differentiation of monocytes into macrophages**. *Infect.Immun.* 2002;70:2392-8.

33. Gerard HC, Kohler L, Branigan PJ, Zeidler H, Schumacher HR, Hudson AP. **Viability and gene expression in Chlamydia trachomatis during persistent infection of cultured human monocytes**. *Med.Microbiol.Immunol.(Berl)* 1998;187:115-20.

34. Rothfuchs AG, Gigliotti D, Palmblad K, Andersson U, Wigzell H, Rottenberg ME. **IFN-alpha beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection**. *J.Immunol.* 2001;167:6453-61.

35. Huang J, DeGraves FJ, Lenz SD, Gao D, Feng P, Li D *et al.* **The quantity of nitric oxide released by macrophages regulates Chlamydia-induced disease**. *Proc.Natl.Acad.Sci.U.S.A* 2002;99:3914-9.

36. Vehmaan-Kreula P, Puolakkainen M, Sarvas M, Welgus HG, Kovanen PT. **Chlamydia pneumoniae proteins induce secretion of the 92-kDa gelatinase by human monocyte- derived macrophages**. *Arterioscler.Thromb.Vasc.Biol.* 2001;21:E1-E8.

37. Kaukoranta-Tolvanen SS, Teppo AM, Laitinen K, Saikku P, Linnavuori K, Leinonen M. **Growth of Chlamydia pneumoniae in cultured human peripheral blood mononuclear cells and induction of a cytokine response**. *Microb.Pathog.* 1996;21:215-21.

38. Ghaem-Maghami S, Bailey RL, Mabey DC, Hay PE, Mahdi OS, Joof HM *et al.* **Characterization of B-cell responses to Chlamydia trachomatis antigens in humans with trachoma**. *Infect.Immun.* 1997;65:4958-64.

39. Pate MS, Hedges SR, Sibley DA, Russell MW, Hook EW, III, Mestecky J. **Urethral cytokine and immune responses in Chlamydia trachomatis- infected males**. *Infect.Immun.* 2001;69:7178-81.

40. Brunham RC, Peeling R, Maclean I, McDowell J, Persson K, Osser S. **Postabortal Chlamydia trachomatis salpingitis: correlating risk with antigen-specific serological responses and with neutralization**. *J.Infect.Dis.* 1987;155:749-55.

41. Cui ZD, Tristram D, LaScolea LJ, Kwiatkowski T, Jr., Kopti S, Ogra PL. **Induction of antibody response to Chlamydia trachomatis in the genital tract by oral immunization**. *Infect.Immun.* 1991;59:1465-9.

42. Pal S, Fielder TJ, Peterson EM, de la Maza LM. **Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of Chlamydia trachomatis**. *Infect.Immun.* 1994;62:3354-62.

43. Rank RG,.Batteiger BE. **Protective role of serum antibody in immunity to chlamydial genital infection**. *Infect.Immun.* 1989;57:299-301.

44. Johansson M, Schon K, Ward M, Lycke N. **Studies in knockout mice reveal that anti-chlamydial protection requires TH1 cells producing IFN-gamma: is this true for humans?** *Scand.J.Immunol.* 1997;46:546-52.

45. Johansson M,.Lycke N. **Immunological memory in B-cell-deficient mice conveys long-lasting protection against genital tract infection with Chlamydia trachomatis by rapid recruitment of T cells** . *Immunology* 2001;102:199-208.

46. Su H, Feilzer K, Caldwell HD, Morrison RP. **Chlamydia trachomatis genital tract infection of antibody- deficient gene knockout mice**. *Infect.Immun.* 1997;65:1993-9.

47. Moore T, Ananaba GA, Bolier J, Bowers S, Belay T, Eko FO *et al.* **Fc receptor regulation of protective immunity against Chlamydia trachomatis**. *Immunology* 2002;105:213-21.

48. Johansson M, Ward M, Lycke N. **B-cell-deficient mice develop complete immune protection against genital tract infection with Chlamydia trachomatis**. *Immunology* 1997;92:422-8.

49. Morrison SG,.Morrison RP. **Resolution of secondary Chlamydia trachomatis genital tract infection in immune mice with depletion of both CD4+ and CD8+ T cells.** *Infect.Immun.* 2001;69:2643-9.

50. Bailey RL, Holland MJ, Whittle HC, Mabey DC. **Subjects recovering from human ocular chlamydial infection have enhanced lymphoproliferative responses to chlamydial antigens compared with those of persistently diseased controls.** *Infect.Immun.* 1995;63:389-92.

51. Ortiz L, Demick KP, Petersen JW, Polka M, Rudersdorf RA, Van der Pol B *et al.* **Chlamydia trachomatis major outer membrane protein (MOMP) epitopes that activate HLA class II-restricted T cells from infected humans**. *J.Immunol.* 1996;157:4554-67.

52. Ortiz L, Angevine M, Kim SK, Watkins D, DeMars R. **T-cell epitopes in variable segments of Chlamydia trachomatis major outer membrane protein elicit serovar-specific immune responses in infected humans**. *Infect.Immun.* 2000;68:1719-23.

53. Kim SK, Angevine M, Demick K, Ortiz L, Rudersdorf R, Watkins D *et al.* **Induction of HLA class I-restricted CD8+ CTLs specific for the major outer membrane protein of Chlamydia trachomatis in human genital tract infections**. *J.Immunol.* 1999;162:6855-66.

54. Kim SK, Devine L, Angevine M, DeMars R, Kavathas PB. **Direct detection and magnetic isolation of Chlamydia trachomatis major outer membrane protein-specific CD8+ CTLs with HLA class I tetramers**. *J.Immunol.* 2000;165:7285-92.

55. Levine WC, Pope V, Bhoomkar A, Tambe P, Lewis JS, Zaidi AA *et al.* **Increase in endocervical CD4 lymphocytes among women with nonulcerative sexually transmitted diseases**. *J.Infect.Dis.* 1998;177:167-74.

56. Rank RG, Bowlin AK, Kelly KA. **Characterization of lymphocyte response in the female genital tract during ascending Chlamydial genital infection in the guinea pig model**. *Infect.Immun.* 2000;68:5293-8.

57. Van Voorhis WC, Barrett LK, Sweeney YT, Kuo CC, Patton DL. **Repeated Chlamydia trachomatis infection of Macaca nemestrina fallopian tubes produces a Th1-like cytokine response associated with fibrosis and scarring**. *Infect.Immun.* 1997;65:2175-82.

58. Van Voorhis WC, Barrett LK, Sweeney YT, Kuo CC, Patton DL. **Analysis of lymphocyte phenotype and cytokine activity in the inflammatory infiltrates of the upper genital tract of female macaques infected with Chlamydia trachomatis**. *J.Infect.Dis.* 1996;174:647-50.

59. el Asrar AM, Van den Oord JJ, Geboes K, Missotten L, Emarah MH, Desmet V. **Immunopathology of trachomatous conjunctivitis**. *Br.J.Ophthalmol.* 1989;73:276-82.

60. Morrison RP, Feilzer K, Tumas DB. **Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in Chlamydia trachomatis genital tract infection**. *Infect.Immun.* 1995;63:4661-8.

61. Magee DM, Williams DM, Smith JG, Bleicker CA, Grubbs BG, Schachter J *et al.* **Role of CD8 T cells in primary Chlamydia infection**. *Infect.Immun.* 1995;63:516-21.

62. Su H,.Caldwell HD. **CD4+ T cells play a significant role in adoptive immunity to Chlamydia trachomatis infection of the mouse genital tract**. *Infect.Immun.* 1995;63:3302-8.

63. Starnbach MN, Bevan MJ, Lampe MF. **Protective cytotoxic T lymphocytes are induced during murine infection with Chlamydia trachomatis**. *J.Immunol.* 1994;153:5183-9.

64. Igietseme JU, Magee DM, Williams DM, Rank RG. **Role for CD8+ T cells in antichlamydial immunity defined by Chlamydia-specific T-lymphocyte clones**. *Infect.Immun.* 1994;62:5195-7.

65. Morrison SG, Su H, Caldwell HD, Morrison RP. **Immunity to murine Chlamydia trachomatis genital tract reinfection involves B cells and CD4(+) T cells but not CD8(+) T cells**. *Infect.Immun.* 2000;68:6979-87.

66. Kimani J, Maclean IW, Bwayo JJ, MacDonald K, Oyugi J, Maitha GM *et al.* **Risk factors for Chlamydia trachomatis pelvic inflammatory disease among sex workers in Nairobi, Kenya**. *J.Infect.Dis.* 1996;173:1437-44.

67. Sieper J, Kingsley G, Palacios Boix A, Pitzalis C, Treharne J, Hughes R *et al.* **Synovial T lymphocyte-specific immune response to Chlamydia trachomatis in Reiter's disease**. *Arthritis Rheum.* 1991;34:588-98.

68. Holland MJ, Conway DJ, Blanchard TJ, Mahdi OM, Bailey RL, Whittle HC *et al.* **Synthetic peptides based on Chlamydia trachomatis antigens identify cytotoxic T lymphocyte responses in subjects from a trachoma-endemic population**. *Clin.Exp.Immunol.* 1997;107:44-9.

69. Rasmussen SJ, Timms P, Beatty PR, Stephens RS. **Cytotoxic-T-lymphocyte-mediated cytolysis of L cells persistently infected with Chlamydia spp**. *Infect.Immun.* 1996;64:1944-9.

70. Beatty PR,.Stephens RS. **CD8+ T lymphocyte-mediated lysis of Chlamydia-infected L cells using an endogenous antigen pathway**. *J.Immunol.* 1994;153:4588-95.

71. Starnbach MN, Bevan MJ, Lampe MF. **Murine cytotoxic T lymphocytes induced following Chlamydia trachomatis intraperitoneal or genital tract infection respond to cells infected with multiple serovars**. *Infect.Immun.* 1995;63:3527-30.

