CONTRASTING FEATURES OF MENINGOCOCCAL DISEASE IN BRAZIL AND ETHIOPIA

Thesis submitted in accordance with the
requirements of the University of Liverpool
for the degree of

Doctor in Philosophy

by

Jailson de Barros Correia

Department of Medical Microbiology and
Genitourinary Medicine,

University of Liverpool

December 2004

Declaration

This thesis is the result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The clinical work was carried out at two referral hospitals in Recife, Brazil, and at local health centres and hospitals in the Southern Nations, Nationalities and Peoples Region of Ethiopia. The research work took place at the Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool.

Bacterial loads were measured by M Guiver and J Marsh and the phenotypic characterisation was carried out by S Gray, T Carr and B Lavender, at the Meningococcal Reference Unit, Manchester Medical Microbiology Partnership, Health Protection Agency, Manchester.

Jailson de Barros Correia

December 2004

To José Arnaldo, Clemilda, Yuri, Josilma
and our unborn baby.

Contents

List of Figures

List of Tables

Acknowledgements

Many people have directly or indirectly helped me in this journey and I am extremely grateful to all of them. I would like to thank Professor Tony Hart and Dr Luis Cuevas for the encouragement, guidance and inspiration.

In Liverpool, I would also like to thank Enitan Carrol (now in Malawi), Scott Hackett, James Bunn, Brian Coulter, Michael Chance, Winfred Dove, Claire Kelly, Norma Lowe, Jean Last, Brian Getty, Linda Jones, Debby Sales, Noel Blundel, Jennifer Collett and the friends at the Departments of Medical Microbiology and School of Tropical Medicine. In Manchester, I would like to thank Malcolm Guiver, John Marsh and Tony Carr. In Awassa and Yirgalem, thanks to Mohammed Yassin, Ali Hassen, Yodit Enkossa, Lemma Rabo, Emebet Solomon and the entire laboratory staff at Yirgalem Hospital.

In Recife, my gratitude goes to Miriam Silveira, Solange Ribeiro, Edmilda Carneiro, Maria Eulália Corte Real, Ana Albertina, Deize Matos, Tânia Falcão and special thanks to Nancy Correia, Maria do Carmo Duarte and Fernando Lindoso. I am also grateful to Jairo Correia, Roberto Nunes, Álvaro Vieira de Mello, João Guilherme Alves, Ruben Maggi and Antonio Carlos Figueira, also on befalf of the late Professor Fernando Figueira.

I would also like to thank my sponsors from CAPES and to express my gratitude to IMIP and the University of Pernambuco, for allowing me to come to Liverpool.

Abbreviations

AChE	Acetylcholinesterase
AIDS	Acquired Immunodeficiency Syndrome
BSA	Bovine serum albumin
CFR	Case fatality rate
CGRP	Calcitonin gene related peptide
CPAP	Continuous positive airway pressure
CSF	Cerebrospinal fluid
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ESMD	Enhanced Surveillance of Meningococcal Disease
FAM	6, carboxyfluorescein
GDP	Gross domestic product
GMSPS	Glasgow Meningococcal Septicaemia Prognostic Score
Hb	Haemoglobin
HCP	Correia Picanço Hospital (*Hospital Correia Picanço*)
Hct	Haematocrit
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
IBGE	Instituto Brasileiro de Geografia e Estatística
IL-	Interleukin-
IL-1ra	Interleukin-1 receptor antagonist
IMIP	Pernambuco Mother and Child Health Institute *(Instituto Materno Infantil de Pernambuco)*

IQR	Interquartile range
Km	Kilometre
LACEN	Central Laboratory for the State of Pernambuco (*Laboratório Central de Pernambuco*)
LOS	Lipoolygosaccharide
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MD	Meningococcal disease
MM	Meningococcal meningitis
MRU	Meningococcal Reference Unit
MS	Meningococcal septicaemia
MSM	Meningococcal septicaemia with meningitis
NISBC	National Institute for Biological Standards and Control (UK)
No.	Number
NPV	Negative predictive value
NSB	Non-specific binding
$PaCO_2$	Carbon dioxide arterial pressure
PBS	Phosphate-buffered solution
PCR	Polymerase chain reaction
PHLSMF	Public Health Laboratory Service Meningococcal Forum
PI	Principal investigator
PICU	Paediatric intensive care unit
pNPP	P-nitro phenyl phosphate
PPV	Positive predictive value
RANTES	Regulated upon activation, normal T cell expressed and secreted
RBC	Red blood cell; red blood count
Rpm	Rotations per minute

SD	Standard deviation
SDS	Sequence detection system
SNNPR	Southern Nations, Nationalities and Peoples Region of Ethiopia
SUS	National health system, Brazil *(Sistema Único de Saúde)*
TAMRA	6, carboxy-tetramethylrhodamine
TNF-α	Tumour necrosis factor alpha
UK	United Kingdom
USA	United States of America
WBC	White blood cell; blood cell count
WHO	World Health Organisation

Abstract

CONTRASTING FEATURES OF MENINGOCOCCAL DISEASE IN BRAZIL AND ETHIOPIA

By Jailson de Barros Correia

Meningococcal disease (MD) presents in a wide spectrum of syndromes, including meningitis (MM), septicaemia (MS) and septicaemia with meningitis (MSM). Clinical presentation varies across the world and little is known of why some people have MM and others have MSM or MS. In Brazil MD is endemic, serogroups B and C meningococci are predominant and most cases present with MSM or MS. Ethiopia is within the African 'meningitis belt' and has regular epidemics of MM due to serogroup A meningococcus.

To compare clinical features, polymerase chain reaction (PCR), bacterial loads, patterns of cytokine and neuropeptide response in infected hosts, consecutive cases of suspected MD were enrolled in Brazil and Ethiopia. The inclusion criteria would have missed individuals with meningococcal infection but without rashes or meningeal signs. 132 confirmed cases of MD were enrolled in Ethiopia over two epidemic seasons and 93 were enrolled in Brazil over a period of 2 years. All Ethiopian cases had MM and the case fatality rate (CFR) was 5.6%, whereas in Brazil 31 (33%) had MM, 51 (55%) had MSM and 11 (12%) had MS and the overall CFR was 14%.

PCR targeting the *ctrA* gene was assessed as a diagnostic tool. Sensitivity varied from 94 to 88% and specificity ranged from 29 to 100%, respectively, when *ctrA* PCR was compared to culture alone or to a gold standard with clinical and laboratory criteria. Quantitative *ctrA* PCR was then used to measure bacterial loads in the blood and cerebrospinal fluid (CSF) of cases. Bacterial loads in blood were found to be associated with fatal outcome in Brazilian cases with MS or MSM ($p<0.001$), but not in cases of MM from Brazil or Ethiopia. Bacterial loads in CSF were not found to be associated with fatal outcome. A subgroup of cases from Ethiopia had high bacterial loads in blood despite not having signs of septicaemia. Plasma levels of Interleukin (IL) 1β, IL-6, IL-8 and TNF-α of Brazilian cases were positively correlated with blood bacterial load and higher levels of these cytokines were associated with fatal outcome ($p<0.001$). Ethiopian cases who had high bacterial loads in blood also had higher levels of pro-inflammatory cytokines in plasma, but none was associated with fatal outcome. The levels of RANTES were negatively correlated with bacterial load in blood and lower levels of RANTES were seen in fatal Brazilian cases ($p<0.001$), but not in Ethiopian cases ($p=0.31$). In Brazilian cases, the levels of IL-10 and IL-1ra were higher in the plasma of non-survivors ($p<0.001$, both for IL-10 and IL-1ra), but lower in the CSF of non-survivors ($p=0.032$ and $p=0.001$ for IL-10 and IL-1ra respectively). In most Ethiopian cases, IL-10 was undetectable in the plasma, regardless of outcome. The levels of substance P and calcitonin gene related peptide (CGRP) were higher in plasma than in the CSF of cases of MD, but were not found to be associated with outcome or bacterial load.

Brazilian and Ethiopian cases of MD had different clinical presentations and appear to have different patterns of cytokine response in relation to the bacterial load and outcome. The mechanisms that lead to death in MM and in MS or MSM appear to differ, as each form has compartmentalised responses to infection.

Chapter One

1 GENERAL INTRODUCTION

1.1 MENINGOCOCCAL DISEASE

Nearly 200 years after Viesseux described the first well documented epidemic of what was then called 'cerebrospinal meningitis' (Cartwright, 1995), meningococcal disease (MD) remains a major public health problem with estimates of 500,000 cases occurring annually worldwide, causing around 50,000 deaths (Tikhomirov *et al.*, 1997).

In the developed world and the UK in particular, after a long period of few successful developments in the reduction of mortality in MD, recent progresses have brought new hopes. There have been reports of reduction in case-fatality rates of children admitted to paediatric intensive care units, attributable to a combination of early recognition, better organisation of care and more aggressive treatment regimens (Thorburn *et al.*, 2001). The successful introduction of the conjugate meningococcal C vaccine has dramatically reduced the incidence of disease and mortality due to serogroup C in the UK (Trotter *et al.*, 2004). Moreover, the introduction of a national PCR diagnostic service has enabled an improvement in case-ascertainment (Kaczmarski *et al.*, 1998) enabling a better understanding of the epidemiology of the disease, whereas a large number of studies have addressed the different patterns of host response (see Hackett *et al.*, 2001), enabling a better understanding of the disease pathogenesis. Lately, new

hopes of development of a serogroup B meningococcal vaccine have emerged through new technologies such as reverse vaccinology (Adu-Bobie *et al.*, 2003).

On the other hand, the picture from the developing world has been less favourable. The meningitis belt of Sub-Saharan Africa has been expanding to new areas (Molesworth *et al.*, 2002), the emergence of less common serogroups such as W135, have caused major concern (Decosas & Koama, 2002) and the usual explosive epidemics stretch the health care systems to their limits. In countries like Brazil, meningococcal septicaemia still has very high case-fatality rates (Donalisio *et al.*, 2000).

1.2 THE MENINGOCOCCUS

N. meningitidis is a Gram-negative bacterium, exclusive of humans, which is relatively fragile and fastidious. It has a bean shape and is found in pairs (diplococci), as seen in figure 1.1. Like other gram-negative bacteria, it has two cell membranes, with a rigid peptidoglycan layer in between (fig. 1.2). The outer leaflet of the outer membrane is rich in lipooligossacharide (LOS) molecules. The hydrophobic portion of the LOS molecule is lipid A (the active moiety of endotoxin). The variable portion of the LOS molecule provides a basis for immunotyping, whereas the acidic polysaccharide capsule of *N. meningitidis* forms the basis for serogrouping (there are 13 serogroups currently recognised: A, B, C, D, H, I, K, L, W135, X, Y, Z and Z'). Meningococci can be typed and subtyped on the basis of epitopes on outer-membrane proteins of class 2 or 3 (porB), and class 1 (porA), respectively. In addition, virulent meningococci

express filamentous projections called pili (fig. 1.1.), which enable meningococci to attach to mucosal epithelial cells and to endothelial cells in the central nervous system (see Hart & Rogers, 1993).

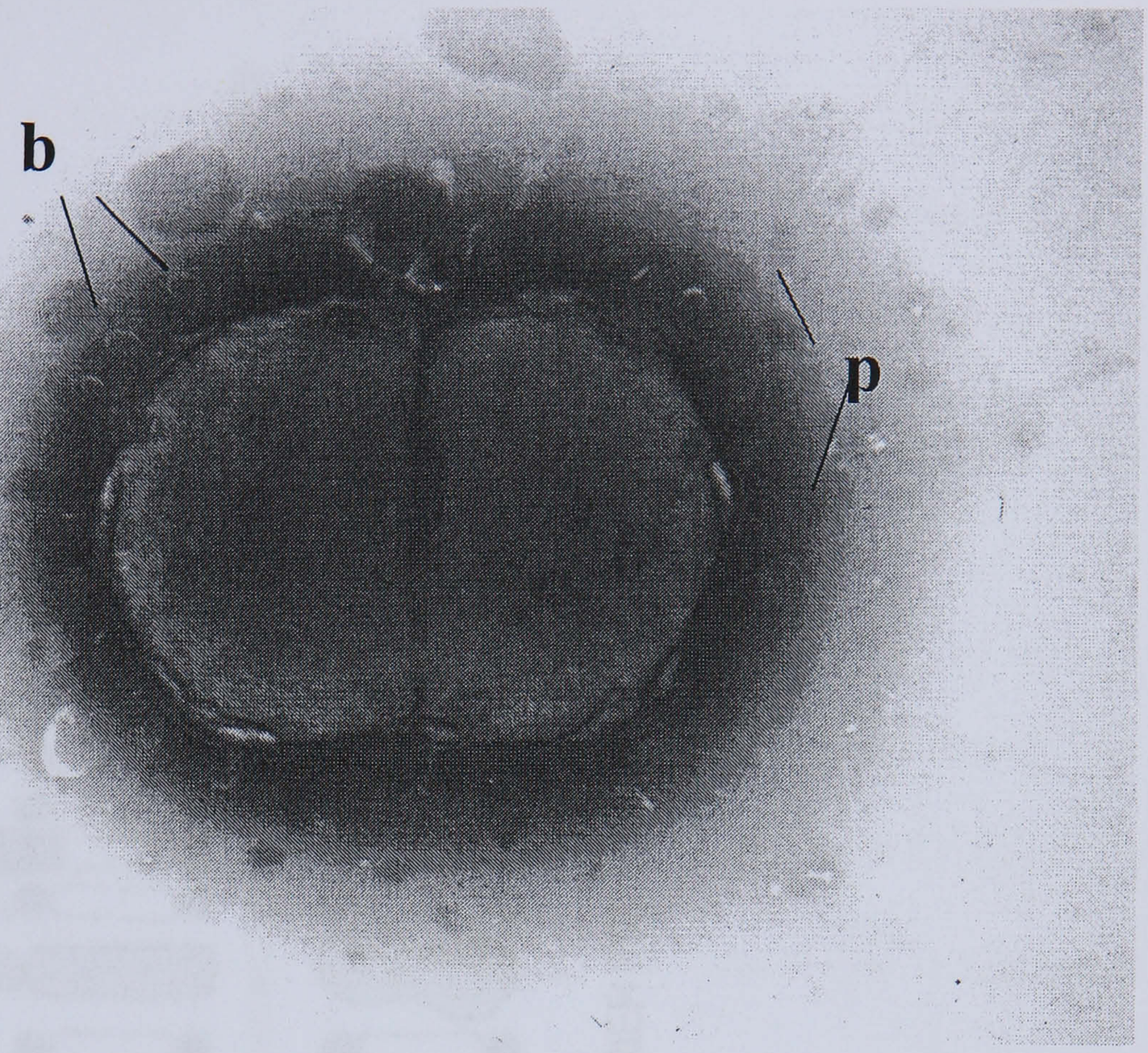


Fig.1.1. Electron photomicrograph of paired meningococci. The outer membrane of the meningococcus continually produces and releases vesicles rich in endotoxin, or 'blebs' (b). Pili in also shown (p).

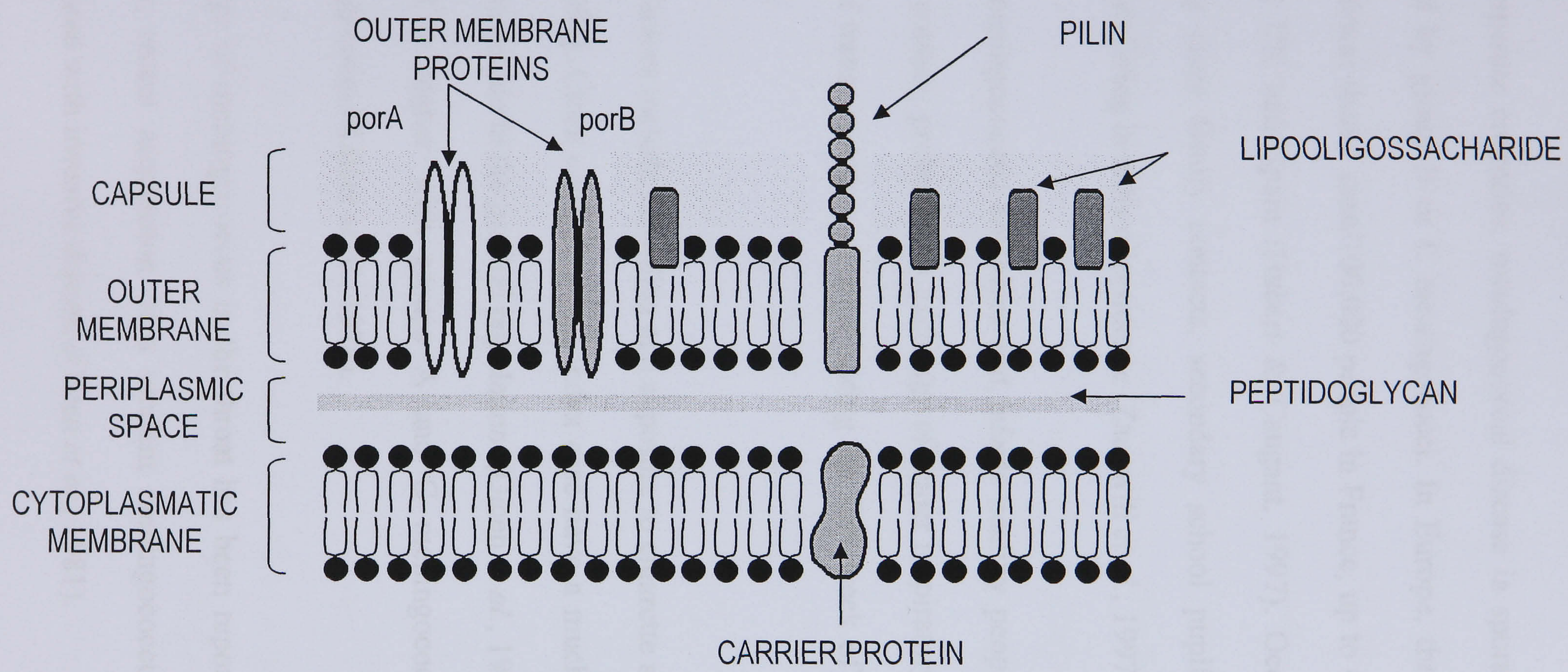


Fig.1.2. Structure of *N. meningitidis* cell wall.

1.3 EPIDEMIOLOGY

In temperate countries, meningococcal disease is sporadic and most commonly caused by group B or C meningococci. In Europe, the annual incidence varies from fewer than 1 case/100,000 people in France, up to 4–5 cases/100,000 people in the UK and Spain (Hubert & Caugant, 1997). Occasional outbreaks occur among close family contacts, secondary school pupils, military recruits, and students living in halls of residence (Zangwill *et al.*, 1997).

The meningococcus colonises and infects healthy people, and is transmitted by close contact, probably by exchange of upper respiratory tract secretions and the risk of transmission is greatest during the first week of contact (De Wals *et al.*, 1981).

Risk factors include crowding and exposure to cigarette smoke (Stanwell-Smith *et al.*, 1994). Close contacts of an index case have a much higher risk of infection than do people in the general population. (Olcen *et al.*, 1981). The risk of epidemic spread is higher with groups A and C meningococci than with group B meningococci (Kaiser *et al.*, 1974).

Carriage of meningococcus in the throat has been reported in up to 10–15% of people; recent acquisition of a virulent meningococcus is more likely to be associated with invasive disease (Olcen *et al.*, 1981).

Sub-Saharan Africa has regular epidemics in countries lying in the expanded "meningitis belt", reaching 500 cases/100 000 people during epidemics, which are

usually due to serogroup A, although recent outbreaks of serogroup W135 cause concern. (Molesworth *et al.*, 2002, Hart & Cuevas, 1997, Decosas & Koama, 2002).

In Brazil, MD is a relevant public health issue. Disease occurs all-year-round, although it is more common in the winter. Focal and relatively unpredictable upsurges and outbreaks occur from time to time, and important epidemics were described in many large cities in the seventies. The recent introduction of routine immunisation for type B *Haemophilus influenzae* (Hib) has decreased the number of cases of meningitis caused by this agent, bringing the meningococcus to the top of the list of the commonest causes of bacterial meningitis in that country (Donalisio *et al.*, 2000, Sacchi *et al.*, 2001).

1.4 PATHOGENESIS

The interaction between *N. meningitidis* and the human host can lead to an array of situations. Most people are colonised and become carriers of the meningococcus in the nasopharynx for a variable amount of time, without having symptoms of disease. In the other end of the spectrum, a few individuals develop meningitis, septicaemia or both. These can lead to death or significant sequelae such as hearing impairment or limb amputations (Carrol *et al.*, 1999).

The release of inflammatory mediators by host cells in response to the presence of meningococcal lipooligossacharide (LOS) has been extensively studied in experimental models and in human cases of MD (see Hackett *et al.*, 2001). An

increasing number of reports have been showing association of extensive inflammation and fatal outcome in MD (van Deuren *et al.*, 2000) and other types of endotoxic shock. These studies led to a number of trials of novel therapies aimed at modulating host response, most of which have yielded disappointing but perhaps not unexpected results given the complexity of the interactions between the cytokines in the infected host (Carrol *et al.*, 2001).

Please see section 6.1 for a more detailed review on host inflammation in the pathogenesis of MD.

1.5 CLINICAL SPECTRUM OF MENINGOCOCCAL DISEASE

Clinical presentation of meningococcal disease varies widely, from transient bacteraemia to fulminant septicaemia with septic shock (Thomson *et al.*, 1990). Although there is some degree of overlapping between these clinical forms, it is acceptable to categorise cases into meningococcal meningitis (MM), meningococcal septicaemia with meningitis (MSM) and meningococcal septicaemia without meningitis (MS) (Riordan *et al.*, 1995a, Hackett *et al.*, 2002b).

The clinical presentation of meningococcal disease in the African meningitis belt differs from what is found elsewhere serogroup B *N. meningitidis* predominates. The purpuric meningococcal rash, which indicates septicaemia and results in a high case-fatality rate is observed in up to 75 % of cases in the UK, but only in less than 5 % of cases in sub-Saharan Africa (Hart & Cuevas, 1997), where most

cases occur as meningitis. In other regions of the developing world, such as in Brazil, the presentation resembles what is observed in industrialised countries (Donalisio *et al.*, 2000). Whether these differences are due to environmental, bacterial or host factors remain unclear.

Please see section 3.1 for more detailed review of clinical features in MD.

1.6 MORTALITY AND PROGNOSTIC SCORES

Mortality is usually highest in infants and adolescents, and is related to disease presentation and availability of therapeutic resources (Hart & Cuevas, 1997). In developed countries, case fatality rates have been around 19–25% for septicaemia, 10–12% for meningitis plus septicaemia, and less than 1% in meningitis alone, but an overall reduction in mortality was observed in recent years in people admitted to paediatric intensive care units (Thorburn *et al.*, 2001).

A number of scores to predict outcome of meningococcal disease have been developed. Riordan (2002) prospectively evaluated several scoring systems and found that the Glasgow Meningococcal Septicaemia Prognostic Score (GMSPS)(Sinclair *et al.*, 1987) was the best predictor of survival. However, this score assumes availability of probes to measure skin temperatures and was only validated for cases with septicaemia and is of little relevance in lower resourced settings.

1.7 RATIONALE AND AIMS OF THIS STUDY

1.7.1 Rationale

Little is known of why MD presents as meningitis alone in some circumstances and as septicaemia in others. Although meningococcal disease in a major public health concern worldwide, the burden of disease is hardest felt in the developing nations, but little is known about host response to MD in patients from these settings. The recent availability of quantitative PCR methods has enabled measurement of bacterial loads in clinical specimens and thus there was an opportunity to correlate levels of bacterial load with cytokines and other markers of inflammation in MD.

To date, no data are available concerning the performance of PCR in developing countries or in areas where meningococci other than B or C are predominant. There is also no information on meningococcal DNA loads from settings outside Western Europe and the amount of information on bacterial loads in the CSF is scarce.

Despite the extensive literature on the associations between cytokines and outcome in MD, few studies have addressed a large number of pro and anti-inflammatory cytokines at the same point in time in the same cohort of patients. Data on cytokines from patients from settings other than Western developed countries are scarce and to date there are no reports on substance P and calcitonin gene related peptide (CGRP) in the context of MD. Moreover, no reports have yet been published on the correlations of cytokines and neuropeptides and the levels of bacterial load in MD.

1.7.2 Aims

The general aims of this study are to describe some bacterial and host factors potentially involved in the pathogenesis of MD, their effect on disease presentation and outcome; and to contrast these observations between cases of MD from divergent developing world settings.

1.7.2.1 Specific aims

- To describe the clinical features of cases with suspected MD in Brazil and Ethiopia, the relative frequency of confirmed cases amongst these, their clinical presentation and outcome.
- To assess the performance of the *ctrA* PCR assay for the diagnosis of meningococcal disease in Brazil and Ethiopia, according to the type of clinical sample and disease presentation and to describe the performance of *siaD* and *myn* PCR assays for determining meningococcal serogroup.
- To describe bacterial loads in blood and in CSF of Brazilian and Ethiopian patients with confirmed meningococcal disease, according to clinical presentation and outcome.
- To describe pro and anti-inflammatory cytokines, chemokines and neuropeptides in Brazilian and Ethiopian confirmed cases of meningococcal disease according to outcome, bacterial load and compartment (blood versus CSF).

Chapter Two

2 SUBJECTS AND METHODS

2.1 OVERVIEW

In this descriptive study, patients with meningococcal disease were recruited prospectively from two disparate developing world settings. The study locations were selected for convenience and operational ease amongst sites with links with the University of Liverpool. The Brazilian study site illustrates an urban setting from a middle-income Latin American country, where meningococcal disease is endemic, most cases have group B *N. meningitidis* and at least half of the cases present with signs of septicaemia. In contrast, the Ethiopian study site exemplifies the situation in the highly endemic, epidemic-prone Sub-Saharan African meningitis belt, where most cases present with group A *N. meningitidis*, meningitis alone and health resources are even scarcer. Differences and similarities between the two study sites are outlined here. In every other chapter of this thesis, data are presented separately for each location, and then comparisons between the two sites are made based on trends and patterns.

2.2 DEFINITIONS

2.2.1 Case definitions

Case definitions for *confirmed*, *probable*, *possible* and *not* meningococcal disease were adapted[1] from the "Guidelines for Public Health Management of Meningococcal Disease in the UK" (PHLSMF, 2002) and the "Enhanced Surveillance of Meningococcal Disease Project Group" (Davison *et al.*, 2002).

Confirmed case of meningococcal disease: clinical diagnosis of meningitis and/or septicaemia (see definitions in section 2.2.2) and confirmatory laboratory evidence of *N. meningitidis* infection, either by isolation or detection of meningococcal DNA from a normally sterile site.

Probable case of meningococcal disease: clinical diagnosis of meningitis and/or septicaemia where the public health physician, in consultation with the clinician and the microbiologist, considers that meningococcal infection is the most likely diagnosis. This includes cases without confirmatory evidence but with typical petechial or purpuric rash or secondary cases when the index has been confirmed. The laboratory findings include presence of Gram-negative diplococci or a positive antigen or rising antibody test, if available.

---

[1] This thesis is primarily concerned with acute, invasive forms of meningococcal disease (notably meningitis and septicaemia with a rash); therefore, presentations such as chronic meningococcaemia or localised infections (such as isolated conjunctivitis, orbital cellulitis and septic arthritis) were not included in the working case definition. Moreover, availability and quality of Gram staining and antigen tests were uneven in the study, thus, cases were considered as *confirmed* only when the presence of *N. meningitidis* was ascertained by isolation or identification of meningococcal DNA from a normally sterile site.