72. Lampe MF, Wilson CB, Bevan MJ, Starnbach MN. **Gamma interferon production by cytotoxic T lymphocytes is required for resolution of Chlamydia trachomatis infection**. *Infect.Immun.* 1998;66:5457-61.

73. Byrne GI, Grubbs B, Marshall TJ, Schachter J, Williams DM. **Gamma interferon-mediated cytotoxicity related to murine Chlamydia trachomatis infection**. *Infect.Immun.* 1988;56:2023-7.

74. Carlin JM, Borden EC, Byrne GI. **Interferon-induced indoleamine 2,3-dioxygenase activity inhibits Chlamydia psittaci replication in human macrophages**. *J.Interferon.Res.* 1989;9:329-37.

75. Beatty WL, Morrison RP, Byrne GI. **Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis**. *Microbiol.Rev.* 1994;58:686-99.

76. Zhong G, Fan T, Liu L. **Chlamydia inhibits interferon gamma-inducible major histocompatibility complex class II expression by degradation of upstream stimulatory factor 1**. *J.Exp.Med.* 1999;189:1931-8.

77. Caspar-Bauguil S, Puissant B, Nazzal D, Lefevre JC, Thomsen M, Salvayre R *et al.* **Chlamydia pneumoniae induces interleukin-10 production that down-regulates major histocompatibility complex class I expression**. *J.Infect.Dis.* 2000;182:1394-401.

78. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. **Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins**. *J.Immunol.* 1986;136:2348-57.

79. Street NE,.Mosmann TR. **Functional diversity of T lymphocytes due to secretion of different cytokine patterns**. *FASEB J.* 1991;5:171-7.

80. Romagnani S. **Human TH1 and TH2 subsets: doubt no more**. *Immunol.Today* 1991;12:256-7.

81. Boom WH, Husson RN, Young RA, David JR, Piessens WF. **In vivo and in vitro characterization of murine T-cell clones reactive to Mycobacterium tuberculosis**. *Infect.Immun.* 1987;55:2223-9.

82. Haanen JB, de Waal MR, Res PC, Kraakman EM, Ottenhoff TH, De Vries RR *et al.* **Selection of a human T helper type 1-like T cell subset by mycobacteria**. *J.Exp.Med.* 1991;174:583-92.

83. Yamamura M, Uyemura K, Deans RJ, Weinberg K, Rea TH, Bloom BR *et al.* **Defining protective responses to pathogens: cytokine profiles in leprosy lesions**. *Science* 1991;254:277-9.

84. Boom WH, Liebster L, Abbas AK, Titus RG. **Patterns of cytokine secretion in murine leishmaniasis: correlation with disease progression or resolution**. *Infect.Immun.* 1990;58:3863-70.

85. Urban JF, Jr., Katona IM, Paul WE, Finkelman FD. **Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice**. *Proc.Natl.Acad.Sci.U.S.A* 1991;88:5513-7.

86. Pearlman E, Hazlett FE, Jr., Boom WH, Kazura JW. **Induction of murine T-helper-cell responses to the filarial nematode Brugia malayi**. *Infect.Immun.* 1993;61:1105-12.

87. Morrison SG,.Morrison RP. **In situ analysis of the evolution of the primary immune response in murine Chlamydia trachomatis genital tract infection**. *Infect.Immun.* 2000;68:2870-9.

88. Mabey DC, Bailey RL, Dunn D, Jones D, Williams JH, Whittle HC *et al.* **Expression of MHC class II antigens by conjunctival epithelial cells in trachoma: implications concerning the pathogenesis of blinding disease**. *J.Clin.Pathol.* 1991;44:285-9.

89. Yang X, HayGlass KT, Brunham RC. **Genetically determined differences in IL-10 and IFN-gamma responses correlate with clearance of Chlamydia trachomatis mouse pneumonitis infection**. *J.Immunol.* 1996;156:4338-44.

90. Cain TK,.Rank RG. **Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of Chlamydia trachomatis**. *Infect.Immun.* 1995;63:1784-9.

91. Tseng CT,.Rank RG. **Role of NK cells in early host response to chlamydial genital infection**. *Infect.Immun.* 1998;66:5867-75.

92. Perry LL, Feilzer K, Caldwell HD. **Immunity to Chlamydia trachomatis is mediated by T helper 1 cells through IFN-gamma-dependent and -independent pathways**. *J.Immunol.* 1997;158:3344-52.

93. Netea MG, Selzman CH, Kullberg BJ, Galama JM, Weinberg A, Stalenhoef AF *et al.* **Acellular components of Chlamydia pneumoniae stimulate cytokine production in human blood mononuclear cells**. *Eur.J.Immunol.* 2000;30:541-9.

94. Netea MG, Kullberg BJ, Galama JM, Stalenhoef AF, Dinarello CA, van der Meer JW. **Non-LPS components of Chlamydia pneumoniae stimulate cytokine production through Toll-like receptor 2-dependent pathways**. *Eur.J.Immunol.* 2002;32:1188-95.

95. Trinchieri G. **Interleukin-12: a cytokine at the interface of inflammation and immunity**. *Adv.Immunol.* 1998;70:83-243.

96. Geng Y, Berencsi K, Gyulai Z, Valyi-Nagy T, Gonczol E, Trinchieri G. **Roles of interleukin-12 and gamma interferon in murine Chlamydia pneumoniae infection**. *Infect.Immun.* 2000;68:2245-53.

97. Vuola JM, Puurula V, Anttila M, Makela PH, Rautonen N. **Acquired immunity to Chlamydia pneumoniae is dependent on gamma interferon in two mouse strains that initially differ in this respect after primary challenge**. *Infect.Immun.* 2000;68:960-4.

98. Cotter TW, Ramsey KH, Miranpuri GS, Poulsen CE, Byrne GI. **Dissemination of Chlamydia trachomatis chronic genital tract infection in gamma interferon gene knockout mice**. *Infect.Immun.* 1997;65:2145-52.

99. Williams DM, Bonewald LF, Roodman GD, Byrne GI, Magee DM, Schachter J. **Tumor necrosis factor alpha is a cytotoxin induced by murine Chlamydia trachomatis infection**. *Infect.Immun.* 1989;57:1351-5.

100. Darville T, Laffoon KK, Kishen LR, Rank RG. **Tumor necrosis factor alpha activity in genital tract secretions of guinea pigs infected with chlamydiae**. *Infect.Immun.* 1995;63:4675-81.

101. Toth M, Jeremias J, Ledger WJ, Witkin SS. **In vivo tumor necrosis factor production in women with salpingitis** . *Surg.Gynecol.Obstet.* 1992;174:359-62.

102. Conway DJ, Holland MJ, Bailey RL, Campbell AE, Mahdi OS, Jennings R *et al*. **Scarring trachoma is associated with polymorphism in the tumor necrosis factor alpha (TNF-alpha) gene promoter and with elevated TNF-alpha levels in tear fluid**. *Infect.Immun.* 1997;65:1003-6.

103. Abu el-Asrar AM, Geboes K, Tabbara KF, Al Kharashi SA, Missotten L, Desmet V. **Immunopathogenesis of conjunctival scarring in trachoma**. *Eye* 1998;12:453-60.

104. Williams DM, Magee DM, Bonewald LF, Smith JG, Bleicker CA, Byrne GI *et al*. **A role in vivo for tumor necrosis factor alpha in host defense against Chlamydia trachomatis**. *Infect.Immun.* 1990;58:1572-6.

105. Shemer-Avni Y, Wallach D, Sarov I. **Inhibition of Chlamydia trachomatis growth by recombinant tumor necrosis factor**. *Infect.Immun.* 1988;56:2503-6.

106. Perry LL, Su H, Feilzer K, Messer R, Hughes S, Whitmire W *et al.* **Differential sensitivity of distinct Chlamydia trachomatis isolates to IFN-gamma-mediated inhibition**. *J.Immunol.* 1999;162:3541-8.

107. Darville T, Andrews CW, Rank RG. **Does inhibition of tumor necrosis factor alpha affect chlamydial genital tract infection in mice and guinea pigs?** *Infect.Immun.* 2000;68:5299-305.

108. Caldwell HD, Kromhout J, Schachter J. **Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis**. *Infect.Immun.* 1981;31:1161-76.

109. Bavoil P, Ohlin A, Schachter J. **Role of disulfide bonding in outer membrane structure and permeability in Chlamydia trachomatis**. *Infect.Immun.* 1984;44:479-85.

110. Wyllie S, Ashley RH, Longbottom D, Herring AJ. **The major outer membrane protein of Chlamydia psittaci functions as a porin-like ion channel**. *Infect.Immun.* 1998;66:5202-7.

111. Su H, Zhang YX, Barrera O, Watkins NG, Caldwell HD. **Differential effect of trypsin on infectivity of Chlamydia trachomatis: loss of infectivity requires cleavage of major outer membrane protein variable domains II and IV**. *Infect.Immun.* 1988;56:2094-100.

112. Stephens RS, Tam MR, Kuo CC, Nowinski RC. **Monoclonal antibodies to Chlamydia trachomatis: antibody specificities and antigen characterization**. *J.Immunol.* 1982;128:1083-9.

113. Yuan Y, Zhang YX, Watkins NG, Caldwell HD. **Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 Chlamydia trachomatis serovars**. *Infect.Immun.* 1989;57:1040-9.

114. Stephens RS, Sanchez Pescador R, Wagar EA, Inouye C, Urdea MS. **Diversity of Chlamydia trachomatis major outer membrane protein genes**. *J.Bacteriol.* 1987;169:3879-85.

115. Baehr W, Zhang YX, Joseph T, Su H, Nano FE, Everett KD *et al.* **Mapping antigenic domains expressed by Chlamydia trachomatis major outer membrane protein genes**. *Proc.Natl.Acad.Sci.U.S.A* 1988;85:4000-4.

116. Bas S, Muzzin P, Vischer TL. **Chlamydia trachomatis serology: diagnostic value of outer membrane protein 2 compared with that of other antigens**. *J.Clin.Microbiol.* 2001;39:4082-5.

117. Qu Z, Cheng X, de la Maza LM, Peterson EM. **Analysis of the humoral response elicited in mice by a chimeric peptide representing variable segments I and IV of the major outer membrane protein of Chlamydia trachomatis**. *Vaccine* 1994;12:557-64.

118. Pal S, Cheng X, Peterson EM, de la Maza LM. **Mapping of a surface-exposed B-cell epitope to the variable sequent 3 of the major outer-membrane protein of Chlamydia trachomatis**. *J.Gen.Microbiol.* 1993;139:1565-70.

119. Maclean IW, Peeling RW, Brunham RC. **Characterization of Chlamydia trachomatis antigens with monoclonal and polyclonal antibodies**. *Can.J.Microbiol.* 1988;34:141-7.

120. Caldwell HD,.Perry LJ. **Neutralization of Chlamydia trachomatis infectivity with antibodies to the major outer membrane protein**. *Infect.Immun.* 1982;38:745-54.

121. Buzoni Gatel D, Bernard F, Andersen A, Rodolakis A. **Protective effect of polyclonal and monoclonal antibodies against abortion in mice infected by Chlamydia psittaci**. *Vaccine* 1990;8:342-6.

122. Zhang YX, Stewart S, Joseph T, Taylor HR, Caldwell HD. **Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of Chlamydia trachomatis**. *J.Immunol.* 1987;138:575-81.

123. Knight SC, Iqball S, Woods C, Stagg A, Ward ME, Tuffrey M. **A peptide of Chlamydia trachomatis shown to be a primary T-cell epitope in vitro induces cell-mediated immunity in vivo**. *Immunology* 1995;85:8-15.

124. Igietseme JU,.Murdin A. **Induction of protective immunity against Chlamydia trachomatis genital infection by a vaccine based on major outer membrane protein-lipophilic immune response-stimulating complexes**. *Infect.Immun.* 2000;68:6798-806.

125. Rank RG. **Models of immunity.** In Stephens RS, ed. *Chlamydia: intracellular biology, pathogenesis and immunity*, pp 239-95. Washington, DC: American Society for Microbiology, 1993.

126. Zhang D, Yang X, Berry J, Shen C, McClarty G, Brunham RC. **DNA vaccination with the major outer-membrane protein gene induces acquired immunity to Chlamydia trachomatis (mouse pneumonitis) infection**. *J.Infect.Dis.* 1997;176:1035-40.

127. Pal S, Barnhart KM, Wei Q, Abai AM, Peterson EM, de la Maza LM. **Vaccination of mice with DNA plasmids coding for the Chlamydia trachomatis major outer membrane protein elicits an immune response but fails to protect against a genital challenge**. *Vaccine* 1999;17:459-65.

128. Everett KD,.Hatch TP. **Architecture of the cell envelope of Chlamydia psittaci 6BC**. *J.Bacteriol.* 1995;177:877-82.

129. Hatch TP. **Developmental Biology**. In Stephens RS, ed. *Chlamydia: Intracellular Biology, Pathogenesis and Immunity*, pp 29-67. Washington DC: ASM Press, 1999.

130. Watson MW, Lambden PR, Everson JS, Clarke IN. **Immunoreactivity of the 60 kDa cysteine-rich proteins of Chlamydia trachomatis, Chlamydia psittaci and Chlamydia pneumoniae expressed in Escherichia coli**. *Microbiology* 1994;140:2003-11.

131. Coles AM, Crosby HA, Pearce JH. **Analysis of the human serological response to Chlamydia trachomatis 60-kDa proteins by two-dimensional electrophoresis and immunoblotting**. *FEMS Microbiol.Lett.* 1991;65:299-303.

132. Wagar EA, Schachter J, Bavoil P, Stephens RS. **Differential human serologic response to two 60,000 molecular weight Chlamydia trachomatis antigens**. *J.Infect.Dis.* 1990;162:922-7.

133. Sanchez-Campillo M, Bini L, Comanducci M, Raggiaschi R, Marzocchi B, Pallini V *et al*. **Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera**. *Electrophoresis* 1999;20:2269-79.

134. Mygind P, Christiansen G, Persson K, Birkelund S. **Analysis of the humoral immune response to Chlamydia outer membrane protein 2**. *Clin.Diagn.Lab Immunol.* 1998;5:313-8.

135. Mygind P, Christiansen G, Birkelund S. **Topological analysis of Chlamydia trachomatis L2 outer membrane protein 2**. *J.Bacteriol.* 1998;180:5784-7.

136. Beatty PR,.Stephens RS. **Identification of Chlamydia trachomatis antigens by use of murine T-cell lines**. *Infect.Immun.* 1992;60:4598-603.

137. Goodall JC, Beacock-Sharp H, Deane KH, Gaston JS. **Recognition of the 60 kilodalton cysteine-rich outer membrane protein OMP2 by CD4(+) T cells from humans infected with Chlamydia trachomatis**. *Clin.Exp.Immunol.* 2001;126:488-93.

138. Kaufmann SH. **Heat shock proteins and the immune response**. *Immunol.Today* 1990;11:129-36.

139. Brunham RC,.Peeling RW. **Chlamydia trachomatis antigens: role in immunity and pathogenesis**. *Infect.Agents Dis.* 1994;3:218-33.

140. Peeling RW, Bailey RL, Conway DJ, Holland MJ, Campbell AE, Jallow O *et al.* **Antibody response to the 60-kDa chlamydial heat-shock protein is associated with scarring trachoma**. *J.Infect.Dis.* 1998;177:256-9.

141. Hessel T, Dhital SP, Plank R, Dean D. **Immune response to chlamydial 60-kilodalton heat shock protein in tears from Nepali trachoma patients**. *Infect.Immun.* 2001;69:4996-5000.

142. Bulut Y, Faure E, Thomas L, Karahashi H, Michelsen KS, Equils O *et al.* **Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway**. *J.Immunol.* 2002;168:1435-40.

143. Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. **Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells**. *J.Immunol.* 2000;164:13-7.

144. Kol A, Bourcier T, Lichtman AH, Libby P. **Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages**. *J.Clin.Invest* 1999;103:571-7.

145. Retzlaff C, Yamamoto Y, Hoffman PS, Friedman H, Klein TW. **Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures**. *Infect.Immun.* 1994;62:5689-93.

146. Chen W, Syldath U, Bellmann K, Burkart V, Kolb H. **Human 60-kDa heat-shock protein: a danger signal to the innate immune system**. *J.Immunol.* 1999;162:3212-9.

147. Kol A, Sukhova GK, Lichtman AH, Libby P. **Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression**. *Circulation* 1998;98:300-7.

148. Svanholm C, Bandholtz L, Castanos-Velez E, Wigzell H, Rottenberg ME. **Protective DNA immunization against Chlamydia pneumoniae**. *Scand.J.Immunol.* 2000;51:345-53.

149. Longbottom D, Russell M, Jones GE, Lainson FA, Herring AJ. **Identification of a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of Chlamydia psittaci**. *FEMS Microbiol.Lett.* 1996;142:277-81.

150. Tanzer RJ, Longbottom D, Hatch TP. **Identification of polymorphic outer membrane proteins of Chlamydia psittaci 6BC**. *Infect.Immun.* 2001;69:2428-34.

151. Shirai M, Hirakawa H, Ouchi K, Tabuchi M, Kishi F, Kimoto M *et al.* **Comparison of outer membrane protein genes omp and pmp in the whole genome sequences of Chlamydia pneumoniae isolates from Japan and the United States**. *J.Infect.Dis.* 2000;181 Suppl 3:S524-S527.

152. Christiansen G, Pedersen AS, Hjerno K, Vandahl B, Birkelund S. **Potential relevance of Chlamydia pneumoniae surface proteins to an effective vaccine**. *J.Infect.Dis.* 2000;181 Suppl 3:S528-S537.

153. Stothard, D. R., Toth, G. A., and Batteiger, B. E. **Sequence diversity among the polymorphic membrane proteins of *Chlamydia trachomatis*.** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-2002. p. 575-578.

154. Struyve M, Moons M, Tommassen J. **Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein**. *J.Mol.Biol.* 1991;218:141-8.

155. Campbell LA, Kuo CC, Grayston JT. **Structural and antigenic analysis of Chlamydia pneumoniae**. *Infect.Immun.* 1990;58:93-7.

156. Birkelund, S., Christiansen, G., Vandahl, B., and Pedersen, A. S. **Are the pmp proteins beta-helices?** Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 2002. p. 551-554.

157. Kajava AV, Cheng N, Cleaver R, Kessel M, Simon MN, Willery E *et al.* **Beta-helix model for the filamentous haemagglutinin adhesin of Bordetella pertussis and related bacterial secretory proteins**. *Mol.Microbiol.* 2001;42:279-92.

158. Cevenini R, Donati M, Brocchi E, De Simone F, La Placa M. **Partial characterization of an 89-kDa highly immunoreactive protein from Chlamydia psittaci A/22 causing ovine abortion**. *FEMS Microbiol.Lett.* 1991;65:111-5.

159. Longbottom D, Russell M, Dunbar SM, Jones GE, Herring AJ. **Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the Chlamydia psittaci subtype that causes abortion in sheep**. *Infect.Immun.* 1998;66:1317-24.

160. Knudsen, K., Madsen, A. S., Mygind, P., Christiansen, G., and Birkelund, S. **Surface localized proteins of *Chlamydia pneumoniae*.** 1998. San Francisco. Chlamydial Infections - Proceedings of the 9th International Symposium on Human Chlamydial Infection. P. 267-270.