Possible case of meningococcal disease: clinical diagnosis of meningitis and/or septicaemia where the public health physician, in consultation with the clinician and the microbiologist, considers that diagnoses other than meningococcal disease are at least as likely.

Not meningococcal disease: any suspected case in whom after investigation their illness was thought not to be due to *N. meningitidis* and an alternative diagnosis was given. This includes cases of meningitis or septicaemia with a positive culture or a positive PCR for an alternative bacterial cause and cases thought to be due to a viral aetiology.

2.2.2 Clinical presentations

Working definitions for meningococcal *meningitis* and *septicaemia* (with or without meningitis) were derived[2] from the literature (Thomson *et al.*, 1990, Riordan *et al.*, 1995a, Hackett *et al.*, 2002a).

Meningococcal meningitis (MM): a case with clinical signs of meningitis (fever, vomiting, nuchal rigidity), but no skin rash; together with one or more abnormalities in the CSF, such as: a) white cell count > 10 x 10^6 cells/L, b) presence of Gram-negative diplococci, c) positive culture, PCR or antigen test.

Meningococcal septicaemia (MS): a case with systemic signs and symptoms of infection (sepsis) but no nuchal rigidity; plus a skin rash, which can be purpuric

---

[2] Meningococcal disease presents itself in a spectrum of clinical forms, which may overlap with each other. However, distinct patterns of morbidity and mortality have been associated with each of the forms defined here and this categorisation has been chosen for its simplicity and comparability with previous studies.

(petechial, ecchymotic) or, less often, maculopapular; along with laboratory evidence of meningococcal infection, such as isolation of meningococci or detection of *N. meningitidis* DNA by PCR.

Meningococcal septicaemia + meningitis (MSM): a case presenting with features of septicaemia and meningitis at the same time. There are systemic signs and symptoms of infection (sepsis) plus a purpuric or maculopapular skin rash, along with clinical features of concomitant meningitis, such as nuchal rigidity, and / or laboratory evidence of CSF involvement, which can be characterised by a CSF WBC count > 10×10^6 cells/L.

Septicaemic forms of meningococcal disease: refers to all cases with septicaemia, both with and without concomitant meningitis.

2.2.3 Other relevant definitions

Carrier: individual in whom *N. meningitidis* can be retrieved from the nasopharynx by swabbing. Most carriers are asymptomatic and unaware of their carriage status (PHLSMF, 2002).

Contact: those recently exposed to an index case of meningococcal disease and who might have a higher risk of developing the disease when compared with the general population. To require public health actions, this exposure is either a close, prolonged contact in the same household or a direct contact with respiratory secretions (PHLSMF, 2002).

Suspected case of meningococcal disease: any person presenting with symptoms where clinicians think their illness might be due to *N. meningitidis* (Davison *et al.*, 2002).

Suspected case of acute bacterial meningitis: cases presenting with sudden onset of fever (>38.5°C rectal or 38.0°C axillary) accompanied by nuchal rigidity (or, in children under one year of age, a bulging fontanelle) plus a turbid CSF (WHO, 1998).

Suspected case of meningococcal septicaemia: a case with systemic signs and symptoms of infection (i.e. fever, malaise, patient "unwell") plus a skin rash, which can be purpuric (petechial, ecchymotic) or, less often, maculopapular (Hackett *et al.*, 2002a).

Sepsis: the systemic response to infection, manifested by two or more of the following: a) hyper or hypothermia; b) high heart rate; c) high respiratory rate (or low $PaCO_2$); d) high or low white blood cell count (or >10% immature forms). Defining values are age dependent (Bone *et al.*, 1992).

Severe sepsis: when sepsis is associated with organ dysfunction, hypoperfusion, or hypotension (Bone *et al.*, 1992).

Septic shock: is sepsis with hypotension, despite adequate fluid resuscitation, along with the presence of perfusion abnormalities (Bone *et al.*, 1992).

2.3 INCLUSION AND EXCLUSION CRITERIA AND CATEGORISATION OF CASES FOR ANALYSIS

Inclusion and exclusion criteria were the same in Brazil and Ethiopia. These were criteria for enrolment on admission, thus set to be sensitive to allow most cases of meningococcal meningitis and / or septicaemia (with a skin rash) to be included.

2.3.1 Inclusion criteria

Patients with suspected bacterial meningitis or meningococcal septicaemia (with a skin rash, as defined in section 2.2.3) who attended one of the participating hospitals during the study period.

2.3.2 Exclusion criteria

Patients were excluded from the study if presented one or more of the following conditions:

- had a ventriculo-peritoneal shunt at the time of enrolment;
- had a known diagnosis of HIV-AIDS at the time of enrolment;
- were less than 28 days of age;
- had no blood and / or CSF sample collected on admission or in the first 12 hours of hospital stay;
- had refused to participate or decided to withdraw from the study.

2.3.3 Categorisation of cases for analysis

After all laboratory results (including PCR) became available, cases were retrospectively categorised into *confirmed*, *probable*, *possible* and *not* meningococcal disease (as defined in section 2.2.1), as well as grouped according to their clinical presentation into *meningococcal meningitis* (MM), *septicaemia* (MS) or *meningitis + septicaemia* (MSM) (as in section 2.2.2).

Cases were also categorised according to outcome into survivors and non-survivors, taking into account the mortality observed during hospital stay.

2.4 SAMPLE SIZE CALCULATION

The sample size was calculated using the version 2.0.20 of the Sample Size Determination in Health Studies software (World Health Organisation, Geneva). For each laboratory variable to be studied, an attempt was made to find reference ranges in the literature. Then, each of the available reference values and their respective standard deviations were entered in the software for the calculation of sample sizes, aiming to detect a 3-fold difference (2-sided) between population means of survivors and non-survivors, according to the formula:

$$n = \frac{2\sigma^2\left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{\left(\mu_1 - \mu_2\right)^2}$$

Where n is the minimum required sample size per group; σ is the standard deviation; α is the desired level of significance; $1\text{-}\beta$ is the desired power; μ is the

population mean and *z* is the z-value or percentage point in the normal distribution corresponding to the power or the 2-sided significance level.

The level of significance was set to 5% and the power to 80%. Slightly diverse results for *n* were obtained when different cytokines were entered because each cytokine had a unique variability around the reference value, which affected the sample size. The median value obtained for *n* was 9, meaning that at each location, there should be at least 9 cases in each group of survivors and non-survivors in order to be able to detect 3-fold differences between the groups in most of the cytokines studied. Assuming an overall case fatality rate of 10% in Ethiopia and 20% in Brazil and adjusting for a 20% loss to follow up, the minimum sample sizes required were 108 *confirmed* cases in Ethiopia and 54 in Brazil.

2.5 ETHICAL CONSIDERATIONS

In this observational study, interferences with the management routines of the participating hospitals and health centres were kept to a minimum. During the sessions carried out to explain the research protocol and as part of the interaction between the research team and the local staff, issues on current best practice in the management of meningococcal disease were discussed, with an emphasis on locally relevant information.

Informed consent was obtained from patients or their guardians. English, Portuguese and Amharic versions of the consent form can be found in the appendices 1 to 3.

The research ethics committee in the Liverpool School of Tropical Medicine, as well as local and national committees in Brazil and Ethiopia (National Committee for Science and Technology) approved the study.

2.6 THE BRAZILIAN STUDY SITE

2.6.1 Background

Brazil is the largest country in South America, with an area of 8.5 million km^2 and a population of 176 million inhabitants, 80 % of whom live in urban areas (IBGE, 2004). According to the World Health Report (WHO, 2003), in the year 2001 Brazil had a per capita gross domestic product (GDP) of 7.5 thousand international dollars[3], of which 7.6% (equivalent to 570 international dollars per capita) were spent on health. In 2002, life expectancy at birth was 68.9 years and the child mortality rate (risk of dying before the age of 5 years, per 1000) was 42 for males and 34 for females. These core health indicators have improved over the last few decades, but these improvements were not evenly distributed and considerable regional inequalities remain a major problem. Northeast Brazil, for instance, is comparably less developed than the wealthier South or Southeast, both economically and in terms of health indicators.

---

[3] Gross domestic product (GDP) per capita is the per capita market value of the total final output of goods and services produced in a country over a specific period. The international dollar is a common currency unit that takes into account differences in the relative purchasing power of various currencies. Source: WHO (2003) *The World health report: 2003: shaping the future*, World Health Organisation, Geneva..

The city of Recife, capital of the state of Pernambuco, is one of the largest in the northeast region. It houses nearly 3 million inhabitants in its metropolitan area, most of whom live in unplanned overcrowded urban settlements called *favelas* (shantytowns). More than three quarters of the population of Recife rely upon the national health system (*SUS - Sistema Único de Saúde*) for their health needs. Since the epidemics of meningococcal disease in the 1980s, state health authorities have centralised the diagnostic facilities and management of cases under the SUS at two referral centres: Correia Picanço Hospital (HCP) and the Pernambuco Mother and Child Health Institute (IMIP). Thus, suspected cases attending any health centre or hospital in the metropolitan region are regularly referred to either HCP or IMIP, usually after a brief assessment and initial treatment. Fig. 2.1 shows maps of Brazil and Pernambuco.

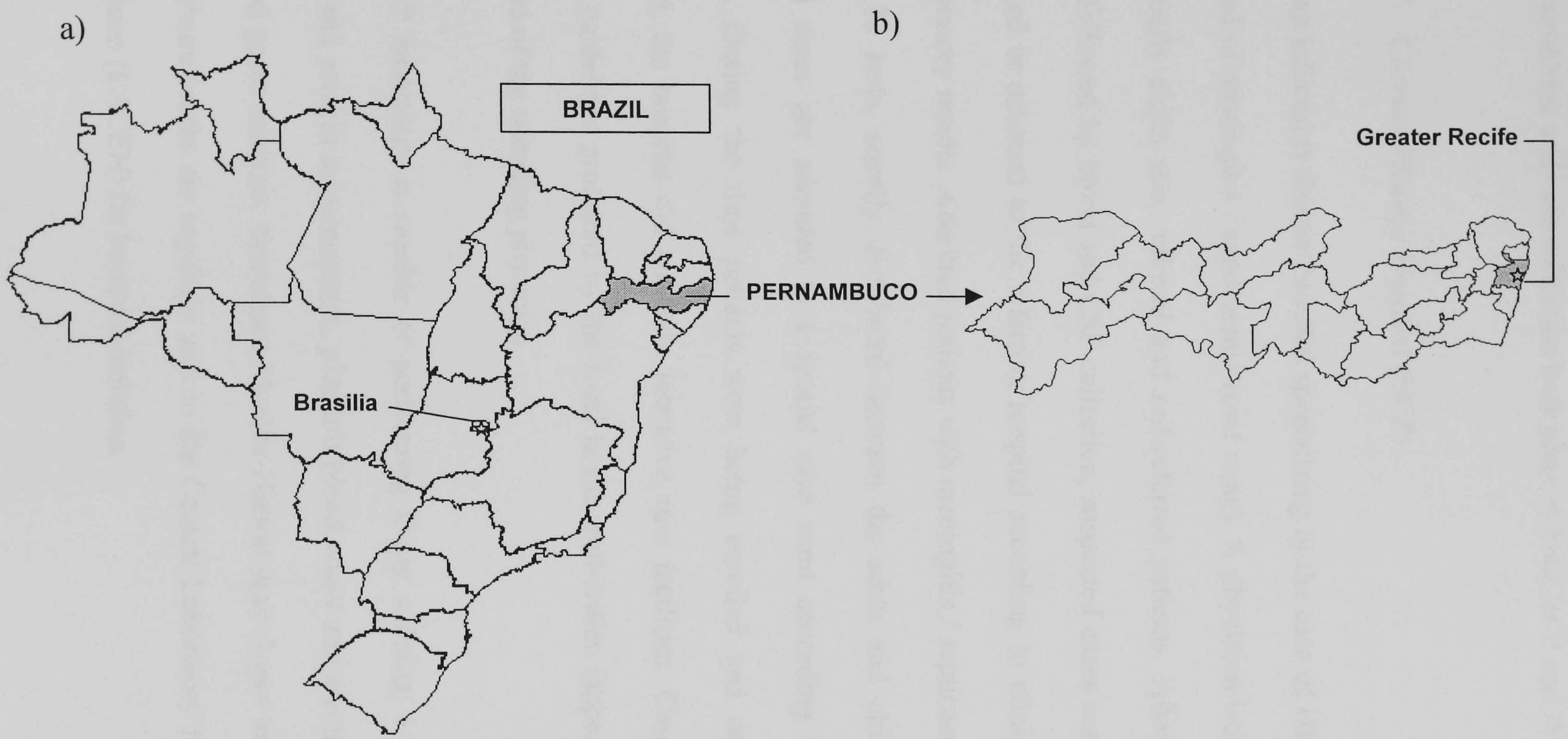


Fig. 2.1. Maps of Brazil and Pernambuco. a) The state of Pernambuco is situated in the tropical north-eastern region of Brazil; b) and its capital city, Recife, where the study took place, is located in the Atlantic coast.

2.6.1.1 Participating hospitals

Patient enrolment and data collection took place at both HCP and IMIP.

2.6.1.1.1 Correia Picanço Hospital (HCP)

HCP is an infectious disease hospital specializing in the care of HIV-AIDS (west wing) and of meningitis / septicaemia (east wing). A physician-led team working in 12 hourly shifts sees referred and self-referred patients. After initial triage, usually followed by blood and CSF collection, suspected cases may be admitted, discharged or returned to the referring hospital according to clinical assessment and laboratory results. Admitted patients with meningitis / septicaemia are sent to one of 32 beds, equally distributed between the adult and children's wards. Selected cases are admitted to a special care ward according to the disease severity. During the time patients were being enrolled and data was being collected, the hospital did not have intensive care facilities. Case management follows guidelines produced by the local health authorities (appendix 4), at the discretion of the attending physician.

The HCP laboratory is capable of performing Gram staining, CSF cell count, glucose and protein measurements, plus full blood count and electrolytes, though not blood gases analysis. Inoculated Mueller-Hinton agar slopes and bottles with blood culture broths are regularly sent to the Central Laboratory for the State of Pernambuco (LACEN) for bacterial isolation.

2.6.1.1.2 Pernambuco Mother and Child Health Institute (IMIP)

IMIP is a 550-bedded tertiary teaching hospital specializing in paediatrics and maternal health. Doctors from its busy paediatric emergency department see self-referred and referred patients from the metropolitan region of Recife and beyond. IMIP is a reference centre for treatment of bacterial meningitis and meningococcal septicaemia in children and has a dedicated ward with 9 beds (one of which in a cubicle for initial isolation of cases). It is the only public hospital in the metropolitan region with intensive care facilities available for cases of meningococcal disease. However, its Paediatric Intensive Care Unit (PICU) is not able to cope with the demand from all areas of the hospital, as it has been estimated that only one in 10 cases with an indication for PICU admission eventually get a bed in the Unit. Therefore, although meningococcal disease is considered a priority, cases with similar severity may or may not be admitted, depending solely on the availability of a place at the time. IMIP has guidelines for the treatment of meningococcal disease in children, which is available in the appendix 5.

The 24-hour laboratory service is capable of performing Gram staining, CSF cell count, glucose and protein measurements, plus full blood count, electrolytes and blood gases. Its own microbiology laboratory performs bacterial isolation for patients with suspected meningitis.

2.6.2 Patient recruitment and follow-up

Between July 2001 and June 2002, consecutive patients attending IMIP or HCP with suspected meningococcal disease were enrolled.

2.6.2.1 Phase one

From July to September 2001, the principal investigator (PI) was in Recife. During these first three months of patient enrolment and data collection, staff from both hospitals was instructed to contact the PI on the arrival of a suspected case. The PI went immediately to the hospital, obtained consent from patients or their guardians, collected blood and CSF samples (or processed samples if already collected) and clinically assessed patients on admission. The PI went back to the hospital on a daily basis to perform clinical follow-up until the patient was discharged or died. This initial period served as a pilot study, testing the procedures, instruments and routines but also as a period of sensitisation of the hospital staff and training of a research assistant. During this time, the study protocol was explained and discussed in seminars with the medical, nursing and laboratory staff of both hospitals, but also on an individual basis with those willing to collaborate. In an agreement with the medical staff and directors of HCP, the admission forms of the hospital were updated to comply with the information requirements for assessing severity scores, which could improve quality of recorded data for the study but also facilitate case management. At IMIP, similar forms were made available to the paediatricians from the emergency department.

2.6.2.2 Phase two

After these initial three months, the doctors on-duty, collaborating on a volunteer basis, enrolled the patients themselves, performing the initial clinical assessment and arranging for the samples to be properly collected and stored.

Algorithms with inclusion criteria (appendix 6), along with instructions for the collection of study samples were made available at the triage room (HCP) and at every consulting cubicle at the emergency department (IMIP).

Demographic data, clinical history, physical examination and information on treatment, follow-up and outcome were recorded on a standardised form (see appendix 7).

Although the PI returned to Recife for further 6 months to oversee the study, for most of the time data collection was supervised by Dr Maria Duarte, head of the Paediatric Intensive Care Unit at IMIP, with the assistance of Ms Nancy Correia, who obtained consent, handled samples and made sure the records were kept appropriately. These investigators kept regular exchange of information on the study progress and any issues raised during the collection of data were discussed between them.

2.6.3 Sample collection, processing and storage

2.6.3.1 Blood

On admission, peripheral blood was collected by venepuncture and distributed into tubes with and without an anti-coagulant for full blood count and electrolytes; a bottle with broth for blood culture and an extra tube with ethylenediaminetetra-acetic acid (EDTA), marked 'research', which was immediately put in the laboratory fridge. Within 12 hours of collection, the EDTA tube was gently mixed and an aliquot of 500 µL of whole blood put in a screw cap tube for further PCR testing. The EDTA tube was then centrifuged at 10,000 rpm for 10 minutes and the separated plasma distributed in 250 µL aliquots into screw cap tubes. All screw cap tubes were labelled and stored at -70°C until testing.

On day 2 and around day 5 of hospital stay, whenever clinical conditions required a blood sample, further EDTA blood samples were obtained for the study and processed in the same way.

2.6.3.2 Cerebrospinal fluid

On admission, CSF was collected by lumbar puncture with a sterile spinal needle. Around 1 mL of CSF was put into a universal tube (for Gram staining, cell count and biochemistry); three to five drops of CSF were placed into a tube with a slope of Mueller-Hinton medium and an extra 1 mL of CSF into a universal plastic tube marked 'research', which was immediately stored in the hospital fridge. Within 12 hours of collection, the CSF research tubes were mixed gently and transferred to

screw cap tubes in 250 μL aliquots. The screw caps tubes were labelled and frozen at -70°C until testing.

2.6.4 Laboratory procedures performed locally

Full blood count, CSF cell count and differential, Gram staining, measurement of glucose and proteins were performed locally, following standard routine procedures in both IMIP and HCP laboratories.

Procedures for identification of bacterial isolates from blood or CSF cultures followed local versions of the World Health Organisation guidelines (WHO, 1998). Once the laboratory technician identified a *N. meningitidis*, the isolate was swabbed from a chocolate-agar plate and placed into a tube with beads and bacterial preservatives, then frozen at –70°C until transported to the UK.

2.7 THE ETHIOPIAN STUDY SITE

2.7.1 Background

Ethiopia is located south of the Sahara, in East Africa, and occupies an area of 1.1 million km^2. It has a population of nearly 69 million inhabitants, with 85% living in rural areas. It ranks amongst the poorest countries in the world, with a per capita GDP of just 382 international dollars a year (in 2001) and a total health expenditure of only 14 international dollars per capita (or 3.6% of the GDP). In 2002 life expectancy at birth was 48 years and the child mortality rate (risk of

dying before the age of 5 years, per 1000) reached 185 for males and 168 for females (WHO, 2003).

Ethiopia has had epidemics of meningococcal disease reported since as early as 1935, gaining its place in Lapeyssonnie's classical description of the 'meningitis belt' (Lapeyssonnie, 1963), though these have been traditionally associated with the north-western districts of the country. In more recent years, however, the Southern Nations, Nationalities and Peoples Region (SNNPR) has experienced large epidemics with marked seasonality, with peak incidence observed in the dry months between January and March. Fig. 2.3 shows maps of Africa and Ethiopia, highlighting the SNNPR location.

Doctors from the Health Bureau in Awassa, SNNPR, had collaborative links with the Liverpool School of Tropical Medicine and this facilitated the selection of this region for the Ethiopian field study, which took place over the two epidemic seasons of 2003 and 2004.

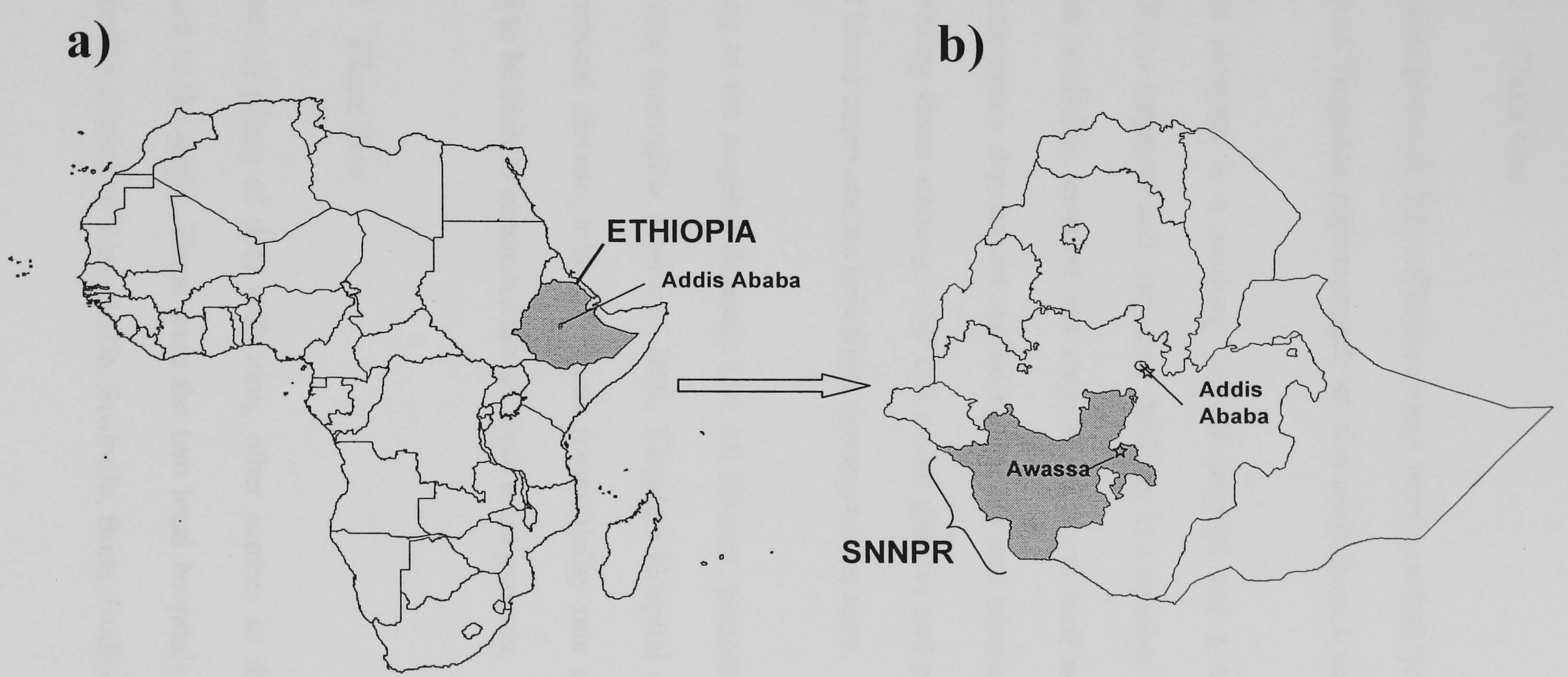


Fig. 2.2. Maps of Africa and Ethiopia. a) Ethiopia is situated in East Africa, south of the Sahara. b) The town of Awassa is the administrative centre for the Southern Nations, Nationalities and People's Region (SNNPR), where the participating hospitals were located.

2.7.1.1 Participating hospitals and health centres

2.7.1.1.1 Phase One

In the initial phase of data collection, cases were enrolled from a single hospital in the town of Yirgalem, approximately 40 Km south from Awassa.

Yirgalem Hospital is a teaching general hospital and a referral centre for the region. It has inpatient and outpatient facilities in a number of specialties and has adult and paediatric, medical and surgical wards, as well as a maternity wing. It has an emergency department staffed by doctors and laboratory facilities capable of performing Gram staining, CSF cell count, glucose and protein measurements, plus full blood count and malaria films, amongst other tests.

According to the hospital database (Dr Ali Hassen, personal communication) in the previous meningitis season of 2002, Yirgalem Hospital received 437 cases of meningococcal disease, with 27 deaths (case-fatality rate of 6.2%). Temporary tents had to be built to accommodate all these extra patients.

2.7.1.1.2 Phase Two

In the second phase of data collection, other centres in the SNNPR area also contributed to the study. These were the two local hospitals in Dilla and Gidole, plus the health centres of Yirgacheffe, Bushullo, Bona, Bodity and Shone.

2.7.2 Patient recruitment and follow-up

2.7.2.1 Phase One

From January to March 2003, consecutive patients with suspected meningococcal disease were enrolled on admission. The principal investigator (PI) performed the initial assessment and daily follow-up of cases, together with Dr Ali Hassen, head of the department of paediatrics, and with the assistance of nursing staff, who also helped as interpreters. The attending physicians collected CSF (as part of the management routine) and laboratory personnel collected blood samples. Demographic data, history, findings of physical examination, information on hospital treatment, follow-up and outcome were recorded on a standardised form (appendix 8).

2.7.2.2 Phase Two

As the number of cases enrolled in the first phase of data collection was lower than expected, the collaborative research group discussed a number of alternatives. There were time and funding constraints for another field study with the same characteristics to be repeated in the following dry season. However, one of the members of the research team (Dr Mohammed Yassin) was also involved with another project in the same region of Ethiopia, which had different objectives but shared the same inclusion criteria and was due to be in progress during the meningitis season. An agreement was then established between the two research teams.

So, from January to May 2004, patients were recruited by a team of local collaborators, under the supervision of Dr Mohammed Yassin, from 3 hospitals and 5 health centres in the SNNPR. Information on clinical history, disease presentation and outcome were made available for each patient.

2.7.3 Sample collection, processing and storage

2.7.3.1 Blood

On admission, peripheral blood was collected by venepuncture and placed into 2 tubes containing EDTA, one for full blood count and the other for the study. This was immediately put in the laboratory fridge. Within 12 hours of collection, the EDTA tube was gently mixed and an aliquot of 500 μL of whole blood put in a screw cap tube for further PCR testing. The EDTA tube was then centrifuged at 10,000 rpm for 10 minutes and the separated plasma placed in a screw cap tube, where it was stored at -20°C until transported to Liverpool.

In phase one of patient enrolment; EDTA blood samples were also obtained for the study on day 2 and around day 5 of hospital stay, whenever clinical conditions required a further blood sample. In phase 2 of data collection, only plasma was kept for the study.

2.7.3.2 Cerebrospinal fluid

On admission, CSF was collected by lumbar puncture with a sterile spinal needle and distributed into a universal tube (for Gram staining, cell count and

biochemistry); a trans-isolate medium bottle and an extra universal plastic tube marked 'research', which was immediately stored in the hospital fridge. Within 12 hours of collection, the CSF research tubes were mixed gently and stored in labelled screw cap tubes, which were frozen at -20°C until transported to Liverpool.

2.7.4 Laboratory procedures performed locally

Full blood count, CSF cell count and differential, Gram staining, measurement of glucose and proteins were performed at Yirgalem Hospital, following standard routine procedures. Other tests, requested at the discretion of the attending physicians, such as malaria thick blood films were also recorded. These tests were only available for patients enrolled in the first phase of data collection.

Procedures for identification of bacterial isolates from CSF cultures followed the World Health Organisation guidelines (WHO, 1998). Once the laboratory technician identified *N. meningitidis*, the isolate was swabbed from a chocolate-agar plate and placed into a tube with beads and bacterial preservers, then frozen at –70°C until transported to Liverpool.

2.8 LABORATORY METHODS

2.8.1 Immunoassays

2.8.1.1 Detection of IL-1β, IL-1ra, IL-6, IL-8, IL-10, RANTES and TNF-α

DuoSet® ELISA development kits (R&D Systems Europe, Abingdon, UK) were used for the measurement of IL-1β, IL-1ra, IL-6, IL-8, IL-10, RANTES and TNF-α in plasma and CSF samples. These development kits provide cytokine-specific standards, as well as capture and detection antibodies, along with a set of instructions for the preparation of the remainder reagents needed. All these kits share similar principles and general protocols.

2.8.1.1.1 Principle of the assay

The assay is based on the antibody sandwich principle. Initially, a mouse anti-human capture antibody specific to the analyte of interest is bound to a microtitre plate by passive adsorption, thus creating the solid phase. Unbound antibody is removed by washing the plate. A blocking reagent is added to reduce non-specific binding. Following a wash, samples, standards, and controls are then incubated with the solid phase antibody, which captures the analyte. After washing away unbound analyte, a biotin-conjugated, goat[4] anti-human detection antibody is added. This detection antibody binds to a different epitope of the molecule being measured, completing the sandwich.

---

[4] The species of serum for the coating and detecting antibodies must be different, as they must not react with each other. Likewise, the anti-species conjugate cannot react with the coating antibody.

Following a wash to remove unbound detection antibody, horseradish peroxidase (HRP) conjugated streptavidin is added, as a detection reagent. The plate is washed again to remove excess conjugate and a 1:1 mixture of Tetramethylbenzidine (TMB) and hydrogen peroxide is added as a substrate solution. As TMB/peroxide turns blue when modified by HRP, colour develops in proportion to the amount of bound analyte. Finally, an acidic solution (sulphuric acid) is added to stop the reaction, turning the solution yellow. The intensity of the yellow colour is measured by a spectrophotomoter, at a test wavelength of 450 nm.

2.8.1.1.2 General Protocol

Vials with standards, capture and detection antibodies were reconstituted in either PBS or distilled water, according to the manufacturer's instructions. Wash buffer, block buffer, reagent diluents and other solutions were prepared as described in the appendix 9.

2.8.1.1.2.1 Plate Preparation: coating the capture antibody

For each cytokine, the capture antibody stock solution was diluted to the working concentration in PBS without carrier protein (the various concentrations are shown in table 2.1). Immediately after that, 100 µL of the diluted capture antibody were transferred with a multi-channel pipette to each of the 96 wells of the flat-bottom, transparent, polystyrene microplates (Corning Life Sciences, Corning, USA). The plates were then sealed and incubated overnight at room temperature until being washed in the following day.

Wash procedure: plates were placed in an automated plate washer (OpsysMW, Dynex Technologies, Chantilly, USA) programmed for aspirating the wells and then dispensing 400 µL of wash buffer to each of them. This was repeated twice for a total of 3 cycles, with a final aspiration at the end of the third cycle. Plates were then inverted and blotted against a clean paper towel to remove any remaining excess fluid from the wells.

After being washed, plates were then blocked by adding 300 µL of the block buffer to each well, and incubated at room temperature for a minimum of 1 hour. This was followed by 3 further cycles of the wash procedure as described above and, after blotting against clean towels, the plates were sealed and kept in a container with desiccant (silica gel), at +8°C until the assays were run, within the following two weeks.

2.8.1.1.2.2 Assay Procedure

All reagents were brought to room temperature prior to the assay. The standard stock solutions were diluted to the recommended higher standard concentration and two-fold serial dilutions were prepared. Plasma and CSF samples were vortexed for 15 seconds each, before dilution in one of two types of reagent diluent (table 2.1). Diluted samples and standards were then added to the plate (100 µL, per well) in a pre-designed layout; the plates were covered and incubated for 2 hours at room temperature, then washed as described above.

In the meantime, the detection antibody solution was diluted to the working concentration in reagent diluent (table). After plates were washed, 100 µL of the detection antibody solution were added to each well, plates were covered with a

new adhesive strip, incubated for 2 hours at room temperature and then washed, as above.

Horseradish peroxidase (HRP) conjugated streptavidin was then added to the plate wells (100 μL per well). Plates were incubated (away from direct light) for 20 minutes at room temperature, and washed again.

For colour development, a 1:1 mixture of H_20_2 and Tetramethylbenzidine was then added to the wells (100 μL per well) as a substrate solution. Plates were incubated in the dark for another 20 minutes, when 50 μL of a stop solution (2 N H_2S0_4) were added to the wells. Plates were gently tapped to ensure thorough mixing and were ready to go to the microplate reader.

Table 2.1. Concentration of antibodies and standards in DuoSet® ELISA development kit assays for the measurement of IL-1β, IL-1ra, IL-6, IL-8, IL-10, RANTES and TNF-α.

Analyte	Capture antibody working concentration (µg/mL)	Detection antibody working concentration (ng/mL)	Standard concentrations (pg/mL)		Reagent diluent*
			Maximum	Minimum	
IL-1β	4	100	500	7.8	A
IL-1ra	10	100	2500	39.1	A
IL-6	2	200	600	9.4	B
IL-8	4	20	2000	31.3	B
IL-10	4	400	4000	62.5	A**
RANTES	1	10	1000	15.6	A
TNF-α	4	300	1000	15.6	B

*Reagent diluents: A = 1% BSA in PBS, pH 7.2 – 7.4; B = 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2 – 7.4. See appendix 9 for preparation instructions. ** Reagent diluent with 2% heat inactivated normal goat serum.

2.8.1.1.2.3 Determining optical densities

Immediately after adding the stop solution, the optical density of each well was determined in a microplate reader (Multiskan MCC340, Titertek, Finland). The test wavelength was set to 450 nm, and a reference (correction) filter set to 540 nm, to adjust for optical imperfections in the plate, as recommended by the manufacturer. The reader was programmed to deduct the readings of each well to those of the blank well(s).

2.8.1.1.3 Curve fitting

The absorbance readings were expressed as optical density (OD) and were manually entered into a Microsoft Excel spreadsheet. Values of OD were plot in the *y* axis against the concentration in the *x* axis. The concentration of each analyte in each well was calculated from the standard curve derived from each plate, using linear regression as reference, according to the function:

$$y = a + bx$$

Where *y* is the measured OD, *a* is the y-intercept defining the line, *b* is the slope of the line and *x* is the value for the concentration of the analyte.

The best fit for the data was obtained when both the *x* and *y* axes were in logarithmic scale, so OD readings and concentrations were log transformed prior to curve fitting. Thus, the formula can be expressed as:

$$concentration = anti\log\left(\frac{y-a}{b}\right)$$

Because samples were usually tested in variable dilutions, the result given by the equation above was then multiplied by the dilution factor to find the final concentration of the analyte in each sample.

2.8.1.1.4 Quality control

Each plate assay was assessed for quality control criteria. Individual samples were re-diluted and re-assayed if their OD readings were above the OD of the maximum standard. Entire plates were rejected and every sample re-assayed if: a) the linear regression coefficient (R^2) of the standard curve was < 0.90; b) blank well(s) OD readings were >0.2 or c) more than half the samples in the plate had readings above the reading of the maximum standard.

2.8.1.2 Substance P

Concentrations of Substance P in plasma and CSF samples were determined by enzyme immunoassay (R&D Systems Europe, Abingdon, UK). All reagents needed were provided in the kit. See appendix 10 for details.

2.8.1.2.1 Principle of the assay

This assay is based on the competitive binding technique. Substance P present in a sample competes with a fixed amount of alkaline phosphatase-labelled Substance P for sites on a rabbit polyclonal antibody. During the incubation, the rabbit

polyclonal antibody binds to the goat anti-rabbit antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution (p-nitro phenyl phosphate, pNPP) is added to the wells to determine the bound enzyme activity. Immediately following colour development, the absorbance is read at 405 nm. The intensity of the colour is inversely proportional to the concentration of Substance P in the sample. The sensitivity of the Substance P assay is typically less than 8.0 pg/mL.

2.8.1.2.2 Protocol

All reagents and samples were brought to room temperature prior to the assay. The polystyrene 96-well microplate, pre-coated with a goat anti-rabbit polyclonal antibody, was removed from the pouch. The wash buffer concentrate was diluted in distilled water to prepare the wash buffer. The standard stock solution was diluted to the recommended higher standard concentration (10,000 pg/mL) and four-fold serial dilutions were prepared, with a lower standard of 9.8 pg/mL. All samples were vortexed for 15 seconds before being diluted (1:2) in assay buffer.

Assay buffer (100 µL) was added to the non-specific binding (NSB) well and 50 µL of standard or sample to the remaining wells. Then 50 µL of Substance P conjugate was added to each well and 50 µL of Substance P antibody solution to all wells but the NSB. Plates were incubated for 2 hours at room temperature on a horizontal orbital microplate shaker.

Plates were then washed in an automated plate washer (OpsysMW, Dynex Technologies, Chantilly, USA) programmed for aspirating the wells and then dispensing 200 µL of wash buffer to each of them. This was repeated twice for a

total of 3 cycles, with a final aspiration at the end of the third wash. Plates were then inverted and blotted against a clean paper towel to remove any remaining excess fluid from the wells.

The substrate solution (pNPP) was then added (200 µL, per well) and left incubating for one hour on the benchtop, at room temperature. Finally, 50 µL of trisodium phosphate was added to stop the reaction.

Immediately after adding the stop solution, the optical density of each well was determined in a microplate reader (OpsysMR, Dynex Technologies, Franklin, USA). The test wavelength was set to 405 nm. The reader was programmed to deduct the readings of each well to those of the NSB blank well(s).

2.8.1.2.3 Curve fitting

The OpsysMR reader was attached to a microcomputer with curve fitting software (Revelation Quicklink, version 4.24). The concentration of Substance P in each well was calculated from the standard curve derived from each plate, using a four-parameter logistic (sigmoid) curve as reference, according to the equation:

$$Y = \frac{[a-d]}{\left[1+\left(\frac{x}{c}\right)^{b}\right]} + d$$

Where y is the measured OD, x is the value for the concentration of the analyte and the four parameters are: a the maximum response, b the shape factor (determines the gradient of the curve), c the response midway between the maximum and minimum responses and d the minimum response.

The software was programmed to run the algorithm for the equation above and then multiply the results by 2 (the dilution factor) to find the final concentration of the analyte in each sample.

2.8.1.3 Calcitonin Gene Related Peptide (CGRP)

Concentrations of CGRP in plasma and CSF samples were determined by enzyme immunoassay (Spi-Bio, Massy, France). All reagents needed were provided in the kit. See appendix 11 for details.

2.8.1.3.1 Principle of the assay

This assay is based on a double-antibody sandwich technique. The kit supplies plates coated with a CGRP-specific monoclonal antibody as a solid phase. The CGRP present in samples or standards binds this capture antibody during incubation. An acetylcholinesterase (AChE) -Fab' conjugate is also added to the plate wells and binds selectively to a different epitope on the CGRP molecule. This allows the formation of an antibody-antigen-antibody sandwich, which is immobilised on the plate so the excess reagents may be washed away. The concentration of the human CGRP is then determined by measuring the enzymatic activity of the AChE using the Ellman's Reagent. The AChE tracer acts on the Ellman's Reagent to form a yellow compound. The intensity of the colour, which is determined in a spectrophotometer, is proportional to the amount of the human CGRP present in the well.

2.8.1.3.2 Protocol

All reagents were brought to room temperature prior to the assay. The assay buffer was reconstituted in distilled water and was used to reconstitute the lyophilised standards and controls. In case of plates assaying plasma samples, both standards and controls were diluted in CGRP-free human plasma instead (for details, see appendix 11). The higher standard concentration was 1000 pg/mL and serial 2-fold dilutions were prepared, with a lower standard of 7.81 pg/mL. Plasma and cerebrospinal fluid samples were vortexed for 15 seconds, diluted in assay buffer and tested directly, without extraction.

As recommend in the kit insert, the pre-coated polystyrene microplate was placed in the automated plate washer (OpsysMW, Dynex Technologies, Chantilly, USA) programmed for aspirating the wells and then dispensing 300 µL of wash buffer to each of them. This was repeated four times for a total of 5 cycles, with a final aspiration at the end of the fifth wash. The plate was then inverted and blotted against clean paper towels to remove any remaining excess fluid from the wells.

Reagents were then dispensed to the wells; 100 µL of assay buffer to non-specific binding (NSB) wells and equal volumes of diluted samples, controls or standard solutions to the remaining wells, following a pre-designed plate layout. Then 100 µL of anti-CGRP AChE tracer was added to each well. The plate was covered with an adhesive strip and incubated for 16-20 hours at +4 °C.

On the following day, the plate was washed again for 3 times with the wash buffer. After that, 200 µL of freshly reconstituted Ellman's Reagent was added to each well, including the blank well(s). The plate was incubated in the dark, at

room temperature for 30 to 60 minutes and then placed into the microplate reader (OpsysMR, Dynex Technologies, Franklin, USA) set to read light absorbance at 405 nm wavelength. The reader was programmed to deduct the readings of each well to those of the blank well(s).

2.8.1.3.3 Curve fitting

Four-parameter logistic curves were use to fit the data and allow calculation of CGRP concentrations, as discussed for Substance P. However, the definitions for parameters *a* and *d* in the sigmoid equation for CGRP are inverted. This is because the shape factor (*b*) is positive in the CGRP assay (i.e. the higher the OD the higher the concentration, as opposed to the negative relation in competitive assays such as Substance P). Please refer to section 2.8.1.2.3.

2.8.2 Bacteriology

Meningococcal isolates from Brazil and Ethiopia were kept at -70°C in bacterial preserver beads until these beads were carefully inoculated into chocolate-agar plates[5]. After streaking, plates were kept in a 5% CO_2 atmosphere incubator at 37°C for 24h.

Pure isolates were then transferred to chocolate-agar slopes so they could be re-incubated and transported to the Meningococcal Reference Unit (MRU) in Manchester, UK, for phenotypic characterisation.

---

[5] Chocolate agar is a rich non-selective medium that is generally used for demanding aerobic bacteria including meningococci (36g GC II agar base, 10g haemoglobin powder, 100 mL horse serum, 10 mL IsoVitalex enrichment and 900 mL of high-quality water).

In cases of mixed colonies, these were sub-cultured in selective media (such as GC medium[6]) and re-incubated for further 24h. Then, an oxidase test was performed to determine the presence of cytochrome oxidase. In this test, the reagent tetramethyl-p-phenylenediamine hydrochloride is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain, as in the case of meningococci (CDC, 1998). Oxidase-positive isolates growing in GC medium were then transferred to chocolate-agar slopes, re-incubated and sent to the MRU.

2.8.2.1 Phenotypic characterization

At the MRU in Manchester, in-house co-agglutination using rabbit polyclonal antibody was used for polysaccharide serogroup determination (A, B, C, X, Y, Z, 29E and W135). Isolates were also screened with serogroup A, B and C mouse monoclonal antibody (MAb) supplied by the UK National Institute for Biological Standards and Control (NISBC) in a dot-blot ELISA format.

Serotypes and serosubtypes were also determined using mouse MAb in the dot-blot ELISA. In brief, this consisted of dotting bacterial suspensions on nitrocellulose filters and incubating with specific MAb and enzyme-labelled secondary antibodies (Kuipers *et al.*, 2001). The NIBSC meningococcal serotyping MAb panel included: serotypes P3.1, P2.2a, P2.2b, P3.4, P3.11, P3.14, P3.15, P3.21, P2.22 and serosubtypes P1.1, P1.2, P1.3, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15, P1.16 and P1.19.

---

[6] GC (Thayer-Martin) medium is used for the selective isolation of meningococci or gonococci from clinical material (GC base: 13g/L agar, 23g/L peptone, 5g/L sodium chloride, 1g/L starch).

2.8.3 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (PCR) procedures were based in the Taqman method and performed in an automated PCR platform (Perkin-Elmer Applied Biosystems Sequence Detection System 7700, Norwalk, USA) at the Meningococcal Reference Unit, Health Protection Agency, Manchester, UK.

2.8.3.1 Principle

As in any PCR, the basic principle of the technique is to use *in vitro* enzyme-catalysed DNA synthesis to produce a large number of identical copies of the target DNA. This is achieved by running several PCR thermo-cycles, each consisting of a heat-induced denaturation of the double-stranded DNA, which allows annealing of pre-designed primers that, in the presence of a thermostable DNA polymerase, extend to create up to twice as many copies of the target DNA at the end of the cycle. By repeating the process several times, an exponential multiplication of the target DNA molecules is obtained.

2.8.3.1.1 Taqman PCR

The Taqman PCR method employs the nucleolytic activity of *Taq* polymerase to release a fluorescent reporter dye linked to an oligonucleotide probe, which hybridises specifically to the target DNA. The sequence of events in each cycle of the meningococcal Taqman PCR is shown in figure 2.3.

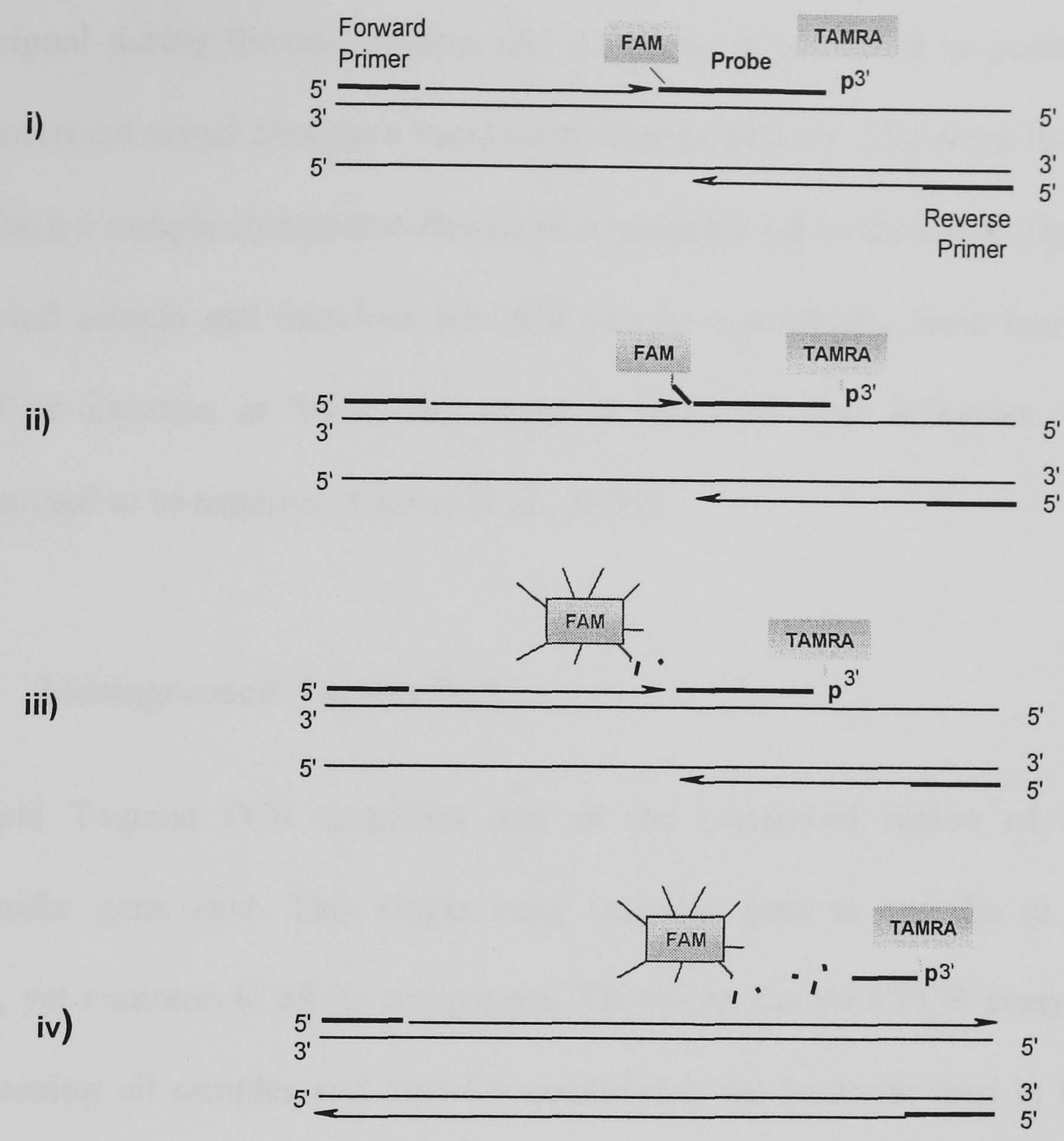


Fig. 2.3. Sequence of events during a cycle in the Taqman PCR. The system employs a dual-labelled fluorescent probe where FAM (6, carboxyfluorescein) is the reporter dye and TAMRA (6, carboxy-tetramethylrhodamine) is the quencher. i) Heat-induced denaturation of the double-stranded DNA allows the probe to hybridise to the target sequence bound by the primers; ii) primers extend causing strand displacement; iii) the probe is digested by the 5' exonuclease activity of *Taq* DNA polymerase, releasing the fluorescent reporter dye; iv) polymerisation is completed.

The automated sequence detection system (SDS) 7700 continuously monitors the fluorescent signal during thermal cycling and a sample is identified as positive when the fluorescent signal exceeds a background threshold level. The point in the cycling at which a sample crosses this threshold is proportional to the DNA copies in the extracted sample and therefore the PCR can be quantifiable from known standards. If no increase in fluorescent signal is observed after 45 cycles, the sample is assumed to be negative (Guiver *et al.*, 2000).

2.8.3.1.1.1 Meningococcal Taqman PCR

Meningococcal Taqman PCR amplifies part of the conserved region of the capsular transfer gene *ctrA*. This single copy capsular gene is specific to *N. meningitidis*, yet common to all its serogroups. Therefore, the *ctrA* PCR assay is used for screening all samples and also for quantifying the bacterial load in the positive ones.

A group-specific sialyltransferase gene probe *siaD* is used to determine the serogroup and confirm the *ctrA* PCR results for groups B, C, Y or W135. For serogroup A, a probe directed against an operon encoding four open reading frames, designated *mynA*, *mynB*, *mynC* and *mynD*, is used (Guiver *et al.*, 2000, Hackett *et al.*, 2002a).

2.8.3.1.1.2 Multiplex Taqman PCR

Taqman technology enables simultaneous testing for different pathogens causing bacterial meningitis / septicaemia by the use of specific DNA targets in a multiplex PCR. For *Streptococcus pneumoniae*, the target to be amplified is in the

pneumolysin gene (*ply*), which encodes the haemolysin species-specific protein toxin produced by all clinically relevant pneumococcal serotypes. For *Haemophilus influenzae*, the *bexA* gene encoding the capsulation-associated BexA protein is used as a target. The *bexA* gene is present in all capsulated *H. influenzae* strains (Corless *et al.*, 2001).

2.8.3.2 Protocol

All samples were screened for meningococci with a *ctrA* assay. Positive meningococcal samples were retested with group specific *siaD* and *myn* assays. Most samples were also screened for pneumococci using the *ply* assay.

2.8.3.2.1 Preparation of controls

The control meningococcal isolates were grown overnight on a blood agar plate at 37°C in an atmosphere of 5% CO_2, then suspended in sterile Elga water. In a spectrophotometer set to read at 650nm wavelength, the suspensions' optical densities (OD) were measured, recorded and standardised by dilution to an OD of 0.1. The meningococcal suspensions were then diluted to 1/1000 (approximately 100 cells per reaction), boiled for 15 minutes and placed at -20°C for 1 minute. The denatured 1/1000 dilutions were then centrifuged for 5 minutes (4°C) at 12000xg and distributed into 25µl aliquots which were stored at -20°C, ready for use.

In addition to the culture positive control, extracted positive and negative plasma controls plus plasmid standards containing 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3, copies /mL were prepared prior to each assay.

2.8.3.2.2 DNA extraction from clinical samples

Nucleic acid was extracted from clinical samples by validated methods prior to the assay. Gentra DNA extraction kits (Flowgen, Lichfield, UK) were used for DNA extraction from whole blood and DNAzol (Molecular Research Center, Cincinnati, USA) for extraction of DNA from plasma and CSF samples. Details can be found in the appendix 12.

2.8.3.2.3 Preparation of the reaction mix

Preparation of master mix was carried out in a PCR clean room using a PCR laminar flow cabinet. It consisted of mixing the appropriate forward and reverse primers, the FAM-labelled probe and the Universal master mix (please see appendix 12). Primers and probes for *ctrA*, *siaD* and *myn* assays were used accordingly.

2.8.3.2.4 Amplification using the automated Sequence Detection System 7700

In each well of the reaction plate, 2 μL of extracted sample, control or standard was added to 23 μL of Taqman PCR reaction mix, following a pre-designed plate layout.

The microwell plate was then placed in the Sequence Detection System 7700 (Perkin-Elmer Applied Biosystems, Norwalk, USA) programmed with the following cycling parameters: heating at 50°C for 5 min, 95°C for 10 min followed by 45 cycles of a two-stage temperature profile of 95°C for 15 seconds and 60°C for 1 minute. The SDS 7700 system continuously measures the amount of PCR product in the sample during cycling and records the cycle in which the amplification plot of a positive sample crosses a signal threshold. This threshold is set at 10 standard deviations above the mean of the background fluorescence emission for all wells between cycle l and 15. The standards should all amplify to at least the 10^4 copies /mL for a valid run.

2.8.3.3 Curve fitting and estimation of bacterial load

As the amount of the target DNA present in a given sample is amplified exponentially during the PCR procedure, a logarithmic transformation was used to obtain a linear relation between the amount of target DNA (plotted in the *x* axis) and the number of PCR cycles (plotted in the *y* axis).

Knowing the cycle (*n*) in which the fluorescent emission crossed the signal threshold and having fitted a regression plot to the standard curve, it is possible to determine the amount of DNA in each sample at the start of the PCR by applying the linear regression equation as seen in section (2.8.1.1.3):

$$P_0 = anti\log\left(\frac{n-a}{b}\right)$$

Where P_0 is the amount of the target DNA product present in the sample at the start of the PCR, a is the y-intercept defining the line, b is the slope of the line and n is the PCR cycle number in which the florescent signal crossed the threshold.

The amount of target DNA in the sample at the start of the PCR procedure is equivalent to the bacterial load, as there is only one copy of the *ctrA* gene per meningococci, and is expressed as number of DNA copies / mL of the sample.

2.9 DATABASE, GRAPHS AND STATISTICAL PROCEDURES

2.9.1 Database

Data from the patient assessment and follow-up forms were entered into a Microsoft Access 97 database, checked for typing errors and entries out of range an then exported to a Microsoft Excel 97 spreadsheet. Using the unique study number as the key identifier, laboratory results were added to the database by cutting, pasting and merging spreadsheets. After checking for inconsistencies, the database was then saved in Microsoft Excel format, which could be read by any statistical package.