161. Birkelund, S., Knudsen, K., Madsen, A. S., Falk, E., Mygind, P., and Christiansen, G. **Differential expression of *Chlamydia pneumoniae* omp4 and omp5 after infection of C57-Black mice.** 1998. San Francisco. Chlamydial Infections - Proceedings of the 9th International Symposium on Human Chlamydial Infection. P. 275-278.

162. Buendia AJ, Salinas J, Sanchez J, Gallego MC, Rodolakis A, Cuello F. **Localization by immunoelectron microscopy of antigens of Chlamydia psittaci suitable for diagnosis or vaccine development**. *FEMS Microbiol.Lett.* 1997;150:113-9.

163. Longbottom D, Findlay J, Vretou E, Dunbar SM. **Immunoelectron microscopic localisation of the OMP90 family on the outer membrane surface of Chlamydia psittaci**. *FEMS Microbiol.Lett.* 1998;164:111-7.

164. Mygind PH, Christiansen G, Roepstorff P, Birkelund S. **Membrane proteins PmpG and PmpH are major constituents of Chlamydia trachomatis L2 outer membrane complex**. *FEMS Microbiol.Lett.* 2000;186:163-9.

165. Kiselev, A. O., Johnson, M. L., Ballweber, L. B., Stamm, W., and Lampe, M. F. **Surface localization of pmpD, a polymorphic membrand protein of *Chlamydia trachomatis serovar* L2**. 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-2002. p. 567-570

166. Lindquist, E. A. and Stephens, R. S. **Transcriptional activity of a sequence variable protein family in *Chlamydia trachomatis*.** 1998. San Francisco. Chlamydial Infections - Proceedings of the 9th International Symposium on Human Chlamydial Infection. p. 259-262.

167. Grimwood, J., Mitchell, W., and Stephens, R. S. **Phylogenetic analysis of a multigene family conserved between *Chlamydia trachomatis* and *Chlamydia pneumoniae*.** 1998. San Francisco. Chlamydial Infections - Proceedings of the 9th International Symposium on Human Chlamydial Infection. p. 263-266.

168. Giannikopoulou P, Bini L, Simitsek PD, Pallini V, Vretou E. **Two-dimensional electrophoretic analysis of the protein family at 90 kDa of abortifacient Chlamydia psittaci**. *Electrophoresis* 1997;18:2104-8.

169. Longbottom, D., Dunbar, S. M., Russell, M., Vretou, E., Jones, G. E., and Herring, A. J. **Characterisation, expression and surface localisation of the OEA *Chlamydia psittact* 90kDa protein family.** 1998. San Francisco. Chlamydial Infections - Proceedings of the 9th International Symposium on Human Chlamydial Infection. p. 255-258.

170. Henderson IR,.Lam AC. **Polymorphic proteins of Chlamydia spp.--autotransporters beyond the Proteobacteria**. *Trends Microbiol.* 2001;9:573-8.

171. Henderson IR, Navarro-Garcia F, Nataro JP. **The great escape: structure and function of the autotransporter proteins**. *Trends Microbiol.* 1998;6:370-8.

172. Buzoni Gatel D, Layachi K, Dubray G, Rodolakis A. **Comparison of protein patterns between invasive and non-invasive ovine strains of Chlamydia psittaci**. *Res.Vet.Sci.* 1989;46:40-2.

173. Souriau A, Le Rouzic E, Bernard F, Rodolakis A. **Differentiation of abortion-inducing and intestinal strains of Chlamydia psittaci isolated from ruminants by the microimmunofluorescence test** . *Vet.Rec.* 1993;132:217-9.

174. Layachi K, Rodolakis A, Buzoni Gatel D. **Identification by western blots of virulence specific antigens of Chlamydia psittaci isolated from ewes**. *Vet.Res.* 1993;24:55-65.

175. Souriau A, Salinas J, De Sa C, Layachi K, Rodolakis A. **Identification of subspecies- and serotype 1-specific epitopes on the 80- to 90-kilodalton protein region of Chlamydia psittaci that may be useful for diagnosis of chlamydial induced abortion**. *Am.J.Vet.Res.* 1994;55:510-4.

176. Campbell LA, Kuo CC, Wang SP, Grayston JT. **Serological response to Chlamydia pneumoniae infection**. *J.Clin.Microbiol.* 1990;28:1261-4.

177. Biendo M, Eb F, Lefebvre JF, Orfila J. **Limits of the microimmunofluorescence test and advantages of immunoblotting in the diagnosis of chlamydiosis**. *Clin.Diagn.Lab.Immunol.* 1996;3:706-9.

178. Iijima Y, Miyashita N, Kishimoto T, Kanamoto Y, Soejima R, Matsumoto A. **Characterization of Chlamydia pneumoniae species-specific proteins immunodominant in humans**. *J.Clin.Microbiol.* 1994;32:583-8.

179. Pedersen AS, Christiansen G, Birkelund S. **Differential expression of Pmp10 in cell culture infected with Chlamydia pneumoniae CWL029**. *FEMS Microbiol.Lett.* 2001;203:153-9.

180. Livingstone, M., Wattegedara, D., Buxton, D., Entrican, G., and Longbottom, D. **Comparison of recombinant pomp and momp serological tests for the analysis of antibody responses in sheep following experimental infection with *Chlamydophila abortus*.** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-2002. p. 285-289.

181. Maass M,.Gieffers J. **Cardiovascular disease risk from prior Chlamydia pneumoniae infection can be related to certain antigens recognized in the immunoblot profile**. *J.Infect.* 1997;35:171-6.

182. Linnanmaki E, Leinonen M, Mattila K, Nieminen MS, Valtonen V, Saikku P. **Chlamydia pneumoniae-specific circulating immune complexes in patients with chronic coronary heart disease**. *Circulation* 1993;87:1130-4.

183. Puolakkainen M, Campbell LA, Kuo CC, Leinonen M, Gronhagen Riska C, Saikku P. **Serological response to Chlamydia pneumoniae in patients with sarcoidosis**. *J.Infect.* 1996;33:199-205.

184. Goodall JC, Yeo G, Huang M, Raggiaschi R, Gaston JS. **Identification of Chlamydia trachomatis antigens recognized by human CD4+ T lymphocytes by screening an expression library**. *Eur.J.Immunol.* 2001;31:1513-22.

185. Tan TW, Herring AJ, Anderson IE, Jones GE. **Protection of sheep against Chlamydia psittaci infection with a subcellular vaccine containing the major outer membrane protein**. *Infect.Immun.* 1990;58:3101-8.

186. Pal S, Theodor I, Peterson EM, de la Maza LM. **Immunization with an acellular vaccine consisting of the outer membrane complex of Chlamydia trachomatis induces protection against a genital challenge**. *Infect.Immun.* 1997;65:3361-9.

187. Betsou F, Sueur JM, Orfila J. **Serological investigation of Chlamydia trachomatis heat shock protein 10**. *Infect.Immun.* 1999;67:5243-6.

188. LaVerda D, Albanese LN, Ruther PE, Morrison SG, Morrison RP, Ault KA *et al.* **Seroreactivity to Chlamydia trachomatis Hsp10 correlates with severity of human genital tract disease**. *Infect.Immun.* 2000;68:303-9.

189. Raulston JE, Davis CH, Paul TR, Hobbs JD, Wyrick PB. **Surface accessibility of the 70-kilodalton Chlamydia trachomatis heat shock protein following reduction of outer membrane protein disulfide bonds**. *Infect.Immun.* 2002;70:535-43.

190. Raulston JE, Davis CH, Schmiel DH, Morgan MW, Wyrick PB. **Molecular characterization and outer membrane association of a Chlamydia trachomatis protein related to the hsp70 family of proteins [published erratum appears in J Biol Chem 1994 Apr 1; 269(13):10184]**. *J.Biol.Chem.* 1993;268:23139-47.

191. Danilition SL, Maclean IW, Peeling R, Winston S, Brunham RC. **The 75-kilodalton protein of Chlamydia trachomatis: a member of the heat shock protein 70 family?** *Infect.Immun.* 1990;58:189-96.

192. Zhong G,.Brunham RC. **Antigenic analysis of the chlamydial 75-kilodalton protein**. *Infect.Immun.* 1992;60:1221-4.

193. Bannantine JP, Stamm WE, Suchland RJ, Rockey DD. **Chlamydia trachomatis IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates**. *Infect.Immun.* 1998;66:6017-21.

194. Bannantine JP, Griffiths RS, Viratyosin W, Brown WJ, Rockey DD. **A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane**. *Cell Microbiol.* 2000;2:35-47.

195. Kubo A,.Stephens RS. **Substrate-specific diffusion of select dicarboxylates through Chlamydia trachomatis PorB** . *Microbiology* 2001;147:3135-40.

196. Kawa DE,.Stephens RS. **Antigenic Topology of Chlamydial PorB Protein and Identification of Targets for Immune Neutralization of Infectivity**. *J.Immunol.* 2002;168:5184-91.

197. Fling SP, Sutherland RA, Steele LN, Hess B, D'Orazio SE, Maisonneuve JF *et al.* **CD8+ T cells recognize an inclusion membrane-associated protein from the vacuolar pathogen Chlamydia trachomatis**. *Proc.Natl.Acad.Sci.U.S.A* 2001;98:1160-5.

198. Comanducci M, Manetti R, Bini L, Santucci A, Pallini V, Cevenini R *et al.* **Humoral immune response to plasmid protein pgp3 in patients with Chlamydia trachomatis infection**. *Infect.Immun.* 1994;62:5491-7.

199. Lundemose AG, Rouch DA, Birkelund S, Christiansen G, Pearce JH. **Chlamydia trachomatis Mip-like protein**. *Mol.Microbiol.* 1992;6:2539-48.

200. Lundemose AG, Birkelund S, Fey SJ, Larsen PM, Christiansen G. **Chlamydia trachomatis contains a protein similar to the Legionella pneumophila mip gene product**. *Mol.Microbiol.* 1991;5:109-15.

201. Watkins NG, Hadlow WJ, Moos AB, Caldwell HD. **Ocular delayed hypersensitivity: a pathogenetic mechanism of chlamydial-conjunctivitis in guinea pigs**. *Proc.Natl.Acad.Sci.U.S.A* 1986;83:7480-4.

202. Taylor HR, Johnson SL, Schachter J, Caldwell HD, Prendergast RA. **Pathogenesis of trachoma: the stimulus for inflammation**. *J.Immunol.* 1987;138:3023-7.

203. Morrison RP, Lyng K, Caldwell HD. **Chlamydial disease pathogenesis. Ocular hypersensitivity elicited by a genus-specific 57-kD protein**. *J.Exp.Med.* 1989;169:663-75.

204. Domeika M, Domeika K, Paavonen J, Mardh PA, Witkin SS. **Humoral immune response to conserved epitopes of Chlamydia trachomatis and human 60-kDa heat-shock protein in women with pelvic inflammatory disease**. *J.Infect.Dis.* 1998;177:714-9.

205. Sziller I, Witkin SS, Ziegert M, Csapo Z, Ujhazy A, Papp Z. **Serological responses of patients with ectopic pregnancy to epitopes of the Chlamydia trachomatis 60 kDa heat shock protein**. *Hum.Reprod.* 1998;13:1088-93.

206. Eckert LO, Hawes SE, Wolner Hanssen P, Money DM, Peeling RW, Brunham RC *et al.* **Prevalence and correlates of antibody to chlamydial heat shock protein in women attending sexually transmitted disease clinics and women with confirmed pelvic inflammatory disease**. *J.Infect.Dis.* 1997;175:1453-8.

207. Peeling RW, Kimani J, Plummer F, Maclean I, Cheang M, Bwayo J *et al.* **Antibody to chlamydial hsp60 predicts an increased risk for chlamydial pelvic inflammatory disease**. *J.Infect.Dis.* 1997;175:1153-8.

208. Money DM, Hawes SE, Eschenbach DA, Peeling RW, Brunham R, Wolner Hanssen P *et al.* **Antibodies to the chlamydial 60 kd heat-shock protein are associated with laparoscopically confirmed perihepatitis**. *Am.J.Obstet.Gynecol.* 1997;176:870-7.

209. Ault KA, Statland BD, King MM, Dozier DI, Joachims ML, Gunter J. **Antibodies to the chlamydial 60 kilodalton heat shock protein in women with tubal factor infertility**. *Infect.Dis.Obstet.Gynecol.* 1998;6:163-7.

210. Toye B, Laferriere C, Claman P, Jessamine P, Peeling R. **Association between antibody to the chlamydial heat-shock protein and tubal infertility**. *J.Infect.Dis.* 1993;168:1236-40.

211. Brunham RC, Maclean IW, Binns B, Peeling RW. **Chlamydia trachomatis: its role in tubal infertility**. *J.Infect.Dis.* 1985;152:1275-82.

212. Taylor HR, Maclean IW, Brunham RC, Pal S, Wittum Hudson J. **Chlamydial heat shock proteins and trachoma**. *Infect.Immun.* 1990;58:3061-3.

213. Morrison RP, Belland RJ, Lyng K, Caldwell HD. **Chlamydial disease pathogenesis. The 57-kD chlamydial hypersensitivity antigen is a stress response protein**. *J.Exp.Med.* 1989;170:1271-83.

214. Kelly KA, Natarajan S, Ruther P, Wisse A, Chang MH, Ault KA. **Chlamydia trachomatis infection induces mucosal addressin cell adhesion molecule-1 and vascular cell adhesion molecule-1, providing an immunologic link between the fallopian tube and other mucosal tissues**. *J.Infect.Dis.* 2001;184:885-91.

215. Kelly KA,.Rank RG. **Identification of homing receptors that mediate the recruitment of CD4 T cells to the genital tract following intravaginal infection with Chlamydia trachomatis**. *Infect.Immun.* 1997;65:5198-208.

216. Azenabor AA,.Mahony JB. **Generation of reactive oxygen species and formation and membrane lipid peroxides in cells infected with Chlamydia trachomatis**. *Int.J.Infect.Dis.* 2000;4:46-50.

217. Matrisian LM. **The matrix-degrading metalloproteinases**. *Bioessays* 1992;14:455-63.

218. Welgus HG, Campbell EJ, Cury JD, Eisen AZ, Senior RM, Wilhelm SM *et al.* **Neutral metalloproteinases produced by human mononuclear phagocytes. Enzyme profile, regulation, and expression during cellular development**. *J.Clin.Invest* 1990;86:1496-502.

219. el Asrar AM, Geboes K, Al Kharashi SA, Al Mosallam AA, Missotten L, Paemen L *et al.* **Expression of gelatinase B in trachomatous conjunctivitis**. *Br.J.Ophthalmol.* 2000;84:85-91.

220. Darville T, Andrews CW, Jr., Laffoon KK, Shymasani W, Kishen LR, Rank RG. **Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response**. *Infect.Immun.* 1997;65:3065-73.

221. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. **Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria**. *Nature* 1994;371:508-10.

222. Bouma G, Crusius JB, Oudkerk PM, Kolkman JJ, von Blomberg BM, Kostense PJ *et al.* **Secretion of tumour necrosis factor alpha and lymphotoxin alpha in relation to polymorphisms in the TNF genes and HLA-DR alleles. Relevance for inflammatory bowel disease**. *Scand.J.Immunol.* 1996;43:456-63.

223. Mozzato-Chamay N, Mahdi OS, Jallow O, Mabey DC, Bailey RL, Conway DJ. **Polymorphisms in candidate genes and risk of scarring trachoma in a Chlamydia trachomatis--endemic population**. *J.Infect.Dis.* 2000;182:1545-8.

224. Conway DJ, Holland MJ, Campbell AE, Bailey RL, Krausa P, Peeling RW *et al.* **HLA class I and II polymorphisms and trachomatous scarring in a Chlamydia trachomatis-endemic population**. *J.Infect.Dis.* 1996;174:643-6.

225. Mahdi OS, Whittle HC, Joof H, Mabey DC, Bailey RL. **Failure to detect HLA-A*6802-restricted CD8+ T cells specific for Chlamydia trachomatis antigens in subjects from trachoma-endemic communities**. *Clin.Exp.Immunol.* 2001;123:68-72.

226. West SK, Munoz B, Lynch M, Kayongoya A, Mmbaga BB, Taylor HR. **Risk factors for constant, severe trachoma among preschool children in Kongwa, Tanzania [see comments]**. *Am.J.Epidemiol.* 1996;143:73-8.

227. Collier LH,.Blyth WA. **Immunogenicity of experimental trachoma vaccines in baboons. II. Experiments with inactivated vaccines.** *J.Hyg.Camb* 1967;65:97-107.

228. Wang SP, Grayston JT, Alexander ER. **Trachoma vaccine studies in monkeys**. *Am.J.Ophthalmol.* 1967;63:Suppl:1615-30.

229. Fraser CE, McComb DE, Murray ES, MacDonald AB. **Immunity to chlamydial infections of the eye. IV. Immunity in owl monkeys to reinfection with trachoma**. *Arch.Ophthalmol.* 1975;93:518-21.

230. Woolridge RL, Grayston JT, Chang IH, Cheng KH, Yang CY, Neave C. **Field trial of a monovalent and of a bivalent mineral oil adjuvant trachoma vaccine in Taiwan school children**. *Am.J.Ophthalmol.* 1967;63:Suppl:1645-50.

231. Dhir SP, Agarwal LP, Gupta SB, Detels R, Wang SP, Grayston JT. **Trachoma vaccine trial in India: results of two-year follow-up**. *Indian J.Med.Res.* 1968;56:1289-94.

232. Rodolakis A,.Souriau A. **Response of ewes to temperature-sensitive mutants of Chlamydia psittaci (var ovis) obtained by NTG mutagenesis**. *Ann.Rech.Vet.* 1983;14:155-61.

233. Andre P, Kim A, Khrapko K, Thilly WG. **Fidelity and mutational spectrum of Pfu DNA polymerase on a human mitochondrial DNA sequence**. *Genome Res.* 1997;7:843-52.

234. Rand KN. **Crystal violet can be used to visualise DNA bands during gel electrophoresis and to improve cloning efficiency**. *Elsevier Trends Journals Technical Tips Online* 1996.

235. Bullock WO, Fernandez JM, Short JM. **XLI-Blue: A high efficiency plasmid transforming recA *Eschericia coli* strain with beta-galactosidase selection**. *Bio Techniques* 1987;5:376.

236. Studier FW,.Moffatt BA. **Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes**. *J Molecular Biology* 1986;189:113.

237. Ornstein L. **Disc electrophoresis – I. Background and theory**. *Ann N Y Acad Sci.* 1964;121:321.

238. Davis BJ. **Disc electrophoresis – II. Method and application to human serum proteins**. *Ann N Y Acad Sci* 1964;121:404.

239. Burnette WN. **"Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A**. *Anal.Biochem.* 1981;112:195.

240. Rosner B. Fundamentals of Biostatistics. Boston: Duxbury Press, 1990.

241. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. **A simple system for the assessment of trachoma and its complications**. *Bull.World Health Organ* 1987;65:477-83.

242. Altman DG. **Relation between two continuous variables: interpretation of correlation.** *Practical Statistics for Medical Research*, pp 296-8. Boca Raton, Fla: CRC Press, 1990.

243. Knight R,.Merrett TG. **Hookworm infection in rural Gambia**. *Ann.Trop.Med.Parasitol* 1981;75:299.

244. Palmer DR,.Bundy DAP. **Epidemiology of human hookworm and *Ascaris lumbricoides* infestations in rural Gambia**. *East.Afr.Med.J.* 1995;72:527-30.