2.9.2 Graphs

Histograms, bar and pie charts, scatter plots, box and whisker plots and other graphs appearing throughout the thesis were prepared in Microsoft Excel 97, Minitab 14.0, SPSS 11.0 or Sigmaplot 8.0. In box and whisker plots, the box represents the distance between the quartiles; the line crossing the box represents

the median and the lower and upper whiskers represent the 10^{th} and 90^{th} percentiles respectively.

2.9.3 Statistical procedures

Analyses were carried out using SPSS for Windows (version 11.0) and Win Episcope 2.0.

For each continuous variable, frequency distribution histograms and inverse normal plots were used to assess the normality of the data. Variables with right-skewed distributions were log-transformed and assessed for lognormal distribution. Where appropriate, normality tests (e.g. Kolmogorov-Smirnov or Shapiro-Wilk) were performed. Variables with non-normal distributions were described and analysed using non-parametric tests.

Associations between categorical variables were assessed using Pearson's chi-square or Fisher's exact test. Two-sample t-tests were used for comparing continuous variables with normally distributed data, whereas its non-parametric equivalent, Mann-Whitney U test, was used for non-normally distributed variables.

In the analysis of paired samples, paired t-test or Wilcoxon matched pairs test were used depending upon the distribution of the data.

When more than two groups were compared, analysis of variance (ANOVA) or Kruskal-Wallis tests were used and correlations between explanatory variables were assessed using either Pearson's or Spearman's rank correlations.

A p value of less than 0.05 was considered significant throughout the analysis. Where multiple subgroup significance tests were performed, the p value was adjusted by multiplying the p value by the number of paired comparisons (Bonferroni method) in order to avoid spurious associations (Bland, 2000, Altman, 1991).

Multivariable analysis for prediction of mortality was attempted. However it is not included here as the number of outcomes of interest (i.e. deaths) was lower than the minimum required for the multivariable procedure (Katz, 1999).

Chapter Three

3 CLINICAL FEATURES, MENINGOCOCCAL PHENOTYPE AND OUTCOME

3.1 INTRODUCTION

3.1.1 Disease presentation

The relative importance of meningococcal disease as a cause of serious community acquired bacterial infection, as well as the clinical pattern of invasive meningococcal disease vary widely across the world (Peltola, 2001, Hart & Rogers, 1993). Environmental factors partly explain differences in the incidence of disease (Molesworth *et al.*, 2003, Haberberger *et al.*, 1990) and certain serogroups are more associated to endemic than to epidemic disease (Tikhomirov *et al.*, 1997). Little is known however of why MD presents as meningitis alone in some circumstances and as septicaemia in others.

3.1.1.1 Proportion of meningococcal aetiology in suspected cases

N. meningitidis is by far the commonest aetiological agent of epidemic bacterial meningitis. This is particularly the case in the Sub-Saharan African meningitis belt. However, the relative importance of *N. meningitidis* in the aetiology of bacterial meningitis in other parts of Africa is variable. Peltola (2001) reviewed reports of bacterial meningitis in Africa since the 1960s and found that outside the

belt, pneumococci and *H. influenzae* type b (Hib) are at least as important as or even more common than meningococcal meningitis. Conversely, in most western nations and middle-income countries such as Brazil, the introduction of the Hib vaccine in the 1990s reduced its relative importance (Bryan *et al.*, 1990) and the high case-fatality rate of meningococcal septicaemia also contributes to give *N. meningitidis* a more prominent role. On the other hand, suspected cases of meningococcal septicaemia presenting with fever and a petechial rash are often due to causes other than meningococcal, including self-limited viral illnesses (Mandl *et al.*, 1997, Brogan & Raffles, 2000). These differences affect the relative burden of the disease in the population as well as the likelihood ratio of positive diagnostic tests (as seen in chapter 4).

3.1.1.2 Meningitis versus septicaemia

The proportions of cases with *N. meningitidis* meningitis alone and with meningococcal septicaemia with or without meningitis vary widely. In most of the meningitis belt, the rash denoting septicaemia is rarely seen (Whittle & Greenwood, 1976). In a prospective study from Lilongwe, Malawi, only 3.3% of the patients had skin rashes (Hart & Cuevas, 1997) and similar figures (3.7%) were found in a large study from the Sudan (Salih *et al.*, 1990). In contrast, septicaemia was found in 85% of cases in an epidemic of serogroup B meningococcal disease in Cape Town, South Africa (Ryder *et al.*, 1987), which resembles the observations from most developed countries where the group B meningococcus is endemic. In Merseyside, UK, 81% of cases had either MSM or MS and the case-fatality rate was significantly higher in MS (19%) compared to

11% in MSM and 1% in MM (Riordan *et al.*, 1995a). In a study from Campinas, Brazil, Donalisio et al (2000) reported 33% of cases presenting with MM, 54% with MSM and 13% with MS, with case-fatality rates, respectively, of 3, 22 and 44%.

3.1.1.3 Sex and age

Most reports have shown that males and females are equally affected by meningococcal disease, but some studies have found that male sex is associated with higher risk of having meningococcal disease (than females), without a difference in the case fatality rates (Greenwood *et al.*, 1979a, Trotter *et al.*, 2002, Harrison *et al.*, 2001).

Meningococcal disease rarely affects neonates (Riordan *et al.*, 1995b), although cases as young as 2 weeks were observed in an epidemic of group A meningococci in Malawi (Hart & Cuevas, 1997). Susceptibility increases after the first month of life, and in endemic situations children between the ages of 2 months and 5 years have higher attack rates than older age groups (Thomson *et al.*, 1990). Some studies have documented another peak in incidence in adolescents, with increased case-fatality rates (Ramsay *et al.*, 1997, Harrison *et al.*, 2001). In the case of epidemics, shifts in the age distribution of disease have been observed, with older children and adults being affected (WHO, 1998). The age-related risk of death also varies in different studies and high case-fatality rates have been described in young children (Kornelisse *et al.*, 1997), in adolescents (Harrison *et al.*, 2001), and in the elderly (Trotter *et al.*, 2002, Barquet *et al.*, 1999).

3.1.2 Serogroup and meningococcal phenotype

A number of studies from developed countries have found that certain meningococcal phenotypes are associated with higher case-fatality rates. Spanjaard et al (1987) reported that serotype 2b (B:2b) was associated with a higher frequency of septicaemic presentation and a higher case-fatality in patients from the Netherlands. Although the specific phenotype associated with higher risk of death varied with the setting, other studies found similar results in cases from Norway (Iversen & Aavitsland, 1996), Australia (Ward *et al.*, 2000) and Denmark (Jensen *et al.*, 2003). In a recent, large population based study from the UK, Trotter *et al* (2002) also found that fatal outcome was associated with meningococcal phenotype, with cases due to strains with phenotypes C:2a and B:2a having 3-fold higher risks of death when compared with the baseline. Interestingly, outcome was not influenced by reduced susceptibility to penicillin in this study.

3.1.3 Aims

The aims of this chapter are to describe the general characteristics of cases enrolled in the two cohorts from Brazil and Ethiopia, the relative frequency of confirmed cases amongst these, their clinical presentation and outcome.

3.2 SUBJECTS AND METHODS

The inclusion and exclusion criteria for the patients selected for the study by the attending physicians were described in section 2.3. Patients fulfilling the general enrolment criteria were reclassified retrospectively once all the test results became available and the Brazilian and Ethiopian patients with confirmed meningococcal disease were described according to sex, age, character and duration of symptoms, clinical presentation, meningococcal phenotype and outcome.

Patients were categorised as having meningitis alone (MM), septicaemia with signs of meningitis (MSM) and septicaemia alone (MS) as described in section 2.3. Outcome was defined as survival or death during hospital stay. Patients who were lost to follow up were included in the general descriptive analysis but excluded from the comparison by outcome.

3.2.1 Laboratory methods

Culture methods with serogroup determination were performed at the site laboratories according to local guidelines as described in sections 2.6.4 and 2.7.4 for Brazil and Ethiopia respectively. Phenotypic serotyping and PCR were performed at the Meningococcal Reference Unit in Manchester, UK, as outlined in sections 2.8.2 and 2.8.3.

3.2.2 Statistics

Pearson's chi-square tests were used to compare categorical variables. Mann-Whitney U or Kruskal-Wallis tests were used for non-normally distributed continuous variables respectively when two or more groups were compared. A p value of less than 0.05 was considered as statistically significant.

3.3 RESULTS

3.3.1 Proportion of patients with *N. meningitidis* infection

3.3.1.1 Brazil

One hundred and sixty nine patients were selected for enrolment by the attending physicians. Fifteen (9%) patients did not fulfil the inclusion criteria. Of these, 5 patients had no blood or CSF samples collected in the first 24h after enrolment; 4 were not admitted to the hospital and their records were not found, 3 cases refused to participate or it was not possible to obtain consent from the patient or the guardian; the samples from two patients were mislabelled and considered to be inadequate for the study and one patient was known to have AIDS and presented with neurological symptoms. One hundred and fifty four Brazilian patients were therefore enrolled, 147 (95%) of these at Correia Picanço Hospital and 7 (5%) at the Mother and Child Health Institute of Pernambuco.

Figure 3.1a shows the final diagnoses of the 154 Brazilian patients after completion of the hospital follow up and laboratory results. Ninety three (60%)

patients had confirmed meningococcal disease and 33 (21%) patients had a bacterial aetiology other than *N. meningitidis*: 22 (14%) patients had *S. pneumoniae*, 4 (3%) *H. influenzae,* 4 (3%) *Staphylococcus aureus* and 1 (<1%) each were due to β-haemolytic streptococcus, *Salmonella* spp. and *Mycobacterium tuberculosis*. A further 16 (10%) patients were considered likely to have bacterial infections according to their clinical evolution and laboratory findings, but it was not possible to establish a specific aetiology by culture or PCR. These included 7 patients (1 of whom died) with a clinical diagnosis of meningitis or septicaemia and non-blanching rashes who were managed as cases of meningococcal disease and 12 (8%) patients with fever, milder signs and symptoms and normal or lymphocytic CSF who recovered without antibiotics. These cases were considered likely to have infections of viral aetiology.

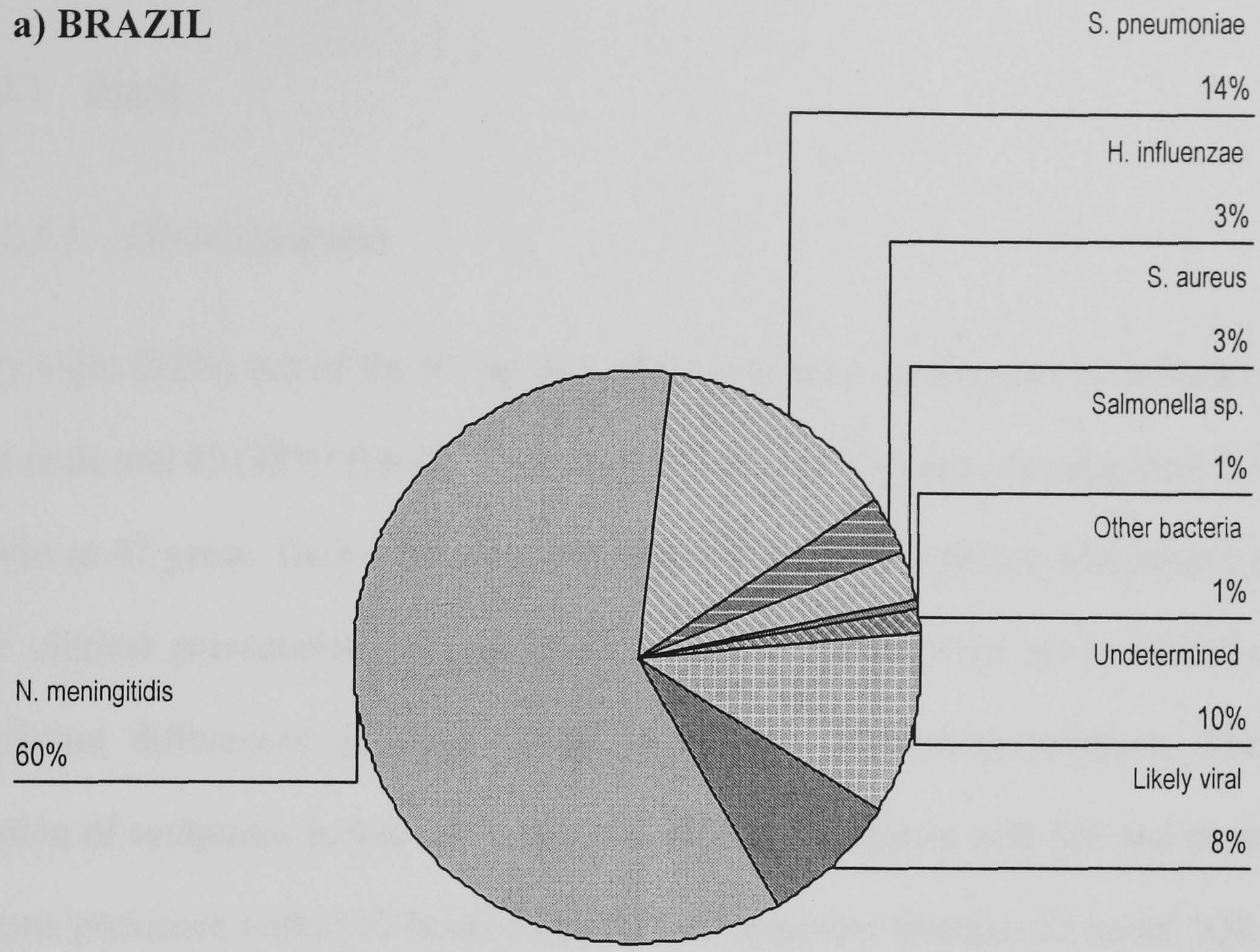
3.3.1.2 Ethiopia

Two hundred Ethiopian patients were selected for inclusion into the study by the attending physicians. Of these, 21 were excluded because they had no samples available for testing and a further 8 patients had no clinical assessment or follow up recorded. Thus, 171 patients were enrolled at the Ethiopian study site, 71 (42%) of these were enrolled at Yirgalem hospital, 69 (40%) at Dilla hospital, 7 (4%) at Gidole Hospital, 12 (7%) at Bushullo Major Health Centre, 7 (4%) at Yirgacheffe Health centre and 5 (3%) at Bona Health Centre.

Figure 3.1b shows the diagnoses of the 171 Ethiopian patients after follow up and completion of all laboratory results. Meningococcal disease was confirmed in 132 (77%) cases whereas 9 (5%) cases were diagnosed as having pneumococcal

infection, 1 (<1%) case had *Plasmodium falciparum* malaria and one patient louse borne relapsing fever. In 28 (16%) cases, it was not possible to reach a specific diagnosis and they were categorised as 'undetermined'. This group includes 1 case with probable tuberculous meningitis and is likely to include patients with other bacterial and viral aetiologies.

a) BRAZIL



b) ETHIOPIA

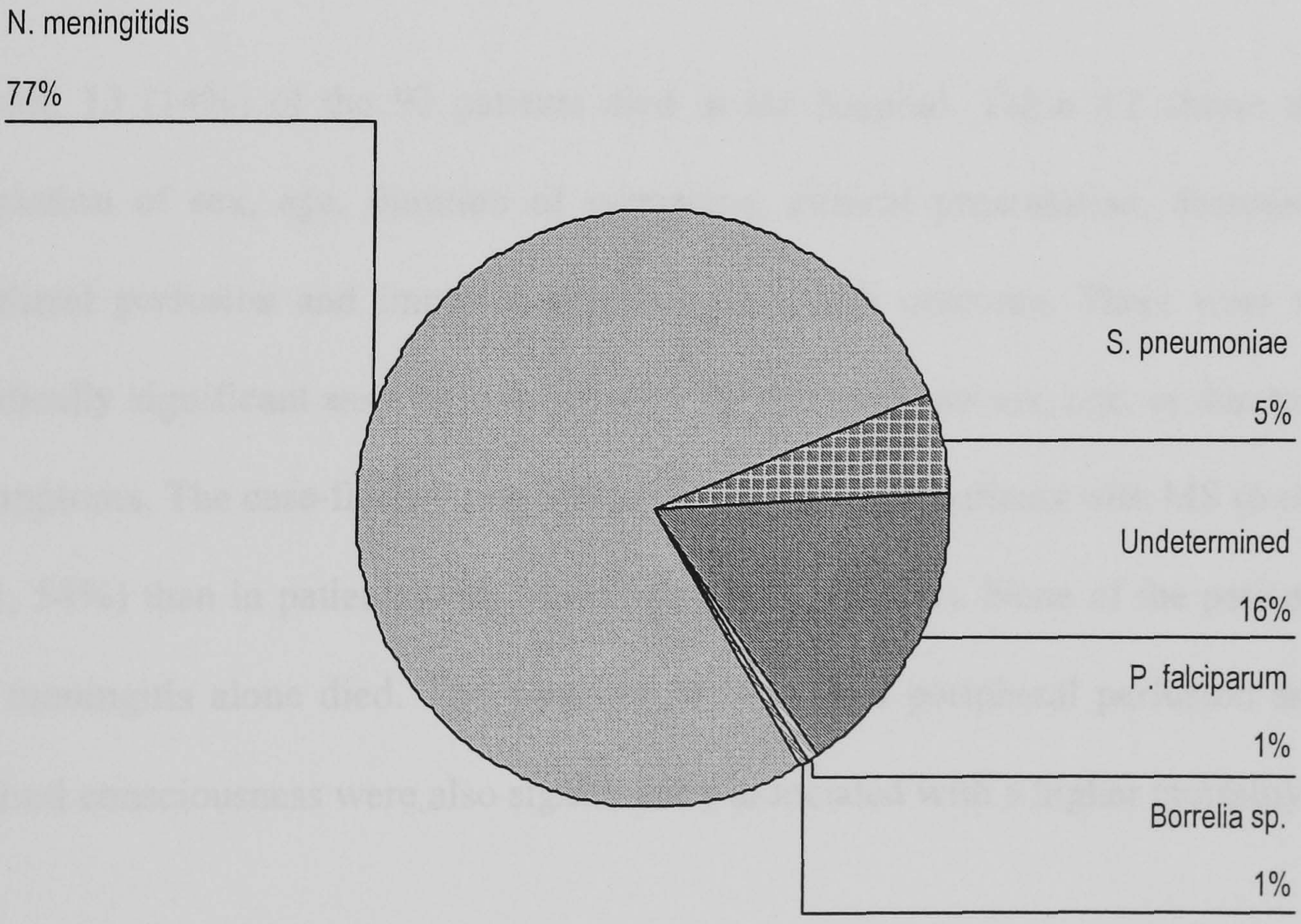


Fig. 3.1. Aetiologies and final diagnoses of patients enrolled in Brazil (a) and Ethiopia (b).

3.3.2 Characteristics of confirmed cases of meningococcal disease

3.3.2.1 Brazil

3.3.2.1.1 Clinical features

Forty eight (52%) out of the 93 patients with confirmed meningococcal infection were male and 45 (48%) female. Their median age was 9.5 years, ranging from 2.5 months to 47 years. Their sex, age and duration of symptoms before admission by their clinical presentation are shown in table 3.1. There were no statistically significant differences in terms of sex or age and clinical presentation. The duration of symptoms before admission was shorter in patients with MS and most patients presented within 12 hours from the first symptom (median 12 hours, IQR 12 hours), whereas patients with MM or MSM had a median duration of symptoms of 24 hours (IQR 24 and 4 hours, respectively).

Overall, 13 (14%) of the 93 patients died in the hospital. Table 3.2 shows the association of sex, age, duration of symptoms, clinical presentation, decreased peripheral perfusion and impaired consciousness with outcome. There were no statistically significant associations between the outcome and sex, age, or duration of symptoms. The case-fatality rate however was higher in patients with MS (6 out of 11, 54%) than in patients with MSM and MM ($p<0.001$). None of the patients with meningitis alone died. The presence of decreased peripheral perfusion and impaired consciousness were also significantly associated with a higher mortality.

3.3.2.1.2 Meningococci

The meningococcal serogroup was established by culture or PCR in 84 (90%) cases. Seventy two (86%) had serogroup B and 12 (14%) serogroup C meningococci. The mortality was higher in cases with serogroup B meningococci (11 out of 72, 15%) than in cases with serogroup C meningococci (0 out of 12, 0%), but this difference was not statistically significant (p=0.35).

The phenotypic characterisation was available for 24 (26%) isolates from the patients enrolled in Brazil. The commonest phenotype was B:4:P1.19/P1.15, with 14 (58%) of the isolates; followed by B:4:P1.7/P1.1 in 3 (13%) of the isolates. Each of the following phenotypes was identified in one each of the isolates: B:15:P1.7/P1.16, B:4:NT/P1.9, B:15:NT/P1.14, B:NT:P1.15/NT, C:4:P1.7/P1.1 and C:2b:NT/P1.10. In one case, the phenotypic characterisation was not possible (reported as *Neisseria* spp).

Table 3.1. Characteristics of 93 **Brazilian** confirmed cases of meningococcal disease according to their **clinical presentation**.

Characteristic		Number of cases (%)				Statistic
		MM	MSM	MS	All	
		n=31	n=51	n=11	n=93	
Male sex		14 (29)	29 (60)	5 (10)	48 (100)	p=0.536 [a]
Median age in years (IQR)		11.0 (11.2)	9.9 (11.8)	4.9 (5.8)	9.5 (11.3)	p=0.123 [b]
Age group	*0 – 4 years*	6 (19)	15 (29)	6 (55)	27 (29)	p=0.453 [c]
	5 – 9 years	8 (26)	12 (24)	3 (27)	23 (25)	
	10 – 19 years	11 (36)	16 (31)	1 (9)	28 (30)	
	≥ 20 years	6 (19)	8 (16)	1 (9)	15 (16)	
	All age groups	31 (100)	51 (100)	11 (100)	93 (100)	
Median duration of symptoms in hours (IQR)		24 (24)	24 (4)	12 (12)	24 (16)	p<0.001 [b]

Comparisons made between the three clinical forms; MM = meningitis, MSM = meningococcal septicaemia with meningitis and MS = meningococcal septicaemia. (a) Pearson's chi-square. (b) Kruskal-Wallis one-way analysis of variance. (c) Fisher's exact test. IQR = interquartile range.

Table 3.2 Characteristics of the 93 **Brazilian** patients with meningococcal disease by **outcome**.

Variable	n	Outcome n (%)		Statistic
		Death	Survival	
Sex				
Male	48	7 (15)	41 (85)	p =0.86 [a]
Female	45	6 (13)	39 (87)	
Clinical presentation				
MM	31	0 (0)	31 (100)	P<0.001 [b]
MSM	51	7 (14)	44 (86)	
MS	11	6 (54)	5 (46)	
Age group				
0 – 4 years	27	4 (15)	23 (85)	p =0.50 [b]
5 – 9 years	23	5 (22)	18 (78)	
10 – 19 years	28	2 (7)	26 (93)	
≥ 20 years	15	2 (13)	13 (87)	
Duration of symptoms				
0 – 24h	66	12 (18)	54 (82)	p =0.10 [b]
> 24h	27	1 (4)	26 (96)	
Level of consciousness				
Alert	52	5 (10)	47 (90)	p =0.045 [b]
Respond to verbal command	31	4 (13)	27 (87)	
Response to painful stimuli or does not respond	10	4 (40)	6 (60)	
Decreased peripheral perfusion				
Present	42	12 (29)	30 (71)	P<0.001 [a]
Absent	45	0 (0)	45 (100)	

(a) Pearson's chi-square. (b) Fisher's exact test. MM = meningitis, MSM = meningococcal septicaemia with meningitis and MS = meningococcal septicaemia. Impaired consciousness defined as no response to external stimuli or response to painful stimuli only. Decreased peripheral perfusion includes slow capillary refill time, cold extremities and feeble pulse.

3.3.2.2 Ethiopia

3.3.2.2.1 Clinical features

There were 132 confirmed cases of meningococcal disease from Ethiopia. Of these, 70 (53%) were male and 62 (47%) female. Their median age was 10 years, ranging from 5 months to 60 years. All patients had a clinical diagnosis of meningitis alone. Six (5%) cases were lost to follow up and 7 (6%) patients out of the remaining 126 with known outcome died in the hospital. Table 3.3 shows the association between sex, age, duration of symptoms and the presence of decreased peripheral perfusion and impaired consciousness with outcome. There were no statistically significant differences in mortality according to sex, age, duration of symptoms or the presence of decreased peripheral perfusion. The presence of impaired consciousness at the time of admission was significantly associated with a higher mortality.

3.3.2.2.2 Meningococci

The meningococcal serogroup was established by culture or PCR in 77 (58%) out of the 132 Ethiopian cases, all of which were due to serogroup A *N meningitidis*. The phenotypic characterisation was available for 46 (35%) of the isolates. Only two phenotypes were identified, 44 (96%) were phenotype A:21:NT/P1.9 and 2 (4%) phenotype A:21:NT/NT.

Table 3.3 Characteristics of the 132 **Ethiopian** patients with meningococcal disease according to **outcome**.

Variable	n	Outcome n (%)		Statistic
		Death	Survival	
Sex				
Male	65	4 (6)	61 (94)	p =1.0 [a]
Female	61	3 (5)	58 (95)	
Age group				
0 – 4 years	28	3 (11)	25 (89)	p=0.10 [b]
5 – 9 years	32	2 (6)	30 (94)	
10 – 19 years	45	2 (4)	43 (96)	
≥ 20 years	21	0 (0)	21 (100)	
Duration of symptoms				
0 – 24h	17	1 (6)	16 (94)	p=1.0 [a]
> 24h	99	5 (5)	94 (95)	
Level of consciousness *				
Alert	7	0 (0)	7 (100)	p=0.026 [a]
Respond to verbal command	7	0 (0)	2 (100)	
Response to painful stimuli or does not respond	9	2 (22)	7 (78)	
Decreased peripheral perfusion *				
Present	8	1 (12)	7 (88)	p=1.0 [a]
Absent	10	1 (10)	9 (90)	

(a) Fisher's exact test. (b) Chi square for trend * Information for the level of consciousness and perfusion is based on 18 cases (first phase of Ethiopian data collection). MM = meningitis, MSM = meningococcal septicaemia with meningitis and MS = meningococcal septicaemia. Decreased peripheral perfusion and impaired consciousness defined as for Brazilian participants.

3.3.2.3 Comparisons between Brazilian and Ethiopian patients

The proportion of confirmed meningococcal disease amongst suspected cases was higher in Ethiopia (77%) than in Brazil (60%, p=0.001), where a larger proportion of diagnoses other than MD were made.

The signs and symptoms presented by Brazilian and Ethiopian patients at the time of admission to hospital are shown in figure 3.2. No skin rashes were observed in the Ethiopian patients, all of whom presented with meningitis alone, whereas 65% of Brazilian cases had a rash denoting septicaemia (with or without meningitis). A petechial or ecchymotic rash was observed in 95% per cent of septicaemic cases in Brazil and in the remaining 5%, the rash was maculopapular. The presence of an impaired level of consciousness was more frequent in Ethiopia (9 out of 18 cases, 50%) than in Brazil (10 out of 93 cases, 11%, p<0.001).

The general characteristics (sex, age, duration of symptoms and outcome) were compared between the Ethiopian cases of meningitis and the 93 Brazilian cases, including patients with septicaemic presentations and with the Brazilian cases presenting with meningitis alone (31 cases). In both scenarios, there were no statistically significant differences by age and sex, but the duration of the symptoms before presentation was more prolonged in the Ethiopian patients (median 60 hours) than in the Brazilian cohort (medians of 24 hours for all Brazilian cases; and 24 hours for Brazilian cases with meningitis alone, p<0.001). When Ethiopian cases were compared with all Brazilian cases, the case-fatality rate was significantly lower in Ethiopia (5.6%) than in Brazil (14%, p=0.032). However, when only Brazilian cases with meningitis were considered, the

mortality in Brazil was lower (0%) than in Ethiopia but this difference was not statistically significant (Fisher's exact test, p=0.35).