245. Bas S, Muzzin P, Ninet B, Bornand JE, Scieux C, Vischer TL. **Chlamydial serology: comparative diagnostic value of immunoblotting, microimmunofluorescence test, and immunoassays using different recombinant proteins as antigens**. *J.Clin.Microbiol.* 2001;39:1368-77.

246. Stagg AJ, Elsley WA, Pickett MA, Ward ME, Knight SC. **Primary human T-cell responses to the major outer membrane protein of Chlamydia trachomatis**. *Immunology* 1993;79:1-9.

247. Cohen CR, Nguti R, Bukusi EA, Lu H, Shen C, Luo M *et al.* **Human immunodeficiency virus type 1-infected women exhibit reduced interferon-gamma secretion after Chlamydia trachomatis stimulation of peripheral blood lymphocytes**. *J.Infect.Dis.* 2000;182:1672-7.

248. Holland MJ, Bailey RL, Conway DJ, Culley F, Miranpuri G, Byrne GI *et al.* **T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of Chlamydia trachomatis in subjects with severe trachomatous scarring**. *Clin.Exp.Immunol.* 1996;105:429-35.

249. Ciervo A, Visca P, Petrucca A, Biasucci LM, Maseri A, Cassone A. **Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of Chlamydia**

pneumoniae in patients with coronary heart disease. *Clin.Diagn.Lab Immunol.* 2002;9:66-74.

250. Bas S, Muzzin P, Fulpius T, Buchs N, Vischer TL. **Indirect evidence of intra-articular immunoglobulin G synthesis in patients with Chlamydia trachomatis reactive arthritis**. *Rheumatology.(Oxford)* 2001;40:801-5.

251. Curry AJ, Portig I, Goodall JC, Kirkpatrick PJ, Gaston JS. **T lymphocyte lines isolated from atheromatous plaque contain cells capable of responding to Chlamydia antigens**. *Clin.Exp.Immunol.* 2000;121:261-9.

252. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. **Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4**. *Ann.Rheum.Dis.* 1995;54:836-40.

253. Sharma SA, Miller GG, Peek RA, Jr., Perez-Perez G, Blaser MJ. **T-cell, antibody, and cytokine responses to homologs of the 60- kilodalton heat shock protein in Helicobacter pylori infection**. *Clin.Diagn.Lab Immunol.* 1997;4:440-6.

254. Debattista J, Timms P, Allan J, Allan J. **Reduced levels of gamma-interferon secretion in response to chlamydial 60 kDa heat shock protein amongst women with pelvic inflammatory disease and a history of repeated Chlamydia trachomatis infections**. *Immunol.Lett.* 2002;81:205-10.

255. Kinnunen A, Molander P, Morrison R, Lehtinen M, Karttunen R, Tiitinen A *et al.* **Chlamydial heat shock protein 60--specific T cells in inflamed salpingeal tissue**. *Fertil.Steril.* 2002;77:162-6.

256. Yi Y, Yang X, Brunham RC. **Autoimmunity to heat shock protein 60 and antigen-specific production of interleukin-10**. *Infect.Immun.* 1997;65:1669-74.

257. Yin Z, Braun J, Neure L, Wu P, Liu L, Eggens U *et al.* **Crucial role of interleukin-10/interleukin-12 balance in the regulation of the type 2 T helper cytokine response in reactive arthritis**. *Arthritis Rheum.* 1997;40:1788-97.

258. Van Voorhis WC, Barrett LK, Sweeney YT, Kuo CC, Patton DL. **Analysis of lymphocyte phenotype and cytokine activity in the inflammatory infiltrates of the upper genital tract of female macaques infected with Chlamydia trachomatis [published erratum appears in J Infect Dis 1996 Nov;174(5):1142]**. *J.Infect.Dis.* 1996;174:647-50.

259. Cohen CR, Plummer FA, Mugo N, Maclean I, Shen C, Bukusi EA *et al.* **Increased interleukin-10 in the the endocervical secretions of women with non-ulcerative sexually transmitted diseases: a mechanism for enhanced HIV-1 transmission?** *AIDS* 1999;13:327-32.

260. Airenne, S., Kinnunen, A., Leinonen, M., Saikku, P., and Surcel, H. M. **Secretion of IL-10 and IL-12 in *Chlamydia pneumoniae* infected human monocytes.** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-2002. p. 77-80.

261. Mosmann TR,.Moore KW. **The role of IL-10 in cross-regulation of TH1 and TH2 responses**. *Immunol.Today* 1991;12:A49-A53.

262. Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. **Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production**. *J.Immunol.* 1993;150:353-60.

263. Igietseme JU, Ananaba GA, Bolier J, Bowers S, Moore T, Belay T *et al.* **Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development**. *J.Immunol.* 2000;164:4212-9.

264. Yang X, Gartner J, Zhu L, Wang S, Brunham RC. **IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection**. *J.Immunol.* 1999;162:1010-7.

265. Kinnunen, A., Lehtinen, M., Koskela, P., Bloigu, A., Karttunen, R., Paavonen, J., and Surcel, H. M. **IL-10 polymorphisms and cell-mediated immunity to chlamydial hsp60 in infertile women.** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. P. 237-240.

266. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. **An investigation of polymorphism in the interleukin-10 gene promoter**. *Eur.J.Immunogenet.* 1997;24:1-8.

267. Zychlinsky A, Prevost MC, Sansonetti PJ. **Shigella flexneri induces apoptosis in infected macrophages**. *Nature* 1992;358:167-9.

268. Rogers HW, Callery MP, Deck B, Unanue ER. **Listeria monocytogenes induces apoptosis of infected hepatocytes**. *J.Immunol.* 1996;156:679-84.

269. Rojas M, Barrera LF, Puzo G, Garcia LF. **Differential induction of apoptosis by virulent Mycobacterium tuberculosis in resistant and susceptible murine macrophages: role of nitric oxide and mycobacterial products**. *J.Immunol.* 1997;159:1352-61.

270. Monack DM, Raupach B, Hromockyj AE, Falkow S. **Salmonella typhimurium invasion induces apoptosis in infected macrophages**. *Proc.Natl.Acad.Sci.U.S.A* 1996;93:9833-8.

271. Perfettini JL, Darville T, Gachelin G, Souque P, Huerre M, Dautry-Varsat A *et al.* **Effect of Chlamydia trachomatis infection and subsequent tumor necrosis factor alpha secretion on apoptosis in the murine genital tract**. *Infect.Immun.* 2000;68:2237-44.

272. Ojcius DM, Souque P, Perfettini JL, Dautry-Varsat A. **Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen Chlamydia psittaci**. *J.Immunol.* 1998;161:4220-6.

273. Jendro MC, Deutsch T, Korber B, Kohler L, Kuipers JG, Krausse-Opatz B *et al.* **Infection of human monocyte-derived macrophages with Chlamydia trachomatis induces apoptosis of T cells: a potential mechanism for persistent infection**. *Infect.Immun.* 2000;68:6704-11.

274. Geng Y, Shane RB, Berencsi K, Gonczol E, Zaki MH, Margolis DJ *et al.* **Chlamydia pneumoniae inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10**. *J.Immunol.* 2000;164:5522-9.

275. Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. **Pathogenic Mycobacterium tuberculosis evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF- alpha**. *J.Immunol.* 1998;161:2636-41.

276. Dean D,.Powers VC. **Persistent Chlamydia trachomatis infections resist apoptotic stimuli**. *Infect.Immun.* 2001;69:2442-7.

277. Bachis A, Colangelo AM, Vicini S, Doe PP, De Bernardi MA, Brooker G *et al.* **Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity**. *J.Neurosci.* 2001;21:3104-12.

278. Schmidt M, Lugering N, Pauels HG, Schulze-Osthoff K, Domschke W, Kucharzik T. **IL-10 induces apoptosis in human monocytes involving the CD95 receptor/ligand pathway**. *Eur.J.Immunol.* 2000;30:1769-77.

279. Roblin, P., Kohlhoff, S, Malay, S., Kutlin, A., Riska, P. F., and Hammerschlag, M. **Effect of acute and chronic *Chlamydia pneumoniae* infection on apoptosis of A-549 cells.** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-0002. p. 89-92.

280. Sabin E, Aranjo M, Carvalho E, Pearce E. **Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with *Schistosoma mansoni*.** *J.Infect.Dis.* 1996;173:269-72.

281. Kullberg MC, Pearce EJ, Hieny SE, Sher A, Berzofsky JA. **Infection with Schistosoma mansoni alters Th1/Th2 cytokine responses to a non-parasite antigen**. *J.Immunol.* 1992;148:3264-70.

282. Pearlman E, Kazura F, Hazlett F, Boom W. **Modulation of murine cytokine responses to mycobacterial antigens by helminth-induced T helper 2 cell responses**. *J.Immunol.* 1993;151:4857-64.

283. Elias D, Wolday D, Akuffo H, Petros B, Bronner U, Britton S. **Effect of de-worming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette- Guerin (BCG) vaccination**. *Clin.Exp.Immunol.* 2001;123:219-25.

284. Bobo L, Munoz B, Viscidi R, Quinn T, Mkocha H, West S. **Diagnosis of Chlamydia trachomatis eye infection in Tanzania by polymerase chain reaction/enzyme immunoassay**. *Lancet* 1991;338:847-50.

285. Katz J, West KP, Khatry SK, LeClerq SC, Pradhan EK, Thapa MD *et al*. **Prevalence and risk factors for trachoma in Sarlahi district, Nepal**. *Br.J.Ophthalmol.* 1996;80:1037-41.

286. Bird, M., Dawson, C., Miao, Y., Schacter, J., and Lietman, T. **Does the clinical exam adequately identify ocular chlamydial infection in trachoma?** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-2002. p. 519-522.

287. Mabey, D., Holland, M., Solomon, A., Alexander, N., Massae, P., Aguirre, A., Li, S., Bailey, R., West, S., and Foster, A. **The epidemiology of ocular *Chlamydia***

trachomatis **infection in a trachoma endemic community determined by quantitative PCR.** 2002. Antalya, International Chlamydia Symposia. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. 16-6-2002. p. 495-498.