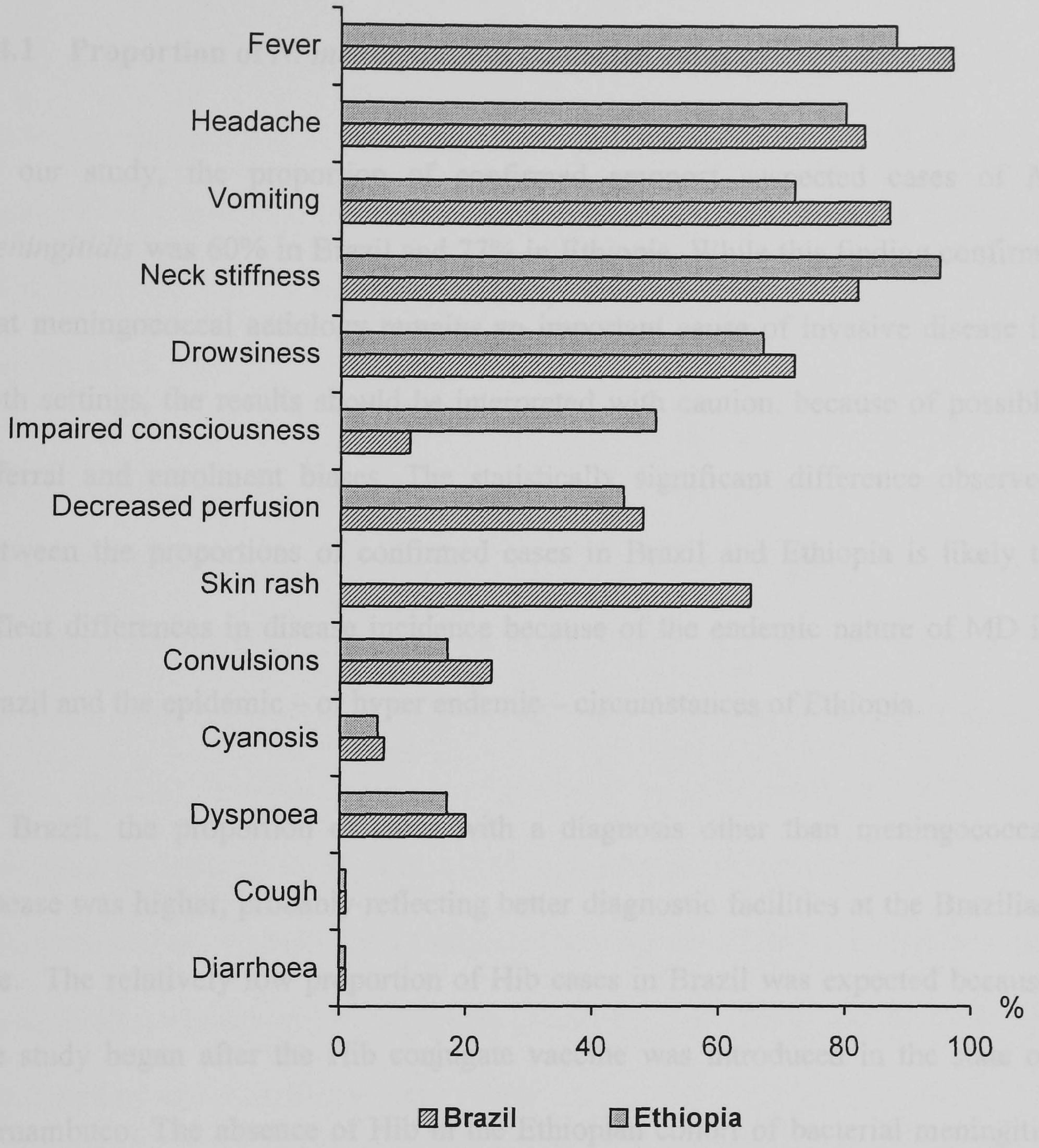


Fig. 3.2. Percentage of signs and symptoms presented by Brazilian and Ethiopian cases with confirmed meningococcal disease.

3.4 DISCUSSION

3.4.1 Proportion of *N. meningitidis* in suspected cases

In our study, the proportion of confirmed amongst suspected cases of *N. meningitidis* was 60% in Brazil and 77% in Ethiopia. While this finding confirms that meningococcal aetiology remains an important cause of invasive disease in both settings, the results should be interpreted with caution, because of possible referral and enrolment biases. The statistically significant difference observed between the proportions of confirmed cases in Brazil and Ethiopia is likely to reflect differences in disease incidence because of the endemic nature of MD in Brazil and the epidemic – or hyper endemic – circumstances of Ethiopia.

In Brazil, the proportion of cases with a diagnosis other than meningococcal disease was higher, probably reflecting better diagnostic facilities at the Brazilian site. The relatively low proportion of Hib cases in Brazil was expected because the study began after the Hib conjugate vaccine was introduced in the state of Pernambuco. The absence of Hib in the Ethiopian cohort of bacterial meningitis however might simply reflect lesser diagnostic capabilities, as culture facilities were limited and the multiplex PCR did not include a Hib gene target. Apart from other bacterial causes of sepsis and meningitis, the differential diagnoses included viral and tuberculous meningitis, and in Ethiopia, included malaria and relapsing fever as well.

3.4.2 Sex and age

In both study sites, the number of cases of confirmed meningococcal disease was higher in males than in females, in agreement with a general trend for infectious diseases in children and as previously reported in the context of MD (Greenwood *et al.*, 1979a, Harrison *et al.*, 2001, Trotter *et al.*, 2002). However, the difference in our study was not statistically significant and did not take into account gender-specific attack rates.

The age distribution was similar between the two sites. The median ages were of 9.5 and 10 years; and the proportions of patients under the age of 5 years were 22% and 29% for Brazil and Ethiopia respectively. Age was not found to be associated with outcome in neither of the sites. Interestingly, in the Brazilian cohort, cases of MS were younger, with 55% of cases below the age of 5 years compared to 29% in those with MSM and 19% in those with MM, but the difference was not statistically significant. These results differ from other hospital-based studies, where cases were younger (Thomson *et al.*, 1990) and mortality was influenced by age (Kornelisse *et al.*, 1997).

3.4.3 Clinical presentation and outcome

As expected, the clinical presentation in Brazil was different from that in Ethiopia. While 67% of Brazilian cases presented with skin rashes, therefore had septicaemia (with or without meningitis), none of the Ethiopian cases had a rash and all presented with meningitis alone. The proportion of septicaemic cases and case-fatality rates seen in Recife were comparable to what has been reported in

other parts of Brazil (Donalisio *et al.*, 2000) and elsewhere serogroup B is predominant (Riordan *et al.*, 1995a). The presentation of cases in Ethiopia matched the description of clinical features observed elsewhere in the meningitis belt, though the case-fatality rate seen in the Ethiopian cohort (5.6%) was relatively low by sub-Saharan African standards (Hart & Cuevas, 1997, Salih *et al.*, 1990). It is possible to speculate that this might reflect the phase in the epidemic in which the study was conducted, as it has been suggested that case-fatality rates (and disease severity) decrease with time during the course of an epidemic (Greenwood *et al.*, 1979b).

Brazilian cases with MS were brought to hospital earlier than cases with MSM or MM. This might indicate the rapid clinical progression in this group of patients and also reflect disease severity, as suggested by previous studies in which a short interval between the first symptom and admission to hospital was associated with dismal outcome (Ansari *et al.*, 1979).

On average, Ethiopian cases were seen in hospital one and a half days later than Brazilian cases, even when only cases of meningitis were considered for comparison. Differences in care-seeking behaviour and access to health care facilities are likely to have influenced these results. The late presentation seen in Ethiopia might explain the higher proportion of cases with impaired consciousness on admission (50%, compared to 10% in Brazil). The presence of impaired consciousness was significantly associated with fatal outcome both in Brazil and Ethiopia, and this is compatible with previous studies that considered coma (with variable comparable definitions) as part of prognostic scores in MD (Kahn & Blum, 1978, Sinclair *et al.*, 1987, Castellanos-Ortega *et al.*, 2002).

The presence of decreased peripheral perfusion on admission was associated with a poor outcome in Brazil but not in Ethiopia. While persistent shock seen in meningococcal septicaemia has long been recognised as a marker for a poor prognosis (Stiehm & Damrosch, 1966), early manifestations of compensated shock (i.e. signs of decreased perfusion without hypotension) may simply be a sign of the systemic response to infection, and might be more easily reversible with the initiation of antibiotic and fluid therapy.

3.4.4 Serogroup and phenotype

No cases of serogroup A meningococcal disease were identified in Brazilian patients. The proportion of serogroups B and C and predominant meningococcal phenotypes found in Recife were similar to what has been described in other parts of Brazil (Sacchi *et al.*, 2001), and the variability of phenotypes is in agreement with endemic, sporadic disease. The relatively low number of isolates with a known phenotype did not allow for comparison by presentation or outcome.

Conversely, there was little variability in the Ethiopian isolates, which were all of serogroup A and all but two had the same phenotype. This finding is in agreement with epidemic disease where most cases are due to a single epidemic clone. The phenotype found in Ethiopia has been shown to belong to the ST-7 sequence type (Norheim *et al.*, 2004).

Chapter Four

4 POLYMERASE CHAIN REACTION AS A DIAGNOSTIC TOOL

4.1 INTRODUCTION

In less than two decades after its development, the polymerase chain reaction (PCR) has increasingly become an important tool both in biomedical research and in medical practice. Its unprecedented ability to exponentially amplify and detect minute amounts of nucleic acid material in biological samples has been widely used in the molecular diagnosis of infectious diseases, with applications ranging from early detection of pathogens to monitoring response to therapy. PCR has evolved to become a simpler and cheaper technique that can increasingly be used in lower-resourced settings. Its technology has also advanced to allow the development of robotized, automated systems that can detect and quantify a number of different target molecules in a single assay, facilitating high throughput of samples (Reischl & Kochanowski, 1999).

Since the first PCR assays for the detection of *N. meningitidis* in clinical samples were described in the early 1990s (Kristiansen *et al.*, 1991, Ni *et al.*, 1992), a number of assays have been developed. These assays use different strategies and gene targets, some of which provide additional information on serogroup (Borrow *et al.*, 1997), serotype (Urwin *et al.*, 1998) and serosubtype (Saunders *et al.*, 1993).

In the UK, in the early to mid 1990s, an increase in the use of pre-admission antibiotics for suspected cases of meningococcal disease coincided with a reduction of the number of patients submitted to a lumbar puncture amid increased perception of the risks involved with the procedure. This resulted in a widening gap between the number of clinically diagnosed and laboratory-proven cases (Newcombe *et al.*, 1996). This contributed to the drive for the development of non-culture diagnostic alternatives, culminating with the establishment of a national PCR service at the Meningococcal Reference Unit (MRU) in Manchester (Kaczmarski *et al.*, 1998). Amongst the successful strategies that evolved to become available for diagnostic use in a larger scale are the PCR enzyme-linked immunosorbent assay (PCR ELISA) and the fluorogenic probe-based 5' exonuclease assay (Taqman) formats. While PCR ELISA-based methods had good sensitivity and specificity and initially allowed more rapid processing of samples, the Taqman method was preferred over time because of its ability to handle a larger throughput of samples with reduced risk of contamination (Guiver *et al.*, 2000).

In both formats, gene targets such as the insertion sequence IS*1106*, the capsular transfer gene *ctrA* and the sialyltransferase gene *siaD* were successfully explored as diagnostic tools. The *siaD* assays were less sensitive than the other gene targets, but their ability to specifically identify and differentiate serogroup B and C meningococci has proven very useful. The IS*1106*-based assays, however, presented problems with specificity in the Taqman format and were later abandoned in favour of the *ctrA* gene as the more sensitive and specific target (Guiver *et al.*, 2000).

Further improvements in the Taqman *ctrA* assay included the development of better primers that allowed the detection of serogroups A, B, C, 29E, W135, X, Y and Z in a multiplex format with gene targets for the two other major causes of bacterial meningitis: *Streptococcus pneumoniae* and *Haemophilus influenzae*. Their respective targets were the pneumolysin (*ply*) and capsulation (*bexA*) genes (Corless *et al.*, 2001).

The improved *ctrA*-based Taqman assay was shown to be highly sensitive in detecting meningococci in CSF, plasma and blood samples of laboratory-confirmed cases. Moreover, despite extensive testing, this assay was not shown to cross-react with either other pathogens or human genomic DNA, thus this assay became the preferred target for initial screening of samples at the MRU (Corless *et al.*, 2001).

The *ctrA* assay has since been assessed for performance as a diagnostic tool in two clinical settings (England and Australia) and in one further laboratory setting (Wales). One study from Merseyside, UK, (Carrol *et al.*, 2000b) prospectively enrolled 319 children presenting with suspected meningococcal disease and categorised 166 as probable and 153 as possible cases, finding that a blood PCR was 47% sensitive and 100% specific for the diagnosis of MD in this population. A subsequent study in the same setting (Hackett *et al.*, 2002a) compared these results with a new cohort of 196 children and found that the use of Gentra columns for the extraction of DNA from whole blood samples allowed the sensitivity of blood PCR to be increased, reaching 87% (with the same 100% specificity). More recently, one study from Melbourne, Australia, enrolled 118 children into groups of 'likely' and 'unlikely' MD, finding that the *ctrA* PCR was 88% sensitive and

100% specific for detecting MD (Bryant *et al.*, 2004). In a laboratory-based study using a central database in South Wales to match and compare results of blood PCR and cultures, an attempt was made to calculate different post-test based on pre-test probabilities, but it did not consider the possibility of PCR being more sensitive than culture to detect true cases of MD (Woerden *et al.*, 2004).

To date, no data are available concerning the performance of *ctrA* PCR in developing countries or in areas where meningococci other than B or C are predominant or according to clinical presentation.

The aim of this chapter is therefore to assess the performance of the *ctrA* PCR assay for the diagnosis of meningococcal disease in Brazil and Ethiopia, according to the type of clinical sample and disease presentation. A secondary aim is to describe the performance of *siaD* and *myn* PCR assays for determining meningococcal serogroup in these two settings.

4.2 SUBJECTS AND METHODS

4.2.1 Participants and categories for analysis

Cases fulfilling the general enrolment criteria (section 2.3) were included in this study when they had at least one clinical sample (whole blood, plasma and / or CSF) available for PCR testing. For the comparison with culture results, only cases that had both a culture result and a PCR sample were included.

In this chapter, the categorisation of cases into 'confirmed', 'probable', 'possible' and 'not' meningococcal disease was blinded for meningococcal PCR results, so modified versions of the definitions in section 2.3 apply. This was done to avoid incorporation bias[7] in the assessment of the performance of *ctrA* PCR.

Performance of *ctrA* PCR was initially assessed by comparing with culture alone. Sensitivity, specificity, positive and negative predictive values were calculated and differences in case ascertainment rates were described as a measure of the added value of PCR testing in these settings. Then the performance of *ctrA* PCR was assessed by comparing with a gold standard that included clinical and laboratory criteria.

Gold standard: culture-confirmed, as well as cases with a clinical diagnosis of 'probable' or 'possible' meningococcal disease who had no alternative diagnoses after laboratory results (but meningococcal PCR) were taken into account were included in the 'gold standard' category. Those cases who were clinically considered unlikely to be of meningococcal aetiology and those in whom a diagnosis other than meningococcal disease was established were grouped to form the 'not meningococcal' category. These categories were used for the calculation of sensitivity, specificity, positive and negative predictive values for *ctrA* meningococcal PCR in three clinical scenarios: a) when only features of meningitis were present; b) when there was a rash indicating septicaemia (with or without meningitis) and c) in any suspected case.

---

[7] Incorporation bias occurs when the results of the test being assessed are included in the case definition, thus affecting the appropriate calculation of sensitivity and specificity (Altman, 2001).

4.2.2 Laboratory methods

The description of the principle, methods and protocols for meningococcal *ctrA* Taqman PCR assay, as well as the *siaD* and *myn* assays, can be found in the methods section 2.8.3.

4.2.3 Statistics

Sensitivity, specificity, positive and negative predictive values and their correspondent 95% confidence intervals were calculated using Win Episcope 2.0.

4.3 RESULTS

4.3.1 Brazil

4.3.1.1 Performance of *ctrA* PCR

4.3.1.1.1 Comparing with culture

A total of 145 out of 154 participants enrolled in Recife had a sample available for PCR testing and also had an available culture result.

Compared to culture alone, when all suspected cases were considered together and blood and CSF results were combined, the *ctrA* PCR had a sensitivity of 98% (95% CI 95-100), a specificity of 67% (95% CI 57-77), a positive predictive value of 66% (95% CI 56-76) and a negative predictive value of 98% (95-100).

Twenty-nine (20%) of the 145 cases had a positive PCR but a negative culture, but 25 (86%) of these 29 cases had characteristic clinical features and would be categorised as 'probable' cases. On all but one occasion, the culture confirmed cases were also PCR positive. This was a 34 year-old female who presented with a 12-hour history of fever, headache and petechiae; had nuchal rigidity on examination and a positive CSF culture for serogroup B meningococcus, tested negative for blood PCR and the CSF sample was not available for PCR testing.

4.3.1.1.2 Comparing with clinical and laboratory 'gold standard'

A total of 151 out of 154 participants enrolled in the Brazilian study had at least one clinical sample available for PCR testing. One hundred cases had both blood and CSF specimens available, 13 had blood only and 38 had CSF only.

On clinical grounds, 91 (60%) of the enrolled cases were initially categorised as 'probable', 43 (29%) as 'possible' and 17 (11%) as 'unlikely' meningococcal disease. Following a positive blood or CSF culture (or pneumococcal PCR), 30 cases were re-categorised to the 'not meningococcal' category, whereas 121 cases with negative cultures and pneumococcal PCR remained in their original group. The distribution of cases into clinical groups and subsequent categorisation for analysis is shown in Fig. 4.1.

One hundred and four (69%) cases met the 'gold standard' criteria and were grouped as meningococcal disease, whereas 47 (31%) cases, who were unlikely meningococcal disease from the outset or had a diagnosis other than MD established, were categorised as 'not MD'. The diagnoses of these patients before

the results of meningococcal PCR, along with age, sex and duration of symptoms in each group are shown in table 4.1.

Table 4.2 describes the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for *ctrA* meningococcal PCR according to type of sample (blood, CSF or both) and to clinical scenario.

The specificity and PPV were the same (100%, 95% CI 100-100) for blood and CSF and in all clinical scenarios evaluated.

For cases presenting with meningitis alone, the sensitivity of the *ctrA* PCR in blood samples was only 31% (95% CI 16-47) but the *ctrA* PCR in CSF samples was 71% (95% CI 57-84) sensitive. When both blood and CSF samples were available for testing, the combined sensitivity was 82% (95% CI 68-96) and NPV was 78% (95% CI 61-95).

Compared with meningitis alone, blood PCR was more sensitive in cases with a rash indicating septicaemia (sensitivity 77%, 95% CI 66-87). In the group of septicaemic patients, the PCR in CSF samples had similar sensitivity (78%, 95% CI 68-88). When both samples were available, the combined sensitivity reached 90% (95% CI 82-98), with a NPV of 29% (95% CI 0-62).

When all suspected cases were considered together, the *ctrA* PCR in blood samples was 60% (95% CI 50-70) sensitive, whereas the PCR in CSF samples had a sensitivity of 75% (95% CI 67-83). Both tests combined had a sensitivity of 88% (95% CI 80-95) with a NPV of 67% (95% CI 50-84).

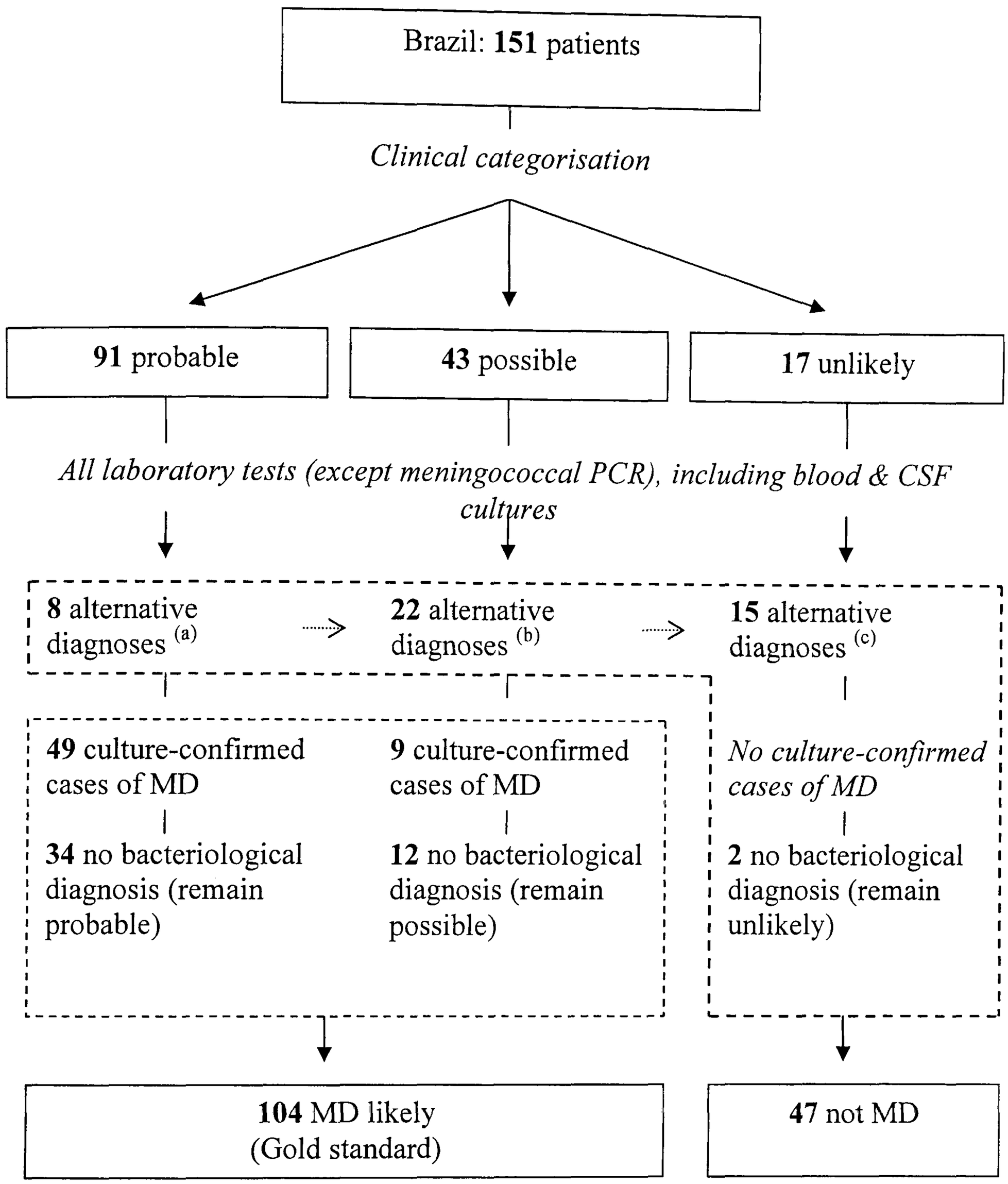


Fig. 4.1. Distribution of cases for the analysis of PCR as a diagnostic tool in Brazil. Cases were initially categorised according to clinical presentation. Once an alternative diagnosis became available, cases were re-categorised to the 'not MD' group. Culture-confirmed, along with probable and possible cases that remained undefined after other laboratory tests, were grouped to form the 'gold standard' category. a) 5 cases of bacterial sepsis with petechial (or vasculitic) rash (3 due to *Streptococcus pneumoniae*, 1 to *Haemophilus influenzae* and 1 to *Staphylococcus aureus*) plus 3 cases of pneumococcal meningitis in whom meningococcal aetiology had been suggested by microscopy. b) Cases of bacterial meningitis due to other organisms (15 *S. pneumoniae*, 4 *H. influenzae*, 1 *S. aureus*, 1 *Salmonella* sp. and 1 Group A β – haemolytic streptococcus). c) 12 cases regarded as likely to be of a viral aetiology (mild presentation, normal or lymphocytic CSF, full recovery without antibiotics), 2 cases of staphylococcal sepsis and 1 mycobacterium infection. MD = meningococcal disease.

Table 4.1. Characteristics of Brazilian patients included in the 'gold standard' and 'not meningococcal disease' groups for the assessment of meningococcal PCR.

Characteristic	MD [a]	Not MD [b]
Median age, years (range)	8.4 (0.21–47)	8.5 (0.13–62)
Male : female ratio	1.04:1	2.62:1
Median duration of symptoms, days (range)	1 (0.4-8)	1 (0.13-10)
Diagnosis [c], no. of patients (%)		
Microbiologically confirmed [d]		
Neisseria meningitidis	58 (56)	0 (0)
Streptococcus pneumoniae	0 (0)	21 (45)
Haemophilus influenzae b	0 (0)	5 (11)
Staphylococcus aureus	0 (0)	4 (8)
β-haemolytic *Streptococcus*	0 (0)	1 (2)
Salmonella sp	0 (0)	1 (2)
Mycobacterium tuberculosis	0 (0)	1 (2)
Viral meningitis likely [e]	0 (0)	12 (26)
Bacterial meningitis, unspecified	12 (11)	0 (0)
Bacterial sepsis/septicaemia, unspecified	34 (33)	2 (4)
Total	104 (100)	47 (100)

a) Meningococcal disease likely (or confirmed). b) Meningococcal disease unlikely from the outset or a diagnosis other than MD established. c) Before meningococcal PCR. d) Includes culture and pneumococcal PCR results. e) Mild presentation, normal or lymphocytic CSF, full recovery without antibiotics.

Table 4.2. Performance of meningococcal *ctrA* PCR compared with clinical and laboratory gold standard in 151 Brazilian patients.

Clinical scenario	Gold standard diagnosis (total no. patients)		%			
	MD [a]	Not MD [b]	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
Meningitis alone [c]						
Blood PCR	11 (35)	0 (15)	31 (16-47)	100 (100-100)	100 (100-100)	39 (23-54)
CSF PCR	31 (44)	0 (28)	71 (57-84)	100 (100-100)	100 (100-100)	68 (54-83)
Both blood / CSF PCR†	23 (28)	0 (18)	82 (68-96)	100 (100-100)	100 (100-100)	78 (61-95)
Septicaemia with or without meningitis [d]						
Blood PCR	46 (60)	0 (3)	77 (66-87)	100 (100-100)	100 (100-100)	18 (0-36)
CSF PCR	49 (63)	0 (3)	78 (68-88)	100 (100-100)	100 (100-100)	18 (0-36)
Both blood / CSF PCR†	47 (52)	0 (2)	90 (82-98)	100 (100-100)	100 (100-100)	29 (0-62)
Any suspected case						
Blood PCR	57 (95)	0 (18)	60 (50-70)	100 (100-100)	100 (100-100)	32 (20-44)
CSF PCR	80 (107)	0 (31)	75 (67-83)	100 (100-100)	100 (100-100)	54 (41-66)
Both blood / CSF PCR†	70 (80)	0 (20)	88 (80-95)	100 (100-100)	100 (100-100)	67 (50-84)

a) Culture-confirmed, probable and possible cases grouped together. b) Meningococcal disease unlikely or diagnosis other than meningococcal disease established. c) Clinical and laboratory features of meningitis but no skin rash. d) Features of septicaemia, with a petechial (or maculopapular) skin rash, with or without accompanying signs of meningitis. † Both blood and CSF samples were available for testing. MD = meningococcal disease. CI = confidence interval. CSF = cerebrospinal fluid.

4.3.1.1.3 Overall case ascertainment

Overall, ninety (87%) cases were ascertained by *ctrA* PCR and culture combined, in contrast with 58 (56%) of the confirmed cases determined by culture alone. This represents an absolute increase of 55% in the number of cases with a confirmatory diagnosis – or a relative increase of 31% in the case ascertainment rate.

4.3.1.2 PCR for serogroup determination

Seventy-six (85%) out of 89 cases with a positive *ctrA* PCR were also tested with the *siaD* PCR assay for serogroup determination. Fifty-eight (76%) cases tested positive for serogroup B *N. meningitidis*, 11 (15%) were positive for serogroup C and in 7 (9%) cases typing was not possible by *siaD* PCR.

4.3.2 Ethiopia

4.3.2.1 Performance of *ctrA* PCR

4.3.2.1.1 Comparing with culture

A total of 112 out of 171 participants enrolled in Ethiopia had a CSF sample available for PCR testing and also had an available CSF culture result (no blood cultures were available from Ethiopia).

Compared to CSF culture alone, when all suspected cases were considered together, the *ctrA* PCR in the CSF had a sensitivity of 94% (95% CI 87-100), a

specificity of 29% (95% CI 17-40), a positive predictive value of 51% (95% CI 40-61), and a negative predictive value of 86% (71-100).

Forty-five (40%) of the 112 cases had a positive PCR but a negative culture, but 43 (96%) of these 45 cases had a Gram-negative diplococci on CSF microscopy and compatible clinical features and would be categorised as 'probable' cases. Out of the 46 cases confirmed by culture, only 3 (6%) tested negative for *ctrA* PCR.

4.3.2.1.2 Comparing with clinical and laboratory 'gold standard'

All 171 participants enrolled in the Ethiopian field study had at least one clinical specimen available for PCR testing, with 132 (77%) having both CSF and a blood (or plasma) sample. Twenty-six cases had only a CSF sample, whereas 13 cases had only a blood (or plasma) sample.

According to clinical assessment at the outset, 142 (83%) of the enrolled cases were categorised as 'probable', 23 (13.5%) as 'possible' and 6 (3.5%) as 'unlikely' meningococcal disease. Following a positive culture (or pneumococcal PCR), 9 cases were re-located to the 'not meningococcal' category, whereas 102 cases with negative cultures and pneumococcal PCR remained in their original group. The distribution of Ethiopian cases and their categorisation for analysis is shown in Fig. 4.2.

Having met the 'gold standard' criteria, 156 (91%) cases were grouped as meningococcal disease, while 15 (9%) cases were categorised as 'not MD' because they were considered 'unlikely meningococcal' from the outset or had a diagnosis other than MD established. Age, sex, duration of symptoms and

diagnoses before meningococcal PCR are described for patients allocated in each of these two groups (table 4.3).

Table 4.4 describes the sensitivity, specificity, positive and negative predictive values for *ctrA* meningococcal PCR in Ethiopian cases. Because all but one case presented as meningitis alone, no breakdown by clinical scenario is reported.

Considering all suspected cases with both a CSF and a blood (or plasma) sample available for testing, the *ctrA* PCR had a sensitivity of 89% (95% CI 83-95), a specificity of 93% (95% CI 79-100), a PPV of 99% (95% CI 97-100) and a NPV of 50% (95% CI 31-69).

When all cases having a CSF sample available for testing were analysed as a group, a sensitivity of 85% (95% CI 80-91) and a specificity of 100% (95% CI 100-100) were achieved.

When cases having any sample from blood (whole blood or plasma) were jointly described, the *ctrA* PCR had a sensitivity of 36% (95% CI 28-44) and a specificity of 93% (95% CI 79-100). However, when each type of sample was assessed separately, the PCR in whole blood tended to outperform the plasma PCR in terms of sensitivity and specificity, with a particularly higher NPV (46% in whole blood against 4% in plasma).

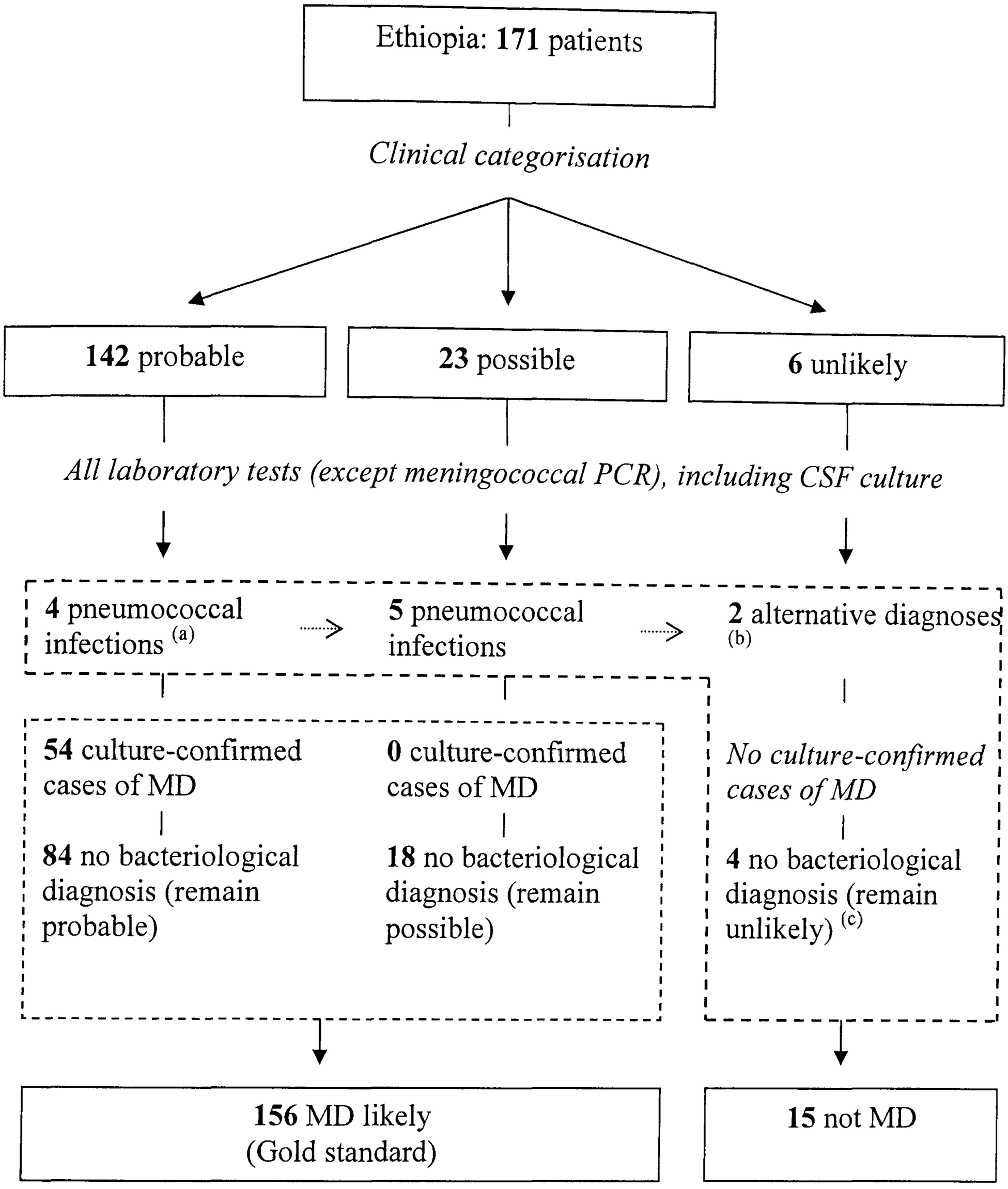


Fig. 4.2. Distribution of cases for the analysis of PCR as a diagnostic tool in Ethiopia. Cases were initially categorised according to clinical presentation. Once an alternative diagnosis became available, cases were re-categorised to the 'not MD' group. Culture-confirmed, along with probable and possible cases that remained undefined after other laboratory tests, were grouped to form the 'gold standard' category. a) 4 PCR-confirmed cases of pneumococcal meningitis in whom meningococcal aetiology had been suggested by microscopy. b) Cases presenting with fever and nuchal rigidity but whose diagnoses were falciparum malaria (1) and relapsing fever (1). c) Three cases with clinical course suggestive of bacterial sepsis but with no characteristic features of MD and one probable case of tuberculous meningitis. MD = meningococcal disease.

Table 4.3. Characteristics of Ethiopian patients according to their diagnoses before meningococcal PCR.

Characteristic	MD [a]	Not MD [b]
Median age, years (range)	10 (0.42–70)	9.5 (0.42–45)
Male : female ratio	1.1:1	6:1
Median duration of symptoms, days (range)	3 (1-14)	3 (0.08-14)
Diagnosis [c], no. of patients (%)		
Confirmed [d]		
Neisseria meningitidis	54 (35)	0 (0)
Streptococcus pneumoniae	0 (0)	9 (60)
Plasmodium falciparum	0 (0)	1 (6.7)
Relapsing fever	0 (0)	1 (6.7)
Tuberculous meningitis likely [e]	0 (0)	1 (6.7)
Bacterial meningitis, unspecified	101 (65)	0 (0)
Bacterial sepsis/septicaemia, unspecified	1 (0.6)	3 (20)
Total	156 (100)	15 (100)

a) Meningococcal disease likely (or confirmed). b) Meningococcal disease unlikely from the outset or a diagnosis other than MD established. c) Before meningococcal PCR. d) Includes culture, pneumococcal PCR and thick blood film results. e) Prolonged course, lymphocytic CSF, chest x-ray findings, lymphadenopathy, history of close contact with a TB patient.

Table 4.4. Performance of meningococcal *ctrA* PCR compared with a clinical and laboratory gold standard in 171 Ethiopian patients.

Clinical scenario	Gold standard diagnosis (total no. patients)		%			
	MD [(a)]	Not MD [(b)]	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
All suspected cases						
CSF PCR	122 (143)	0 (15)	85 (80-91)	100 (100-100)	100 (100-100)	42 (26-58)
Whole blood / plasma sample (together) [(c)]	47 (131)	1 (14)	36 (28-44)	93 (79-100)	98 (94-100)	13 (7-20)
Plasma sample only	38 (110)	1 (4)	35 (26-43)	75 (33-100)	97 (93-100)	4 (0-8)
Whole blood sample only	9 (21)	0 (10)	43 (22-64)	100 (100-100)	100 (100-100)	46 (25-66)
Both CSF and whole blood (or plasma)†	105 (118)	1 (13)	89 (83-95)	93 (79-100)	99 (97-100)	50 (31-69)

a) Culture-confirmed, probable and possible cases grouped together. b) Meningococcal disease unlikely or diagnosis other than meningococcal disease established. c) Either whole blood or plasma sample available. † Both a CSF sample and a sample from blood (either whole blood or plasma) were available for testing. MD = meningococcal disease. CI = confidence interval. CSF = cerebrospinal fluid.

4.3.2.1.3 Overall case ascertainment

One hundred and thirty two (85%) cases of meningococcal disease were ascertained by *ctrA* PCR and culture together, in contrast with 54 (35%) cases determined by culture alone, corresponding to a 2.4 fold increase in the absolute number of cases with a confirmatory test – or a relative increase of 50% in the case ascertainment rate.

4.3.2.2 PCR for serogroup determination

A *myn* PCR assay result was available for 41(32%) of the 127 *ctrA*-positive cases of meningococcal disease. All 41 cases tested positive for *myn* PCR and these cases were therefore determined to be of serogroup A *N. meningitidis*.

4.4 DISCUSSION

4.4.1 Interpretation of results

It is difficult to assess the performance of diagnostic tests in the context of meningococcal disease due to the lack of an ideal 'gold standard'.

Cultures are specific enough to provide definitive confirmation of cases, but have limited sensitivity, leaving a large proportion of true cases to be inappropriately categorised as not having the disease (Carrol *et al.*, 2000b). As in the case of our study, this can be a problem if the test being compared (*ctrA* PCR) is more

sensitive than culture itself, because if culture alone is the gold standard, any positive test in a culture-negative case would be counted as false positive, leading to reports of lower specificity and PPV figures.

The relatively low specificities found by this strategy (comparing with culture alone) in our study (67% in Brazil and 29% in Ethiopia) are likely to reflect a high proportion of true cases missed by culture. This was reinforced by the fact that both clinical characteristics and complementary laboratory evidence such as microscopy (Gram staining) would have categorised between 86 and 96% of these 'false positives' as 'probable' cases. On the other hand, the discrepancy between Brazilian and Ethiopian figures might reflect the fact the standard microbiological diagnostic facilities were superior in the Brazilian study site.

In contrast, gold standard diagnoses based on 'consensual' clinical assessment are helpful, particularly when standardised definitions are used, but this approach also has its limitations and weaknesses. It may be difficult to differentiate people with early features of meningococcal disease from those with self-limiting illnesses (Mandl *et al.*, 1997, Brogan & Raffles, 2000), other pathogens may cause septicaemia with purpuric rash of similar characteristics and – in the case of bacterial meningitis alone – its clinical course may be indistinguishable from other bacterial aetiologies. Besides, the definitions of probable and possible cases (PHLSMF, 2002) include the subjective perception of health staff involved in case management and, arguably, these perceptions may vary in different settings.

Reports in the literature often differ on how cases are categorised as having or not meningococcal disease for the purpose of performance assessment. In two UK

studies (Carrol *et al.*, 2000b, Hackett *et al.*, 2002a), cases considered as 'possible' were allocated as 'not MD' whereas in a recent study from Australia (Bryant *et al.*, 2004), cases falling in this category where added to the gold standard group.

In the Brazilian site of our study, 8 culture-confirmed cases were originally categorised as 'possible'. This reinforced our decision to group probable and possible cases (in both study sites, for consistency) as the gold standard to be compared to the group of unlikely or 'not' MD. We have anticipated this could have an effect on the sensitivity calculations because, by definition, in a possible case a diagnosis other than meningococcal disease is at least as likely and some cases in this group were expected to be non-meningococcal (meningitis due to pneumococcus or *Haemophilus*, for instance). To minimise this potential effect, it was decided to incorporate culture and other laboratory evidence (except meningococcal PCR) as part of the definition of the gold standard.

The weakness of this approach is that the categories used were likely to result in a conservative estimate of the sensitivity of the tests. The overall figures for meningococcal PCR sensitivity observed in Brazil (88%, 95%CI 80-95) and in Ethiopia (89%, 95%CI 83-95) however were comparable to those previously described in the UK (Carrol *et al.*, 2000b, Hackett *et al.*, 2002a) and Australia (Bryant *et al.*, 2004). Likewise, the increase in the number of confirmed cases obtained by adding PCR to culture (31% in Brazil and 50% in Ethiopia) was similar to what has been reported in the literature. Particularly important was the high sensitivity of the *ctrA* PCR assay in Ethiopia, where serogroup A *N. meningitidis* predominates, as problems in detecting this serogroup have been described in earlier *ctrA* assays, which used different primers (Guiver *et al.*, 2000).

In cases presenting as bacterial meningitis alone, meningococcal PCR in CSF samples consistently outperformed blood PCR in terms of sensitivity. In Brazil, CSF PCR in meningitic cases had a sensitivity of 71% (95%CI 57-84), against a sensitivity of 31% (95%CI 16-47) of blood PCR. At the Ethiopian site, where all but one cases presented as meningitis alone, the sensitivities of CSF and blood PCR were 85% (95%CI 80-91) and 36% (95%CI 28-44) respectively. In Brazilian cases presenting with a rash suggestive of septicaemia, CSF and blood samples yielded similar PCR results in terms of sensitivity. However, in any circumstance, having both a CSF and a blood sample increased the chances of having a confirmed result. This is in agreement with the perception in Africa that group A meningococcal disease present less frequently with septicaemia than group B disease elsewhere, although there are very few studies that provide hard evidence for this phenomenon.

In accordance with previous reports (Hackett *et al.*, 2002a), the whole blood PCR tended to perform better than plasma, significantly improving the NPV from 4% (95%CI 0-8) to 46% (95%CI 25-66).

The *ctrA* assay was also highly specific in both study sites and in any of the clinical scenarios evaluated. One single Ethiopian case, categorised in the 'not MD' group, tested positive for *ctrA* PCR. This was a 27 year-old male presenting with bacterial meningitis and negative CSF microscopy and culture, who eventually died in the hospital. This patient had been originally categorised as possible MD, but was re-located to 'not MD' after a positive pneumococcal PCR test. However, when the bacterial DNA meningococcal and pneumococcal loads were compared in the CSF sample, the *ctrA* PCR yielded a load 6.9 x 10^8 DNA

copies / ml whereas the ply (pneumococcal) PCR was only marginally positive. Considering that both *ctrA* and ply tests were shown to be highly specific in previous studies, this finding could suggest a true case of mixed infection (Downs *et al.*, 1987, Corless *et al.*, 2001), with *N. meningitidis* as the predominant pathogen. If this case were re-categorised to the gold standard group, the specificity and positive predictive values would increase to 100%.

Because there were no positive tests in individuals without meningococcal disease in all but one group, it was not possible to calculate the likelihood ratio of a positive test (defined as the proportion of true positives in those diseased divided by the proportion of false positives in those without the disease). This would provide a more informative account of the test usage in clinical practice (Sackett *et al.*, 1991).

The findings of our study reinforce the recommendation of inclusion of the *ctrA* PCR in the gold standard definition to which other future tests might be compared.

4.4.2 Applicability in the developing world

In many countries located in the African meningitis belt, where most of the population live in rural communities, sometimes scattered around vast geographical areas, the diagnosis of meningitis usually is made on clinical grounds and the CSF appearance only. Thus, it is unrealistic to propose that expensive PCR techniques could have any immediate applicability in the management of cases in these settings. However, facilities to process bacteriological samples tend to be restricted to few urban areas, leaving most of the population without access to

confirmatory diagnosis. PCR-based surveillance systems not only have a potential use but have already started to be implemented in locations such as in Niger in west Africa (Sidikou *et al.*, 2003), where the system relies on periodical transport of frozen CSF samples to a central laboratory in Niamey.

In Latin America, this approach would apply to populations of remote areas in the Amazonian rainforest or dry lands of Brazil. Conversely, the use of PCR as a diagnostic tool is on the increase in larger Brazilian cities. If Brazil follows trends observed in developed nations such as the increase of pre-admission antibiotic usage and / or reduction in the number of lumbar punctures because of perceived risks of complications (and consequent drop in number of confirmed cases), there might be a need for a wider use of non-culture methods. If and when operational and reagent costs are reduced, PCR might be an option. Cost effectiveness studies to assess the feasibility of their introduction in reference laboratories of areas with fewer resources should be encouraged.

Chapter Five

5 QUANTITATIVE BACTERIAL DNA LOAD, DISEASE PRESENTATION AND OUTCOME

5.1 INTRODUCTION

The disease presentation and outcome in meningococcal disease have been related to the levels of endotoxin in plasma and in CSF (Brandtzaeg *et al.*, 1989, Brandtzaeg *et al.*, 1992) and endotoxins are major triggers of the inflammatory cascade responsible for disease severity (van Deuren *et al.*, 1995). However, until recently, there was only scanty evidence to demonstrate the association between bacterial load and disease severity (Hackett *et al.*, 2002b) and that bacterial load and endotoxin levels are directly and closely correlated (Ovstebo *et al.*, 2004).

The relationship between the amount of meningococcal antigen in blood and CSF and disease presentation was initially described using bacteriological methods. In one report of two schoolchildren with serogroup B meningococcal disease, one child had septicaemia and one meningitis. The number of colony forming units of *N. meningitidis* was measured by quantitative direct plating. The child with septicaemia had 10^5 organisms/ml of blood and the child with meningitis had 10^4 organisms/ml of CSF (Zwahlen & Waldvogel, 1984). Another study from the USA described bacterial loads ranging from $<10^1$ to $>10^3$ in blood of children with meningococcal meningitis and septicaemia (Sullivan & LaScolea, 1987). In both studies the bacterial load was likely to be underestimated by culture because only

viable bacteria could be counted and the first report using quantitative PCR in cases of meningococcal disease found considerably higher bacterial loads (Hackett *et al.*, 2002b). This higher bacterial load has been recently demonstrated to be in the order of 1 viable bacterium cultured per thousand detected by PCR (Ovstebo *et al.*, 2004). These two studies used the capsular transfer gene *ctrA* as the PCR target. As there is only one *ctrA* gene per meningococcus, the DNA load equals the number of bacteria (cells) in the specimen.

Hackett *et al* (2002b) and Ovstebo *et al* (2004) applied real-time PCR technology to quantify the amount of bacterial DNA in clinical samples. This technique is based upon the continuous measurement of the signal emitted from the PCR reaction, using labelled oligonucleotide probes that fluoresce when cleaved or hybridised (Klein, 2002). Although the technique has many positive attributes such as a wide dynamic range of quantification, high sensitivity and precision and low risk of cross contamination, it also has some limitations. These limitations are mainly related to the exponential nature of PCR amplification (Raeymaekers, 1999), though they can be minimised when known standards are co-amplified in the reaction (Reischl & Kochanowski, 1999).

In Hackett *et al* (2002b), 51 children admitted to a paediatric referral hospital in Merseyside (UK) were prospectively enrolled over a period of one year. The study described bacterial loads in blood but not in CSF. Children with a positive PCR had a median blood bacterial load of 1.6×10^6 DNA copies/ml on admission. The bacterial loads were higher in patients with severe disease (defined as a Glasgow Meningococcal Septicaemia Prognostic Score ≥ 8) and in children who died. The

highest bacterial load in their study (1.8 x 10^9 DNA copies/ml) was found in a child who died.

Ovstebo *et al* (2004) described bacterial loads in 65 patients (age range not given) admitted to an university hospital in Oslo (Norway) over a period of 18 years. Twelve of the patients had CSF samples tested by quantitative PCR. Blood bacterial loads were higher in cases who presented with persistent shock (median 2 x 10^7 copies/ml versus $<10^3$ copies/ml in patients without shock) and there was a close correlation between the meningococcal DNA load and lipopolysaccharide concentrations in plasma ($r = 0.905$) and CSF ($r = 0.964$).

To date, there is no information on meningococcal DNA loads from settings outside Western Europe or from developing countries, where patients may have a different pattern of disease presentation. Patients in these settings are often seen late in the course of the disease and may have higher morbidity and mortality. Moreover, overall, the amount of information on bacterial loads in the CSF is limited to one case series (Ovstebo *et al.*, 2004) and there are no data available on serogroup A *N. meningitidis* meningitis.

This chapter describes the meningococcal bacterial loads in blood (whole blood or plasma) and CSF samples of Brazilian and Ethiopian patients with meningococcal disease, according to clinical presentation and outcome.

5.2 SUBJECTS AND METHODS

5.2.1 Participants

Patients who fulfilled the general enrolment criteria described in section 2.3 were included for quantitative bacterial DNA estimation if they had meningococcal disease confirmed by a positive culture or *ctrA* PCR and had at least one clinical sample (whole blood, plasma and / or CSF) available for PCR testing.

Cases were categorised as having meningitis alone (MM), septicaemia with signs of meningitis (MSM) and septicaemia alone (MS) according to the definitions described in section 2.3. Outcome was defined as survival or death during hospital stay. Patients who were lost to follow up were included in the general descriptive analysis but excluded from comparisons by outcome.

5.2.2 Laboratory methods

The principles, methods and protocol for the quantitative meningococcal *ctrA* Taqman PCR assay are described in the methods section 2.8.3.

Bacterial loads are expressed as genome copies per millilitre. The lower detection limit of the method is 10^3 DNA copies/ml (Guiver *et al.*, 2000, Hackett *et al.*, 2002b). Bacterial loads in plasma from the Ethiopian study were corrected by a factor of 1 log fold to adjust for the lower recovery in plasma as compared to whole blood (M Guiver, personal communication). Corrected values were grouped with whole blood measurements and described as a single variable as blood bacterial loads.

5.2.3 Statistics

The bacterial load values were positively skewed. Results are therefore presented as medians (interquartile range, IQR), with non-parametric tests for statistical analysis throughout the text unless otherwise stated. Box and whisker plots were used for consistency and ease of interpretation.

5.3 RESULTS

5.3.1 Brazil

Ninety out of 93 *confirmed* cases enrolled in the Brazilian study had at least one clinical sample available for PCR testing and were described here. Seventy (78%) of these cases had both blood and CSF specimens available, 5 (6%) had blood only and 15 (16%) had CSF only.

The median bacterial load on admission for all patients was 1.1×10^4 (range $<10^3$ to 3.0×10^9) copies/ml in whole blood and 3.1×10^6 (range $<10^3$ to 3.6×10^9) copies/ml in the CSF (Mann-Whitney, $p < 0.001$).

The bacterial load of the patients according to sex, age, outcome, duration of symptoms, level of consciousness and presence of decreased peripheral perfusion on admission (slow capillary refill time, cold extremities or feeble pulse; with or without hypotension) are described in tables 5.1 for blood and 5.2 for CSF samples. There were no significant differences between these variables and CSF bacterial loads. Blood bacterial loads on admission however were associated with

outcome, duration of symptoms, age and the presence of decreased peripheral perfusion, although the latter did not reach statistical significance. These associations are shown in figs 5.1 to 5.3.

The blood bacterial loads were 3 log-folds higher in non-survivors than in survivors (Mann-Whitney, $p < 0.001$) and this difference remained significant when cases with meningitis alone were excluded from the analysis. The median bacterial load in the 11 septicaemic cases who died was 3.7×10^7 copies/ml compared to a median load of 1.8×10^4 copies/ml in the 41 patients who survived (Mann-Whitney, $p = 0.001$). As illustrated in fig. 5.1, the blood bacterial load in cases of meningitis alone (median $< 10^3$, IQR 1.3×10^4 copies/ml) was significantly lower than the median bacterial load in septicaemic patients (3.9×10^4, IQR 5.6×10^5 copies/ml, Mann-Whitney, $p < 0.001$). None of the patients who had meningitis alone died.

The duration of the first symptom before presentation (usually fever) was also associated with the bacterial load in blood. Bacterial loads in blood were higher in patients presenting in the first 24 hours after initiation of symptoms than patients with later presentation (table 5.1, Mann-Whitney, $p < 0.001$). However, when cases with meningitis alone and those with septicaemia (with or without meningitis) were analysed separately (fig. 5.2), bacterial loads were only higher for patients with septicaemia who presented early but not for patients with meningitis alone who presented early (Mann-Whitney, $p = 0.8$ for meningitis alone, $p = < 0.001$ for patients with septicaemia).

Blood bacterial loads were also significantly associated with age (Kruskal-Wallis, $p = 0.04$). Figure 5.3 illustrates the loads of the patients by age group. Younger children (< 5 years) had significantly higher bacterial loads than patients above this age (Mann-Whitney, Bonferroni corrected $p = 0.028$).

No statistically significant differences were observed when bacterial loads in blood or in CSF were compared according to meningococcal serogroups B and C (fig. 5.4).

Table 5.1. Bacterial loads in the **blood** of **Brazilian** patients.