288. el Shourbagy M, Abd el Maeboud K, Diab KM, el Ghannam A, Nabegh L, Ammar S. **Genital Chlamydia trachomatis infection in Egyptian women: incidence among different clinical risk groups**. *J.Obstet.Gynaecol.Res.* 1996;22:467-72.

289. Marais NF, Wessels PH, Smith MS, Gericke A, Richter A. **Chlamydia trachomatis, Mycoplasma hominis and Ureaplasma urealyticum infections in women. Prevalence, risks and management at a South African infertility clinic**. *J.Reprod.Med.* 1991;36:161-4.

290. Cetin MT, Vardar MA, Aridogan N, Koksal F, Kilic B, Burgut R. **Role of Chlamydia trachomatis infections in infertility due to tubal factor**. *Indian J.Med.Res.* 1992;95:139-43.

291. Garland SM, Lees MI, Skurrie IJ. **Chlamydia trachomatis--role in tubal infertility**. *Aust.N.Z.J.Obstet.Gynaecol.* 1990;30:83-6.

292. De Muylder X, Laga M, Tennstedt C, van Dyck E, Aelbers GN, Piot P. **The role of Neisseria gonorrhoeae and Chlamydia trachomatis in pelvic inflammatory disease and its sequelae in Zimbabwe**. *J.Infect.Dis.* 1990;162:501-5.

293. Ballard RC,.et al. **Urethritis and associated infections in Johannesburg - the role of *Chlamydia trachomatis***. *South African Journal of Sexually Transmitted Diseases* 1981;1:24-6.

294. Mabey DC, Ogbaselassie G, Robertson JN, Heckels JE, Ward ME. **Tubal infertility in the Gambia: chlamydial and gonococcal serology in women with tubal occlusion compared with pregnant controls**. *Bull.World Health Organ* 1985;63:1107-13.

295. Walraven G, Scherf C, West B, Ekpo G, Paine K, Coleman R *et al*. **The burden of reproductive-organ disease in rural women in The Gambia, West Africa**. *Lancet* 2001;357:1161-7.

296. Pleyer U,.Baatz H. **Antibacterial protection of the ocular surface**. *Ophthalmologica* 1997;211 Suppl 1:2-8.

297. Buisman NJ, Ossewaarde JM, Rieffe M, van Loon AM, Stilma JS. **Chlamydia keratoconjunctivitis determination of Chlamydia trachomatis specific secretory immunoglobulin A in tears by enzyme immunoassay**. *Graefes Arch.Clin.Exp.Ophthalmol.* 1992;230:411-5.

298. Vaahtoranta-Lehtonen HH, Lehtonen OP, Forsten Y, Meurman OH, Saari KM. **Tear anti-Chlamydia antibodies in males with chlamydial urethritis**. *Ophthalmic Res.* 1993;25:23-9.

299. Haller EM, Auer-Grumbach P, Stuenzner D, Kessler HH, Pierer K, Muellner K *et al.* **Detection of antichlamydial antibodies in tears: a diagnostic aid?** *Ophthalmology* 1997;104:125-30.

300. Caldwell HD, Stewart S, Johnson S, Taylor H. **Tear and serum antibody response to Chlamydia trachomatis antigens during acute chlamydial conjunctivitis in monkeys as determined by immunoblotting**. *Infect.Immun.* 1987;55:93-8.

301. Holland MJ, Bailey RL, Hayes LJ, Whittle HC, Mabey DC. **Conjunctival scarring in trachoma is associated with depressed cell-mediated immune responses to chlamydial antigens**. *J.Infect.Dis.* 1993;168:1528-31.

302. Witkin SS, Jeremias J, Toth M, Ledger WJ. **Cell-mediated immune response to the recombinant 57-kDa heat- shock protein of Chlamydia trachomatis in women with salpingitis**. *J.Infect.Dis.* 1993;167:1379-83.

303. Arno JN, Xie C, Jones RB, Van Der PB. **Identification of T cells that respond to serovar-specific regions of the Chlamydia trachomatis major outer membrane protein in persons with serovar E infection**. *J.Infect.Dis.* 1998;178:1713-8.

304. Gaston JS, Deane KH, Jecock RM, Pearce JH. **Identification of 2 Chlamydia trachomatis antigens recognized by synovial fluid T cells from patients with Chlamydia induced reactive arthritis**. *J.Rheumatol.* 1996;23:130-6.

305. Abu el-Asrar AM, Geboes K, Missotten L. **Immunology of trachomatous conjunctivitis**. *Bull.Soc.Belge Ophtalmol.* 2001;73-96.

306. Derynck R, Choy L. Transforming growth factor-β and its receptors. In Thomson A, ed. *The Cytokine Handbook*, pp 593-636. London: Academic Press, 1998.

307. Cenni E, Granchi D, Vancini M, Pizzoferrato A. **Platelet release of transforming growth factor-beta and beta- thromboglobulin after in vitro contact with acrylic bone cements**. *Biomaterials* 2002;23:1479-84.

308. Omer, F. M. 2002. 2001.

309. Barral-Netto M, Barral A, Brownell CE, Skeiky YA, Ellingsworth LR, Twardzik DR *et al.* **Transforming growth factor-beta in leishmanial infection: a parasite escape mechanism**. *Science* 1992;257:545-8.

310. Gazzinelli RT, Oswald IP, Hieny S, James SL, Sher A. **The microbicidal activity of interferon-gamma-treated macrophages against Trypanosoma cruzi involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta**. *Eur.J.Immunol.* 1992;22:2501-6.

311. Williams DM, Grubbs BG, Park Snyder S, Rank RG, Bonewald LF. **Activation of latent transforming growth factor beta during Chlamydia trachomatis-induced murine pneumonia**. *Res.Microbiol.* 1996;147:251-62.

312. Rodel J, Straube E, Lungershausen W, Hartmann M, Groh A. **Secretion of cytokines by human synoviocytes during in vitro infection with Chlamydia trachomatis**. *J.Rheumatol.* 1998;25:2161-8.

313. Polo M, Smith PD, Kim YJ, Wang X, Ko F, Robson MC. **Effect of TGF-beta2 on proliferative scar fibroblast cell kinetics**. *Ann.Plast.Surg.* 1999;43:185-90.

314. Igietseme JU, Ramsey KH, Magee DM, Williams DM, Kincy TJ, Rank RG. **Resolution of murine chlamydial genital infection by the adoptive transfer of a biovar-specific, Th1 lymphocyte clone.** Reg Immunol. 1993;5:317-24.

315. Holland MJ, Bailey RL, Conway DJ, Culley F, Miranpuri G, Byrne GI et al. **T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of Chlamydia trachomatis in subjects with severe trachomatous scarring.** Clin.Exp.Immunol. 1996;105:429-35.

316. Shaw AC, Vandahl BB, Larsen MR, Roepstorff P, Gevaert K, Vandekerckhove J et al. **Characterization of a secreted Chlamydia protease.** Cell Microbiol. 2002;4:411-24.

317. Fan P, Dong F, Huang Y, Zhong G. **Chlamydia pneumoniae secretion of a protease-like activity factor for degrading host cell transcription factors is required for major histocompatibility complex antigen expression.** Infect.Immun. 2002;70:345-9.

318. Zhong G, Fan P, Ji H, Dong F, Huang Y. **Identification of a chlamydial protease-like activity factor responsible for the degradation of host transcription factors.** J.Exp.Med. 2001;193:935-42.

319. Weir RE, Morgan AR, Britton WJ, Butlin CR, Dockrell HM. **Development of a whole blood assay to measure T cell responses to leprosy: a new tool for immuno-epidemiological field studies of leprosy immunity**. J.Immunol.Methods 1994;176:93-101.

320. Elliott AM, Hurst TJ, Balyeku MN, Quigley MA, Kaleebu P, French N et al. **The immune response to Mycobacterium tuberculosis in HIV-infected and uninfected adults in Uganda: application of a whole blood cytokine assay in an epidemiological study.** Int.J.Tuberc.Lung Dis. 1999;3:239-47.

Appendix - Immunoblot scores by observer

ID No	MOMP			omp2			hsp60			pmpG			pmpG		
Observer	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	2	2	3	3	3	3	2	2	2	3	3	3	3	2	3
2	1	0	0	3	2	2	3	3	3	0	0	0	0	0	0
3	1	0	0	3	3	3	3	3	3	3	2	3	3	2	3
4	2	1	2	2	1	2	3	2	3	2	1	1	2	1	2
5	3	3	3	3	3	3	3	2	2	1	1	2	3	3	3
6	3	3	3	3	3	3	3	3	3	1	1	1	3	3	3
7	3	2	2	3	3	3	2	1	1	2	0	1	2	1	0
8	2	1	2	3	2	3	3	2	3	1	0	0	2	1	2
9	1	0	0	3	3	3	3	3	3	0	0	0	2	1	2
11	2	1	2	3	2	2	3	3	3	0	0	0	0	0	0
12	3	3	2	3	3	3	3	3	3	2	0	0	2	0	0
13	2	0	0	3	2	2	2	1	2	0	0	0	2	1	2
14	3	2	2	3	3	3	3	2	2	0	0	0	2	1	1
15	3	2	2	3	3	3	2	1	2	0	0	0	2	1	2
16	3	3	2	3	3	3	3	3	3	0	0	0	2	1	2
17	3	2	2	2	1	2	3	1	2	0	0	0	1	0	0
18	3	2	2	3	3	3	3	3	3	1	1	1	2	2	2
19	2	1	2	3	3	3	2	2	2	1	1	1	2	2	2
20	2	1	0	3	2	2	3	2	2	0	0	0	1	0	1
21	1	1	1	3	3	3	3	3	3	0	0	0	2	2	2
22	3	1	1	3	2	3	3	2	3	2	2	2	3	2	3
23	2	1	3	2	1	2	3	3	3	2	2	2	2	2	2
24	1	1	2	3	3	3	2	2	2	0	0	0	2	2	2
25	2	2	3	3	3	3	2	2	3	0	0	0	2	2	2
26	1	1	1	3	3	3	1	1	1	0	0	0	1	1	1
28	1	1	1	0	0	0	3	2	2	3	2	2	2	1	2
29	3	3	3	2	2	2	2	1	2	3	3	3	1	1	1
30	3	2	2	3	3	2	2	2	2	2	1	2	3	2	2
33	2	1	2	3	2	2	2	1	2	2	1	2	2	1	2
34	3	2	2	3	3	3	3	2	2	1	1	1	1	1	1
36	2	2	2	3	3	2	3	3	3	0	0	0	3	2	2
37	2	1	2	3	3	3	3	2	2	0	0	0	2	1	2
38	2	1	2	2	2	2	3	3	3	0	0	0	3	3	2
39	2	1	2	3	3	3	3	3	3	0	0	0	1	1	1