Variable	n	Median load copies/ml of blood (IQR)	Statistic †
All patients	75	1.1×10^4 (3.2×10^5)	
Sex			
Male	39	1.3×10^4 (2.5×10^5)	p = 0.983
Female	36	9.2×10^3 (5.4×10^5)	
Age group			
0 – 4 years	23	8.1×10^4 (2.7×10^6)	p = 0.040
5 – 9 years	16	3.5×10^3 (2.1×10^4)	
10 – 19 years	24	1.3×10^4 (2.5×10^5)	
≥ 20 years	21	4.4×10^3 (3.0×10^5)	
Duration of symptoms			
0 – 24h	51	1.0×10^5 (7.0×10^5)	p < 0.001
> 24h	24	1.1×10^3 (7.3×10^3)	
Level of consciousness			
Alert	41	9.7×10^3 (2.5×10^5)	p = 0.522
Respond to verbal command	25	1.1×10^4 (3.0×10^5)	
Response to painful stimuli or does not respond	9	2.3×10^4 (2.6×10^7)	
Decreased peripheral perfusion			
Present	34	3.8×10^4 (5.0×10^5)	p = 0.052
Absent	36	4.6×10^3 (1.5×10^5)	
Outcome			
Survival	64	6.8×10^3 (2.3×10^5)	p < 0.001
Death	11	6.9×10^6 (3.7×10^7)	

†Mann-Whitney U test for comparison between 2 categories and Kruskal-Wallis one-way analysis of variance for comparing three or more categories. IQR = interquartile range. Decreased peripheral perfusion includes slow capillary refill time, cold extremities and feeble pulse; with or without hypotension.

Table 5.2. Bacterial loads in the **CSF** of **Brazilian** patients.

Variable	n	Median load copies/ml of CSF (IQR)	Statistic †
All cases	85	3.1×10^6 (5.2×10^7)	
Sex			
Male	45	2.2×10^6 (3.1×10^7)	p = 0.260
Female	40	4.6×10^6 (5.8×10^7)	
Age group			
0 – 4 years	26	3.5×10^6 (1.0×10^8)	p = 0.610
5 – 9 years	21	1.5×10^6 (5.1×10^7)	
10 – 19 years	26	2.7×10^6 (5.4×10^7)	
≥ 20 years	12	5.1×10^6 (5.1×10^7)	
Duration of symptoms			
0 – 24h	58	2.5×10^6 (3.6×10^7)	p = 0.343
> 24h	27	3.5×10^6 (8.5×10^7)	
Level of consciousness			
Alert	46	3.8×10^6 (5.6×10^7)	p = 0.245
Respond to verbal command	30	2.0×10^6 (6.1×10^7)	
Response to painful stimuli or does not respond	9	4.6×10^5 (5.1×10^6)	
Decreased peripheral perfusion			
Present	34	2.1×10^6 (5.7×10^7)	p = 0.820
Absent	45	3.5×10^6 (5.2×10^7)	
Outcome			
Survival	76	3.4×10^6 (5.4×10^7)	p = 0.705
Death	09	1.9×10^6 (5.6×10^6)	

†Mann-Whitney U test for comparison between 2 categories and Kruskal-Wallis one-way analysis of variance for comparing three or more categories. IQR = interquartile range. Decreased peripheral perfusion includes slow capillary refill time, cold extremities and feeble pulse; with or without hypotension.

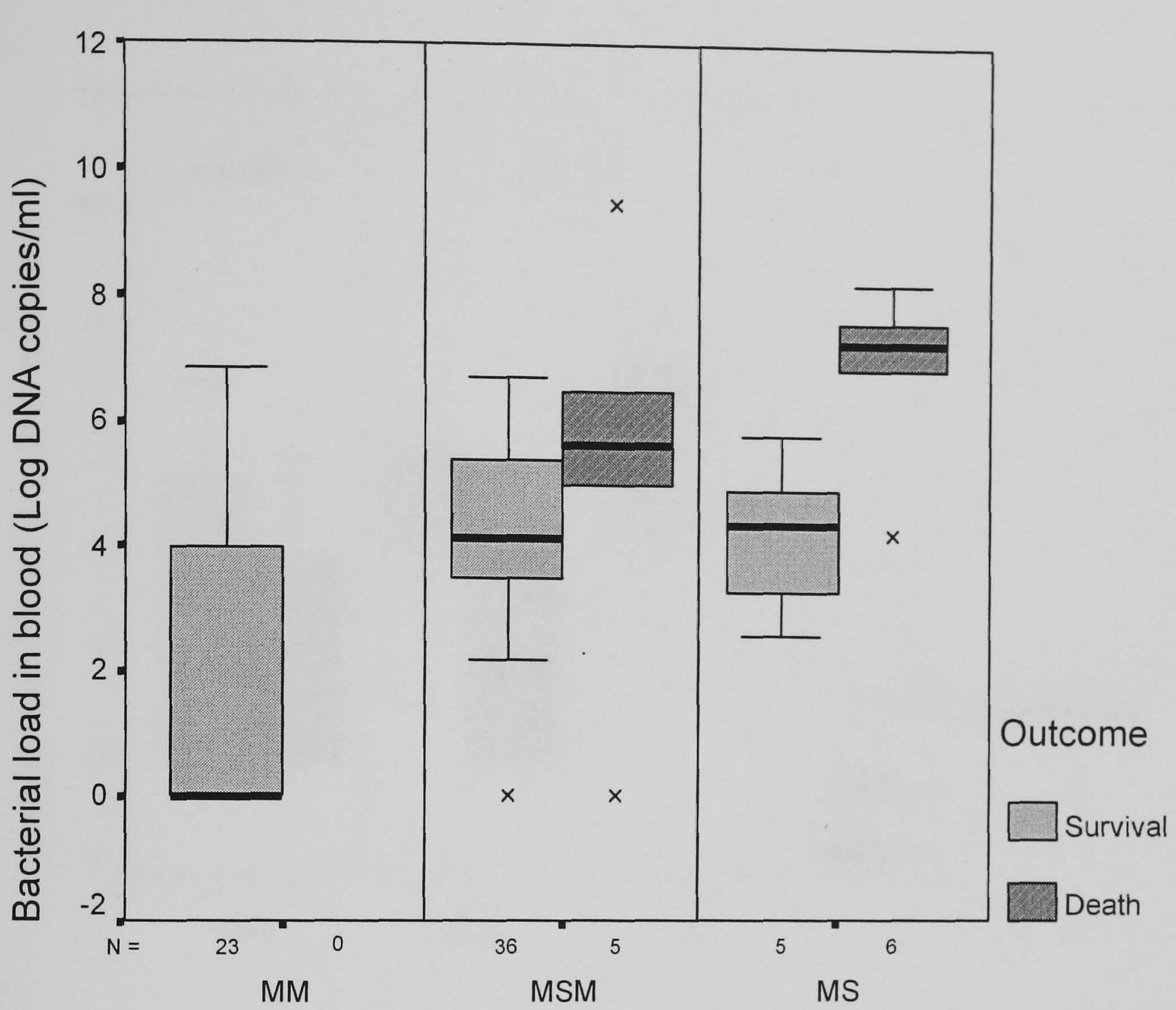


Fig. 5.1. Bacterial load in blood of survivors and non-survivors amongst Brazilian patients according to clinical form. Upper and lower whiskers represent the 90th and 10th percentiles, the box represents the interquartile range (75th and 25th percentiles). The thick line crossing the box represents the median (50th percentile). When upper or lower percentiles coincide, whiskers are omitted. Outliers are shown as "x". MM = meningococcal meningitis, (alone). MSM = meningococcal septicaemia with meningitis. MS = meningococcal septicaemia (alone).

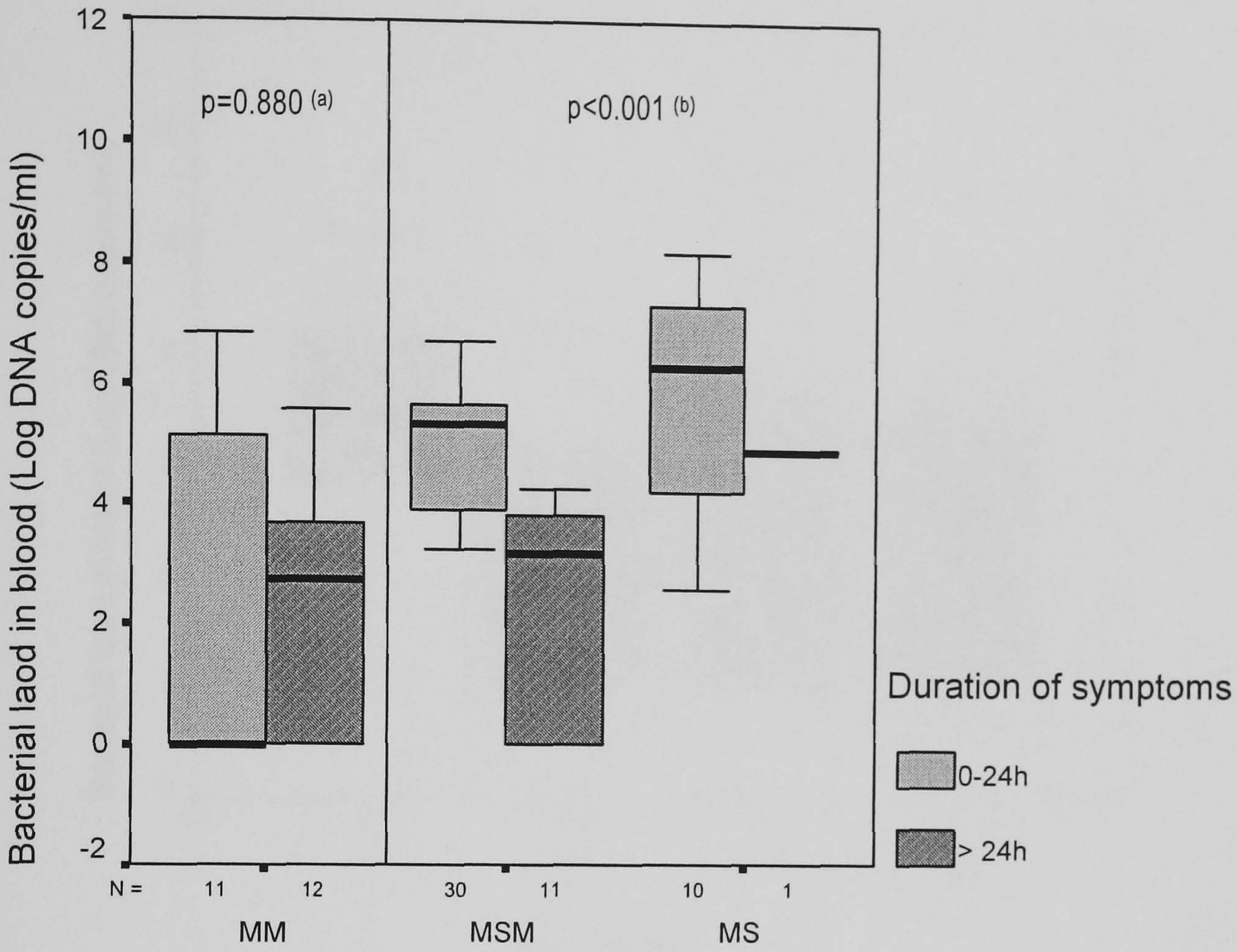


Fig. 5.2. Bacterial load in blood of cases by clinical form, comparing groups according to duration of symptoms (≤24h and >24h) in cases of meningitis alone (a) and the group of septicaemic cases with or without meningitis (b), Mann-Whitney. MM = meningococcal meningitis, (alone). MSM = meningococcal septicaemia with meningitis. MS = meningococcal septicaemia (alone).

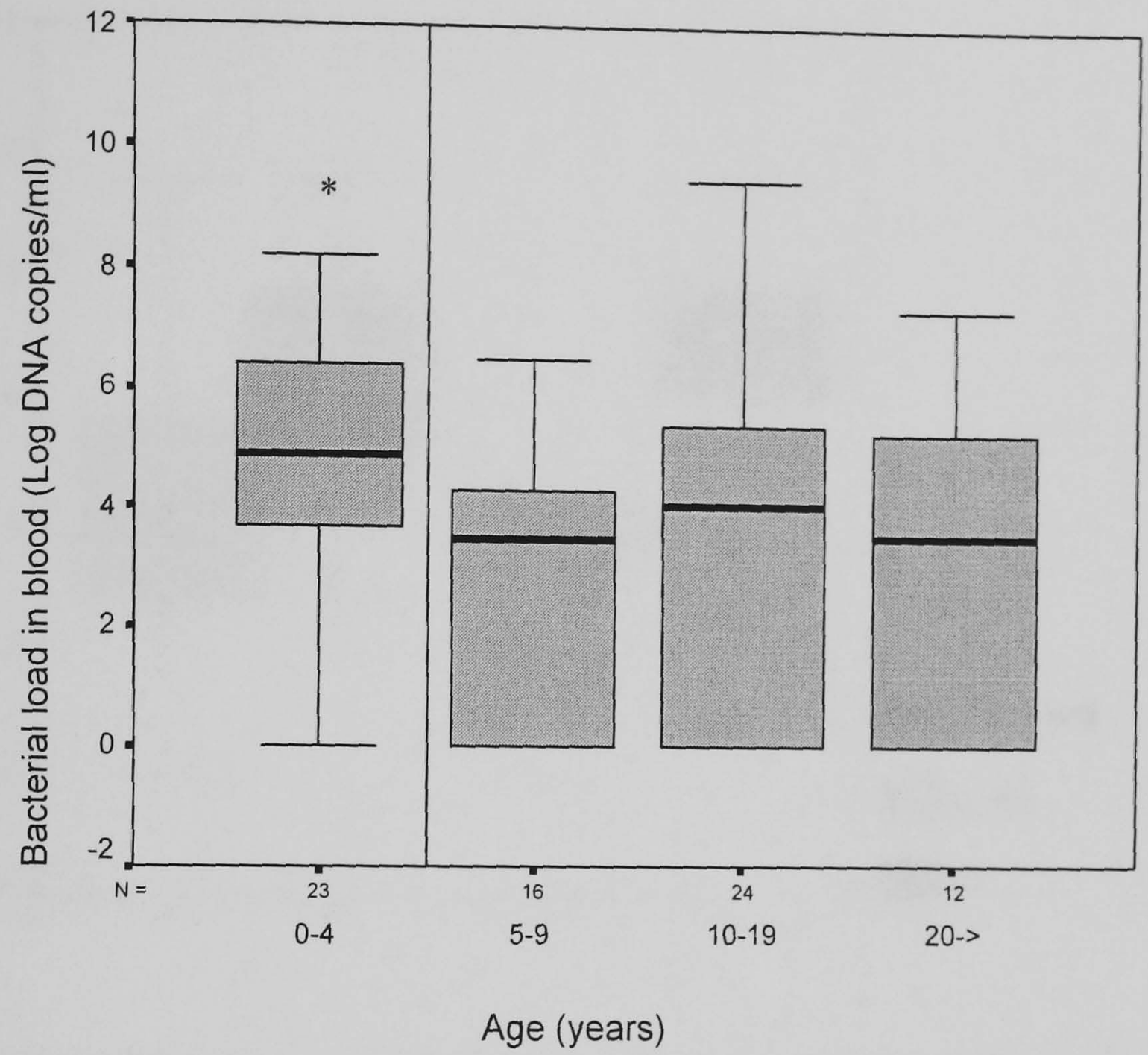


Fig. 5.3. Bacterial load in blood according to age group (years). Bacterial loads were significantly higher in 0-4 year old children compared to the other age groups (Mann-Whitney, Bonferroni corrected $p = 0.028$).

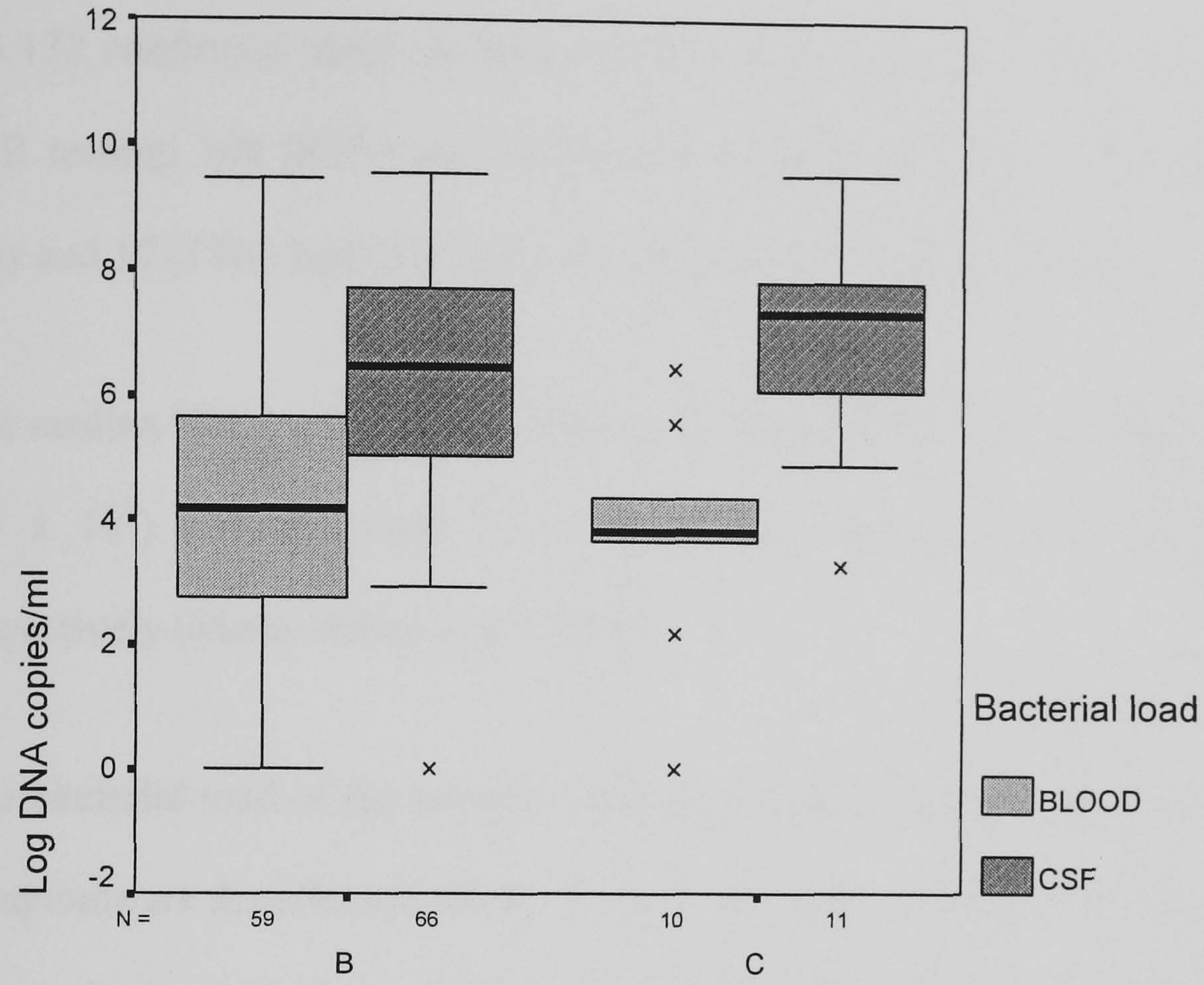


Fig. 5.4. Bacterial load in blood and CSF of Brazilian patients with meningococcal serogroup B or C infection. Comparisons were made between blood loads (Mann-Whitney, p = 0.732) and between CSF loads (Mann-Whitney, p = 0.247) of serogroups B and C.

5.3.2 Ethiopia

All 132 *confirmed* cases enrolled in Ethiopia had a clinical sample available for PCR testing; 108 (82%) had both blood and CSF specimens, 7 (5%) had blood only and 17 (13%) had CSF only. All patients had meningitis alone.

The median blood and CSF bacterial loads on admission were $<10^3$ (range $<10^3$ to 6.3×10^9) copies/ml and 3.0×10^7 (range $<10^3$ to 3.3×10^{10}) copies/ml respectively (Mann-Whitney, $p < 0.001$).

The bacterial load of the patients according to sex, age, outcome and duration of symptoms are described in table 5.3 for blood and in table 5.4 for CSF. Figure 5.5 shows the bacterial loads in blood and CSF according to the patients' outcome. There were no statistically significant differences in blood or CSF loads and outcome or any of the former variables. There were however few cases with complete clinical data and we are unable to describe the bacterial loads according to the level of consciousness or decreased peripheral perfusion at the time of admission.

Table 5.3. Bacterial loads in the **blood** of **Ethiopian** patients.

Variable	n	Median load copies/ml of blood (IQR)	Statistic †
All patients	115	$<10^3$ (8.0×10^5)	
Sex			
Male	60	$<10^3$ (9.2×10^5)	p = 0.278
Female	55	$<10^3$ (6.9×10^5)	
Age group			
0 – 4 years	26	$<10^3$ (1.6×10^5)	p = 0.147
5 – 9 years	30	1.6×10^5 (2.5×10^6)	
10 – 19 years	40	$<10^3$ (4.1×10^5)	
≥ 20 years	19	$<10^3$ (3.3×10^6)	
Duration of symptoms			
0 – 24h	16	$<10^3$ (3.0×10^6)	p = 0.743
> 24h	90	$<10^3$ (6.0×10^5)	
Outcome			
Survival	104	$<10^3$ (7.8×10^5)	p = 0.381
Death	5	$<10^3$ (1.6×10^6)	

†Mann-Whitney U test for comparison between 2 categories and Kruskal-Wallis one-way analysis of variance for comparing three or more categories. IQR = interquartile range.

Table 5.4. Bacterial loads in the **CSF** of **Ethiopian** patients.

Variable	n	Median load copies/ml of CSF (IQR)	Statistic †
All patients	125	3.0×10^7 (2.3×10^8)	
Sex			
Male	65	3.0×10^4 (2.5×10^8)	p = 0.641
Female	60	3.7×10^7 (2.1×10^8)	
Age group			
0 – 4 years	28	4.8×10^7 (1.8×10^8)	p = 0.649
5 – 9 years	32	5.3×10^7 (2.4×10^8)	
10 – 19 years	45	2.3×10^7 (1.7×10^8)	
≥ 20 years	20	3.7×10^7 (6.1×10^8)	
Duration of symptoms			
0 – 24h	18	2.2×10^7 (6.8×10^8)	p = 0.832
> 24h	99	3.6×10^7 (2.0×10^8)	
Outcome			
Survival	112	3.3×10^7 (2.2×10^8)	p = 0.331
Death	7	2.6×10^8 (2.7×10^9)	

†Mann-Whitney U test for comparison between 2 categories and Kruskal-Wallis one-way analysis of variance for comparing three or more categories. IQR = interquartile range.

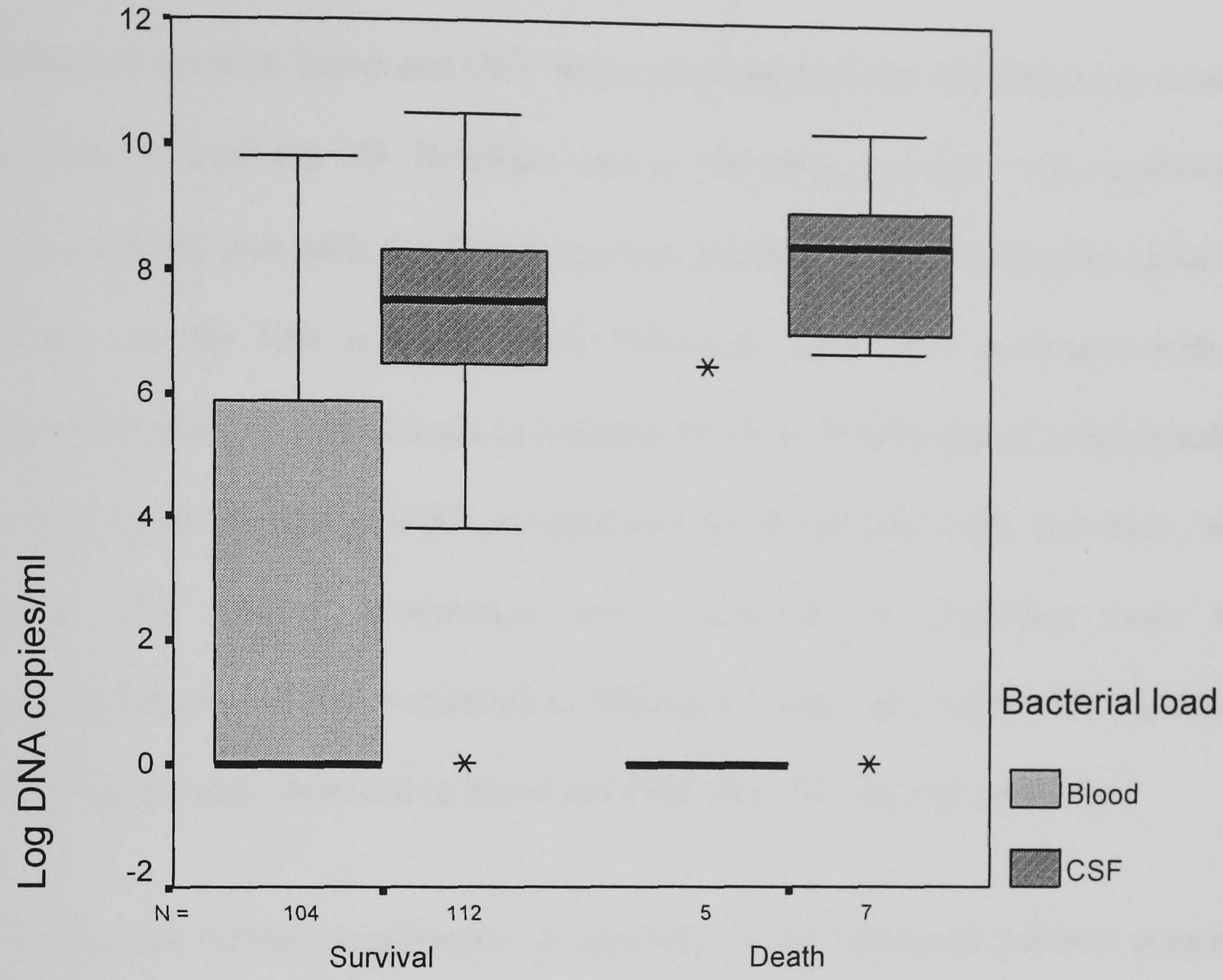


Fig. 5.5. Bacterial loads in blood and CSF of Ethiopian patients according to outcome. Comparisons were made between blood loads (Mann-Whitney, p = 0.381) and between CSF loads (Mann-Whitney, p = 0.331) of survivors and non-survivors. Outliers are shown as "*". All patients had meningitis.

5.3.3 Comparison between Ethiopian and Brazilian cases

Bacterial loads in blood and CSF were compared between the Ethiopian cases of meningitis and the 93 Brazilian cases, including patients with septicaemic presentations and with the Brazilian cases presenting with meningitis alone (31 cases). In the first scenario, when Ethiopian cases were compared with all Brazilian cases, bacterial loads in blood and CSF were both higher in the Brazilian cohort ($p=0.035$ and $p<0.001$, respectively for blood and CSF). However, when cases with similar presentation were compared (i.e. Brazilian cases with meningitis alone were compared to Ethiopian cases), no statistically significant differences were observed in blood and CSF bacterial loads (figure 5.6).

Despite not having septicaemia, a subgroup of 29 Ethiopian patients presented with levels of bacterial load as high as those observed in Brazilian patients with meningococcal septicaemia.