ID No	MOMP			omp2			hsp60			pmpGc			pmpGa		
Observer	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
41	2	2	1	3	3	2	3	3	2	3	3	3	0	0	0
42	2	1	1	1	1	1	2	1	2	1	0	0	0	0	0
43	3	2	3	2	1	2	2	1	2	3	3	3	0	0	0
44	2	2	3	2	2	2	2	2	2	2	2	2	0	0	0
45	3	3	3	3	2	2	3	3	3	2	1	2	1	0	1
47	3	2	2	2	2	2	2	1	2	3	2	3	1	0	0
48	1	1	2	1	1	0	2	2	2	1	0	0	3	2	2
49	2	2	3	1	1	1	3	2	3	0	0	0	3	2	2
50	2	2	3	2	2	2	2	3	3	0	0	0	3	3	3
51	3	1	2	3	1	2	3	2	3	0	0	0	3	2	2
53	2	1	1	3	2	2	0	0	0	0	0	0	0	0	0
54	3	2	2	3	3	3	2	1	2	1	1	1	2	1	2
55	1	1	1	3	2	2	3	2	2	0	0	0	2	1	2
56	2	2	2	3	2	2	2	1	1	0	0	0	0	0	0
57	2	1	2	3	3	3	3	3	3	0	0	0	2	1	2
58	2	1	1	2	1	2	2	1	2	0	0	0	2	2	2
59	2	1	2	3	3	3	3	3	3	1	1	2	3	2	2
61	2	2	3	3	3	3	3	3	3	0	0	0	2	1	2
64	3	2	2	2	1	1	2	1	2	0	0	0	0	1	1
65	2	1	2	2	1	2	3	2	2	0	0	0	2	1	2
66	2	1	2	1	1	1	3	3	3	0	0	0	1	0	1
67	3	2	2	3	3	3	3	3	3	0	0	0	0	0	0
68	1	1	1	3	3	3	3	3	2	0	0	0	1	1	1
69	2	2	2	3	3	3	2	2	2	2	1	2	2	2	2
70	0	0	1	2	1	2	2	1	2	2	1	2	3	2	2
71	1	1	1	2	1	2	2	2	2	3	3	3	3	3	3
72	0	0	0	1	1	1	2	1	2	0	0	0	1	1	1
73	1	1	1	2	1	2	2	2	2	2	2	3	0	0	0
74	2	2	2	3	3	3	3	2	2	2	1	2	2	2	2
75	2	1	2	3	2	2	3	2	2	2	1	2	2	1	2
76	2	2	2	3	3	3	2	2	2	2	1	2	1	1	1
77	1	1	1	1	1	1	2	1	2	0	0	0	1	1	1
79	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0
80	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
81	2	1	2	2	1	2	2	1	2	0	0	0	2	1	2
82	3	2	2	3	2	2	3	3	3	0	0	0	0	0	0
83	3	2	2	3	2	2	3	3	3	2	1	2	2	1	2
84	3	2	2	3	2	2	3	3	3	0	1	0	0	0	0
85	3	2	2	3	2	2	3	3	2	0	0	0	0	0	0

ID No	MOMP			omp2			hsp60			pmpGc			pmpGa		
Observer	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
86	3	3	3	3	3	2	3	3	3	3	3	2	3	3	2
87	3	2	3	3	2	2	3	2	3	2	1	2	2	1	2
88	3	3	3	3	2	2	3	2	2	1	1	1	2	1	2
89	3	1	2	1	1	2	2	1	2	1	0	0	2	1	2
91	2	1	1	9	9	9	3	2	2	0	0	0	2	1	2
92	3	3	3	9	9	9	2	2	2	0	0	0	0	0	0
93	2	1	2	9	9	9	2	1	1	0	0	0	0	0	0
94	3	3	3	1	1	1	2	1	2	2	1	2	1	1	1
95	3	2	2	1	1	1	3	3	3	0	0	0	0	1	1
96	3	2	2	3	2	2	3	2	2	3	2	3	3	3	2
97	2	1	2	3	3	3	3	3	3	2	1	2	2	1	2
98	3	2	2	2	2	2	3	3	2	2	2	2	2	2	2
99	3	3	3	3	3	3	3	3	3	0	0	0	2	2	2
100	1	1	1	3	2	2	3	2	2	0	0	0	2	0	0
101	1	1	1	1	1	1	3	2	2	2	0	0	1	0	0
102	3	1	2	2	1	1	3	2	2	0	0	0	0	0	0
103	3	2	2	3	2	3	1	0	1	0	0	0	0	0	0
104	3	3	3	3	3	3	2	0	2	0	0	0	0	0	0
105	3	3	2	2	2	2	2	1	2	0	0	0	0	0	0
106	3	2	2	3	2	2	2	0	2	0	0	0	0	0	0
111	3	3	2	3	2	2	3	2	2	0	0	0	0	0	0
112	3	1	2	3	2	2	3	2	2	1	0	0	0	0	0
113	2	1	2	2	1	1	2	1	1	0	0	0	2	1	1
114	3	2	2	1	1	1	3	3	3	0	0	0	0	0	0
115	3	3	2	0	1	0	3	3	2	0	0	0	0	1	0
116	3	3	3	1	1	1	3	3	2	0	0	0	1	1	1
117	3	3	2	9	9	9	3	2	3	2	1	1	0	0	0
118	3	3	3	9	9	9	3	3	3	1	1	1	0	0	0
119	3	3	3	9	9	9	3	3	2	2	2	2	2	0	1
120	2	2	2	9	9	9	2	2	2	0	0	0	0	0	0
121	3	2	2	9	9	9	3	3	3	2	2	2	2	1	2
122	1	1	2	9	9	9	3	3	3	3	2	2	2	2	2
123	2	1	2	9	9	9	3	3	3	3	2	2	2	1	2
125	2	1	2	3	3	3	2	2	2	1	0	0	3	1	1
126	2	1	2	1	0	2	2	1	2	1	0	0	2	2	2
128	2	2	2	2	1	2	2	1	2	1	0	0	2	2	2
129	1	1	1	1	0	0	3	3	2	0	0	0	0	0	0
130	3	3	2	2	1	2	3	2	2	0	0	0	0	0	0
131	2	2	2	2	1	1	3	3	3	0	0	0	1	0	0

ID No	MOMP			omp2			hsp60			pmpGc			pmpGa		
Observer	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
132	3	2	2	2	2	2	3	2	2	0	0	0	2	0	0
133	3	3	3	9	9	9	3	3	3	1	1	1	2	1	2
134	1	1	1	9	9	9	2	2	2	2	1	2	2	1	1
135	3	3	2	9	9	9	3	3	3	0	0	0	0	0	0
136	3	3	3	9	9	9	3	2	2	2	1	2	2	1	2
137	2	1	2	9	9	9	2	1	2	0	0	0	1	0	0
138	2	2	2	9	9	9	3	3	3	0	0	0	0	0	0
141	2	1	2	9	9	9	2	2	2	1	1	1	2	1	2
142	3	2	2	9	9	9	3	3	3	1	1	1	2	2	2
143	3	2	2	3	2	2	1	0	0	0	0	0	0	0	0
144	2	1	2	3	3	2	3	2	2	0	0	0	0	0	0
145	2	1	2	0	0	1	0	0	0	0	0	0	0	0	0
146	2	2	2	9	9	9	3	3	2	0	0	0	0	0	0
147	3	3	3	9	9	9	3	3	3	2	0	0	2	1	1
148	3	2	2	9	9	9	3	2	2	2	1	2	2	1	2
149	3	3	3	9	9	9	3	3	3	2	2	2	3	3	3
150	3	3	3	9	9	9	3	3	3	0	0	0	0	0	0
151	3	3	3	9	9	9	3	3	3	3	3	3	3	3	3
152	3	2	2	9	9	9	2	1	2	1	1	1	2	2	2
154	3	3	3	9	9	9	3	3	3	0	0	0	0	0	0
155	3	3	3	9	9	9	2	2	2	0	0	0	2	2	2
156	2	2	2	9	9	9	3	3	3	0	1	1	2	1	2
157	2	2	2	9	9	9	2	2	2	0	1	0	2	2	2
159	3	3	3	1	1	1	3	2	2	2	1	1	0	1	1
160	3	2	2	2	1	1	3	3	3	3	2	3	2	2	2
161	3	2	2	3	2	2	3	2	2	1	1	1	1	0	0
162	3	3	3	2	2	2	3	3	3	3	2	2	2	1	1
171	3	2	2	9	9	9	3	2	2	3	2	2	2	1	2
172	1	1	1	1	1	1	2	2	2	1	1	1	0	0	0
173	1	1	1	3	2	2	2	2	2	1	0	1	0	0	0
175	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
176	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
180	3	2	2	9	9	9	2	2	2	0	0	0	0	0	0

BLANK IN ORIGINAL