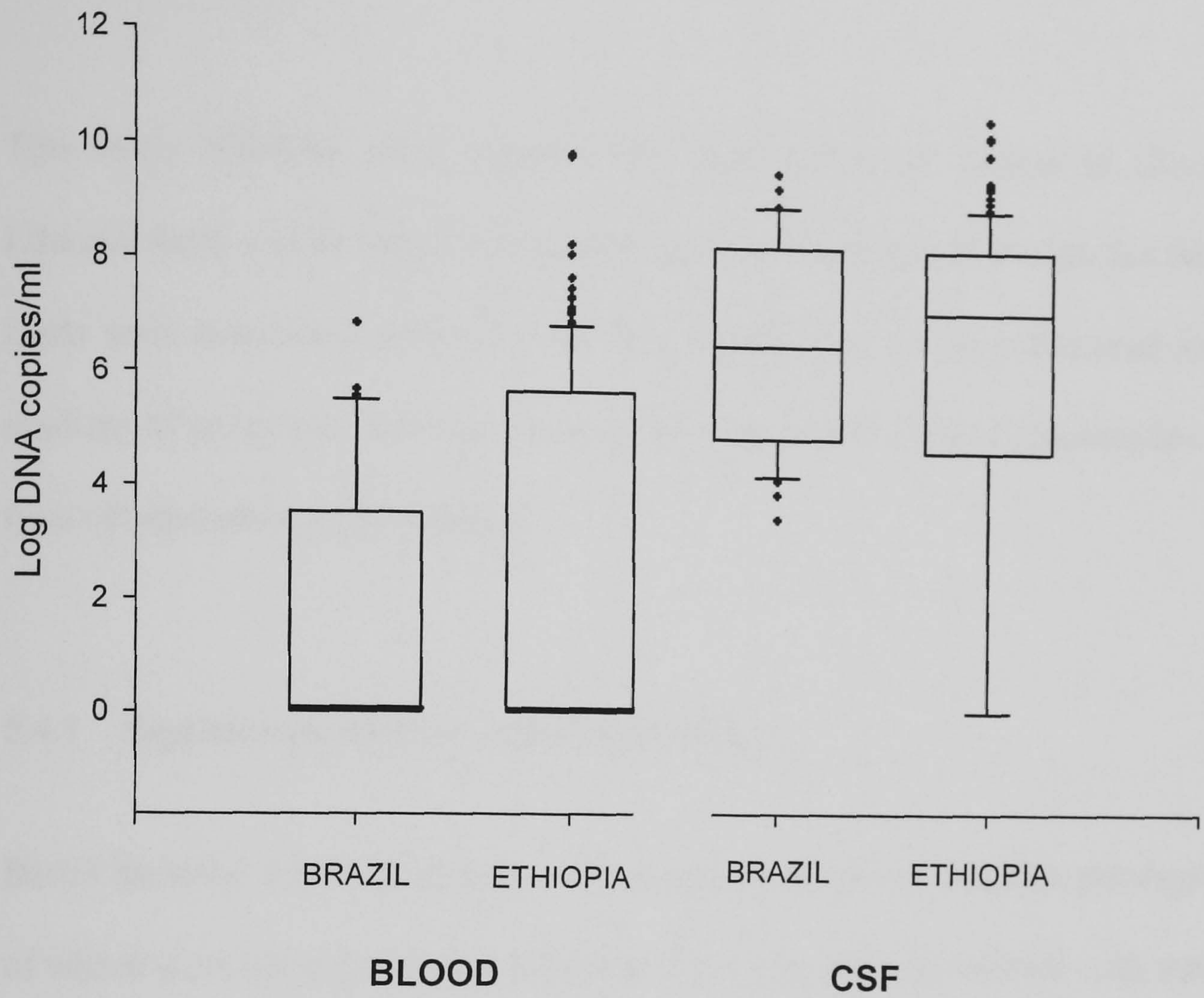


Fig. 5.6. Comparison of bacterial loads in blood and CSF of Brazilian and Ethiopian cases of meningococcal disease presenting with **meningitis** alone (Mann-Whitney, $p = 0.812$ for blood loads, $p = 0.758$ for CSF loads).

5.4 DISCUSSION

This study illustrates that patients with meningococcal disease in Brazil and Ethiopia have a wide range of bacterial loads in blood and CSF and that bacterial loads were associated with outcome. The significance of these bacterial loads as markers of prognosis however varied according to the clinical presentation at the time of admission to the hospital.

5.4.1 Septicaemia with or without meningitis

Blood bacterial loads in patients with septicaemia with or without meningitis, all of whom were identified in the Brazilian study site, were associated with outcome, age and duration of symptoms. In accordance with previous reports, bacterial loads were higher in the blood samples of patients who died compared to the loads of patients who survived (Hackett *et al.*, 2002b, Ovstebo *et al.*, 2004). These findings are in agreement with the assumption that bacterial loads are proportional to the amount of bacterial lipopolysaccharides, which in turn are major triggers for the inflammatory response responsible for shock and ultimately death in patients of severe septicaemia (Brandtzaeg *et al.*, 1989). These findings support the potential of blood quantitative PCR, that the test could not only become an important diagnostic tool if it becomes more widely available in the future, but also serve as a prognostic marker in cases of septicaemia, helping to identify patients with increased risk of death. This could also guide the selection of patients for enrolment in clinical trials of novel or experimental therapies.

The association between blood bacterial load and age and duration of symptoms in septicaemic patients has not been previously described. The age and duration of symptoms of the patients participating in Ovstebo *et al* study (2004) were not reported and Hackett *et al* study (2002b) only included children and no assessment was made for the differences in bacterial loads according to age. The median duration of symptoms in this latter study was just under 12 hours both for mild and severe cases and no differences in bacterial loads were observed between survivors and non-survivors. Conversely, in our study children with less than 5 years of age had higher blood bacterial loads than patients above this age. Although this could reflect variations in disease severity by age, the case-fatality in children under five was not statistically different than in older patients. Younger children may be less able to prevent the multiplication of *N. meningitidis* in the bloodstream, thus yielding higher bacterial loads. However, the extent to which this affects the systemic inflammatory response remains unclear. Patients presenting within 24 hours since the appearance of the first symptoms had higher blood bacterial loads than those presenting later and this may denote differences in disease severity. Early presentation has been associated with disease severity (Ansari *et al.*, 1979), as the disseminating rash and the early development of symptoms of shock may influence the patient and or carers to health seeking behaviour. In our study, the case-fatality of septicaemic patients presenting within 24 hours after initiation of symptoms was 3-fold higher than in patients presenting after this period, although the numbers of children in the subgroups was rather small and the difference was not statistically significant. The time since the perception of the first symptom before admission may also be influenced by the

parent's awareness of the symptoms and signs of the disease and by their accessibility to the health services.

5.4.2 Meningitis alone

Blood and CSF bacterial loads of patients who had only meningitis, (23 Brazilian and all Ethiopian patients), were not associated with outcome. There were however no deaths in the 23 Brazilian patients with meningitis alone, the number of deaths in Ethiopia was relatively low and this study would not have enough power to detect small differences. When the Brazilian cases with septicaemia with signs of meningitis were analysed, blood load – but not CSF load – was associated with a poor outcome. This finding might be explained by the fact that while endotoxin-induced shock is the major cause of death in septicaemic cases, the inflammatory response is compartmentalised to the brain in the case of meningitis and most deaths are due to cerebral oedema with raised intracranial pressure and its consequences (Nugent *et al.*, 1979).

It is also worth highlighting that a number of Ethiopian patients without clinical signs of septicaemia had high bacterial loads in blood. These patients may have different immunological and cytokine responses and a subgroup of 29 patients who had high bacterial loads (above the 75^{th} percentile) will be assessed in more detail in chapter 6.

We were not able to follow our patients for a long time. Further studies therefore are desirable as they might observe more subtle adverse outcomes such as

neurological sequelae (including hearing impairment and learning difficulties) in patients with high or low bacterial loads.

Further studies should include longer term follow up and a more detailed description of disease severity, with validated scores for septicaemia and prognostic scores that are reliable and relevant to patients with meningitis in lower-resourced settings.

Chapter Six

6 HOST INFLAMMATORY RESPONSE, BACTERIAL LOAD AND OUTCOME

6.1 INTRODUCTION

The release of inflammatory mediators by host cells in response to the presence of meningococcal lipooligossacharide (LOS) has been extensively studied in experimental models and in human cases of MD (see Hackett *et al.*, 2001). The description of the central role played by TNF-α in the pathogenesis of endotoxic shock (Beutler & Cerami, 1986) was followed by the demonstration of a strong correlation between fatal outcome of septic shock and high levels of TNF-α (Waage *et al.*, 1987). These early studies led to a an increasing number of reports showing association of extensive inflammation and fatal outcome in MD (van Deuren *et al.*, 2000) and other types of endotoxic shock. These studies led to a number of trials of novel therapies aimed at modulating host response, most of which have yielded disappointing but perhaps not unexpected results given the complexity of the interactions between the cytokines in the infected host (Carrol *et al.*, 2001).

6.1.1 Cytokines

Cytokines can be defined as '*regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include*

numerous effects on cells of the immune system and modulation of inflammatory responses' (Vilcek, 1998). Many such molecules have been described so far and their intricate biological actions include characteristics such as pleiotropy (a cytokine tends to have multiple targets and multiple actions); redundancy (different cytokines may have similar actions); synergism (or antagonism) and a cascade effect (a cytokine may increase or decrease the production of another cytokine). Different classification systems have been suggested, but shared functional properties allow categorisation into groups such as 1) haematopoietic cytokines; 2) cytokines involved in specific immunity; 3) primary inflammatory and secondary inflammatory cytokines of innate immunity; 4) anti-inflammatory/immunosuppressive cytokines (Mantovani *et al.*, 2000).

6.1.1.1 Pro-inflammatory cytokines

The classical representatives of this category of cytokines are TNF-α, IL-1β, and IL-6.

In MD, TNF-α was shown to be the first cytokine to increase after LOS stimulus and to be associated with disease severity, development of shock and mortality (Waage *et al.*, 1989a, van Deuren *et al.*, 1998). Its role is well established in the pathogenesis of meningococcal septicaemia and meningitis (Waage *et al.*, 1989b). TNF-α promoter polymorphisms have been described in studies of genetic susceptibility, but their association with higher risk of severe disease or death by MD in unclear as studies have yielded conflicting results (Nadel *et al.*, 1996, Read *et al.*, 2000). Although anti-TNF therapy has shown promising results in animal models of sepsis, clinical trials in humans have not been successful (Read, 1998).

Raised concentrations of IL-1β have also been demonstrated in plasma and CSF of patients with meningococcal disease, with higher levels described in the plasma of fatal cases than in survivors (Waage *et al.*, 1989a, Waage *et al.*, 1989b, van Deuren *et al.*, 1995). The activity of IL-1β is regulated by a specific receptor antagonist (IL-1ra) and the ratio between the two cytokines might influence outcome (van Deuren *et al.*, 1997). A variation in the IL-1 gene locus was shown to be associated with an increased risk of death in MD in England and Wales, but not with susceptibility to the infection (Read *et al.*, 2003).

IL-6 has also been shown to be raised in the CSF of patients with meningococcal meningitis (Waage *et al.*, 1989b, Hashim *et al.*, 1995) in the plasma of septicaemic cases; and to be associated with poor outcome in MD (Waage *et al.*, 1989a, van Deuren *et al.*, 1995, Frieling *et al.*, 1996). IL-6 has recently been shown to be a mediator of the myocardial depression seen in MD (Pathan *et al.*, 2004).

6.1.1.2 Chemokines

Chemokines are chemotactic proteins that can be classified as secondary inflammatory cytokines for their central role in leukocyte trafficking. The prototype chemokine is IL-8. This and other chemokines have been studied in MD (Hackett *et al.*, 2001).

Increased concentrations of IL-8 are found in plasma and CSF of cases of MD, with higher levels in septicaemic cases, where it correlates with levels of TNF-α and IL-6 (Halstensen *et al.*, 1993). IL-8 also appears to have a role in the skeletal muscle damage seen in septicaemic cases of MD (Carrol *et al.*, 2002).

On the other hand, another chemokine named RANTES (regulated upon activation, normal T cell expressed and secreted) has been shown to be negatively correlated with IL-8 and TNF-α with significantly lower levels seen in fatal cases. RANTES thus also seems to play a role in the pathogenesis of MD (Carrol *et al.*, 2000a).

6.1.1.3 Anti-inflammatory cytokines

Human IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of the major pro-inflammatory cytokines and chemokines such as TNF-α and IL-1 (Opal *et al.*, 1998). Increased levels of IL-10 have been described in the serum of cases of MD, and it has also been correlated with mortality (Riordan *et al.*, 1996, Lehmann *et al.*, 1995). Higher IL-10 to TNF-α ratios were also associated with death, suggesting the overproduction of IL-10 has a major role as predictor of fatal outcome (Gogos *et al.*, 2000).

Another of the anti-inflammatory (or inhibitory) cytokines is the IL-1 receptor agonist (IL-1ra), which has the function of preventing biological response to IL-1, modulating its activity specially in the early stages of meningococcal infections (van Deuren *et al.*, 1997), where it has been found in increased levels in those with the most severe disease.

6.1.2 Neuropeptides

6.1.2.1 Substance P

Substance P is a tachykinin synthesized on ribosomes as a larger protein and then enzymatically converted into the active 11 amino acid peptide. It is released from the central and peripheral endings of primary afferent neurons and functions as a neurotransmitter. It has been implicated in a number of disease processes including neurological, respiratory and intestinal diseases (Harrison & Geppetti, 2001). Substance P has been shown to have potent vasodilator and pro-inflammatory properties and has been studied in the context of sepsis and septic shock. Beer et al (2002) found increased levels of substance P in plasma of cases with postoperative sepsis compared to controls and also found that non-survivors had higher levels, though this did not become apparent until later in the course of sepsis.

6.1.2.2 Calcitonin Gene Related Peptide

CGRP is a 37 amino acid neuropeptide found in sensory peptidergic nerves that, along with substance P, may be released during endotoxic shock. It also has been shown to have potent vasodilator properties and has been postulated to play a role in the pathogenesis of vasoplegic shock. In two cohorts of adult patients with sepsis, it was found to have increased levels in cases compared to controls and in non-survivors compared to survivors, since the outset of the sepsis (Arnalich *et al.*, 1996, Beer *et al.*, 2002).

No studies to date have reported levels of substance P or CGRP in the context of MD.

6.1.3 Bacterial load

The disease presentation and outcome in meningococcal disease have been related to the levels of LOS in plasma and in CSF (Brandtzaeg *et al.*, 1989, Brandtzaeg *et al.*, 1992) and recently bacterial loads have been shown to correlate with LOS levels (Ovstebo *et al.*, 2004) and disease severity (Hackett *et al.*, 2002b).

6.1.4 Rationale and aims

Despite the extensive literature on the associations between cytokines and outcome in MD, the failure of anti-cytokine therapies is a reminder that greater understanding of the complex interactions between cytokines is needed. Few studies have addressed a large number of pro and anti-inflammatory cytokines at the same point in time in the same cohort of patients. Data on cytokines from patients from settings other than Western developed countries are scarce and to date there are no reports on substance P and CGRP in the context of MD. Moreover, no reports have yet been published on the correlations of cytokines and neuropeptides and the levels of bacterial load in MD.

This study describes pro and anti-inflammatory cytokines, chemokines and neuropeptides in Brazilian and Ethiopian confirmed cases of meningococcal disease according to outcome and bacterial load.

6.2 SUBJECTS AND METHODS

6.2.1 Participants

Patients who fulfilled the general enrolment criteria described in section 2.3 and had meningococcal disease confirmed by a positive culture or *ctrA* PCR were included if they had at least one clinical sample (plasma and / or CSF) available for immunoassay testing. Only patients from Brazil were tested for CGRP.

Cases were categorised as having meningitis alone (MM), septicaemia with signs of meningitis (MSM) and septicaemia alone (MS) according to the definitions described in section 2.3. Outcome was defined as survival or death during hospital stay. Patients who were lost to follow up were included in the general descriptive analysis but excluded from comparisons by outcome.

For the comparison of cytokine and neuropeptide responses, Ethiopian patients with meningitis were also categorised according to the levels of blood meningococcal bacterial load in two groups: 'high blood bacterial load' (defined as bacterial load in blood above the 75th percentile, i.e. with levels comparable to Brazilian septicaemic cases) and 'not-high blood bacterial load' (the remaining patients).

6.2.2 Laboratory methods

The principles, methods and protocols for the ELISA assays to measure pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokines (IL-8 and RANTES) as well as anti-inflammatory cytokines (IL-1ra and IL-10) are described

in the methods section 2.8.1.1. The methods for the measurements of neuropeptides are found in sections 2.8.1.2 (substance P) and 2.8.1.3 (CGRP). Quantitative PCR was performed as described in section 2.8.3 and in chapters 4 and 5 and bacterial loads are expressed as genome copies per millilitre.

6.2.3 Statistics

Most quantitative variables were found not to follow a normal distribution. For consistency and ease of interpretation, results are therefore presented as medians (interquartile range, IQR), with non-parametric tests for statistical analysis throughout the text unless otherwise stated. Box and whisker plots were draw to present associations by outcome. Scatter plots were used to present correlations between two continuous variables and logarithmic scales were used when convenient. Ratio statistics were calculated with SPSS for Windows version 11.0 and the 95% confidence intervals given did not assume normal distribution of ratio data.

6.3 RESULTS

6.3.1 Brazil

All 93 *confirmed* cases enrolled in the Brazilian study had at least one clinical sample available for immunoassay testing. Seventy six (82%) of these cases had a blood sample and 88 cases (95%) had a CSF sample.

6.3.1.1 Cytokines

Plasma and CSF concentrations for the various cytokines are shown in table 6.1. Plasma concentrations of IL-1β, IL-6, TNF-α, IL-8, IL-1ra and IL-10 were all higher in non-survivors ($p<0.001$). The exception to this was RANTES, for which levels in non-survivors were lower than in survivors ($p<0.001$).

On the other hand, in the CSF of Brazilian patients statistically significant differences between survivors and non-survivors were only found in concentrations of anti-inflammatory cytokines. Both IL-10 and IL-1ra were lower in the CSF of non-survivors ($p=0.032$ and $p=0.001$, respectively for IL-10 and IL-1ra).

The anti: pro inflammatory ratios are shown in the bottom of table 6.1. In non-survivors, the IL-10: TNF-α ratio was higher in blood and lower in CSF compared to survivors, whereas the IL-10: IL-1β ratio was lower in blood and similar in CSF and the IL-1ra: IL-1β ratio was lower in blood and in CSF. None of these differences however were statistically significant, as their 95% confidence intervals overlapped.

Figure 6.1 shows box-and-whisker plots for pro-inflammatory cytokines and chemokines by outcome, whereas figure 6.2 shows concentrations of anti-inflammatory cytokines in plasma and in CSF of survivors and non-survivors.

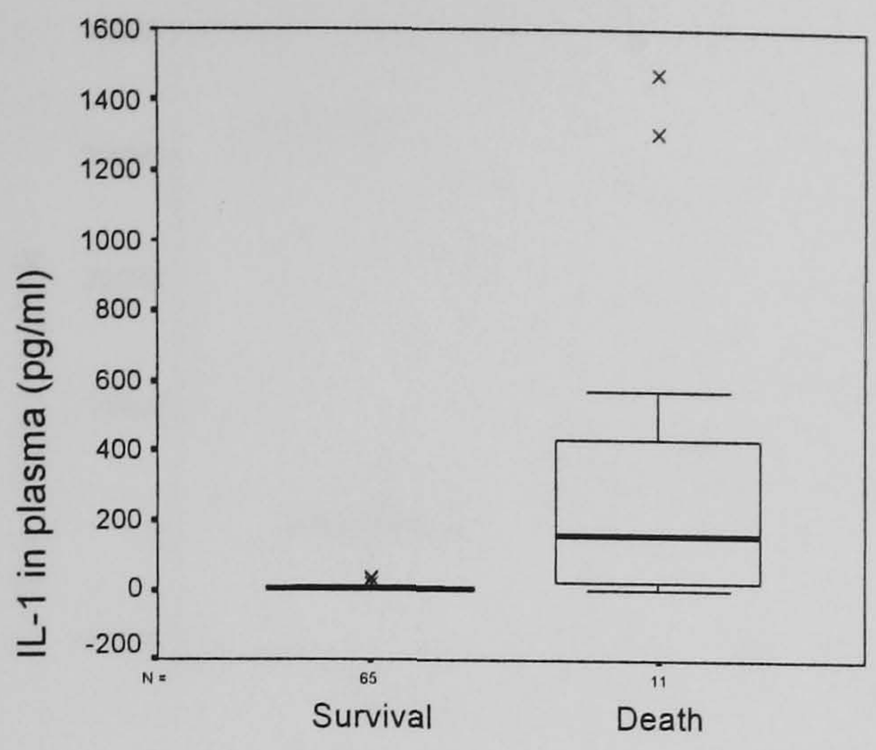
Correlations between concentrations of cytokines and levels of bacterial loads in each of the body compartments (peripheral blood and CSF) are shown in table 6.2. All cytokines but RANTES showed a statistically significant positive correlation

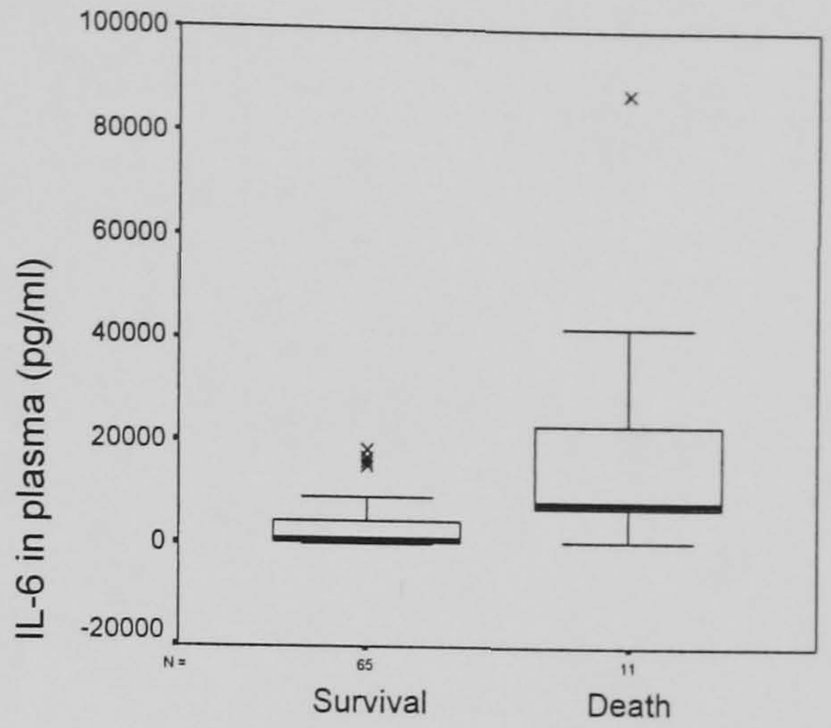
with bacterial load in each body compartment ($p<0.001$). RANTES in plasma showed a negative correlation with blood bacterial load (Spearman's rho –0.258. $p=0.025$) but RANTES in CSF showed a positive correlation with CSF bacterial load (correlation coefficient 0.368, $p=0.001$). In figure 6.3, scatter plots for IL-6 (a) and RANTES (b) and bacterial loads in blood are shown as examples of positive and negative correlations, respectively.

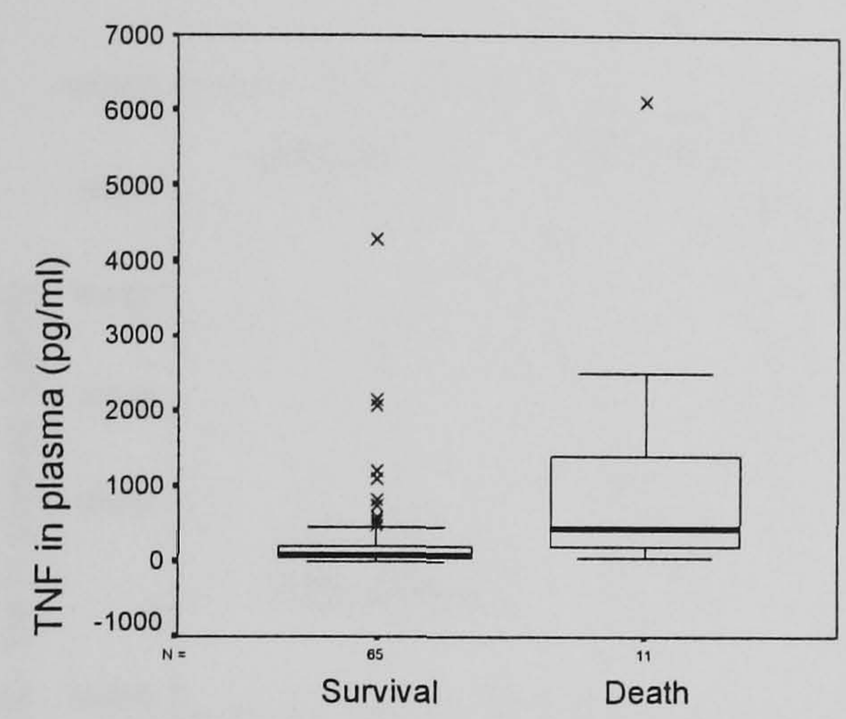
Table 6.1 Levels of pro–inflammatory cytokines and chemokines, anti-inflammatory cytokines and anti: pro inflammatory cytokine ratios in **Brazilian** patients by **outcome**.

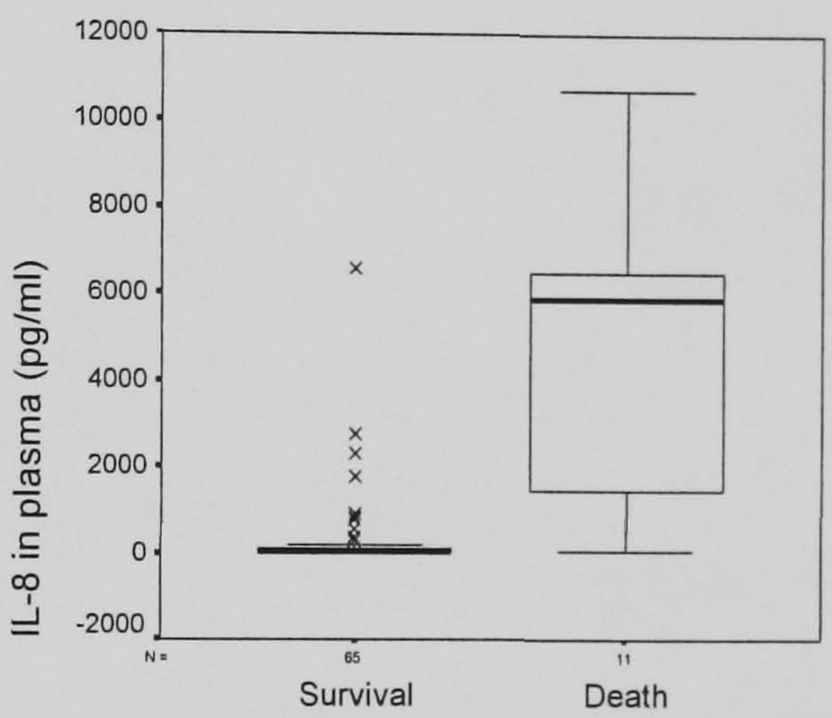
Cytokine		Median concentration in pg/ml (IQR)					
		Blood			CSF		
		Dead (n=11)	Alive (n=65)	p*	Dead (n=10)	Alive (n=78)	p*
Pro-inflammatory	IL-1β	161 (549)	3 (8)	<0.001	159 (3797)	1784 (3494)	0.39
	IL-6	8176 (24845)	957 (4565)	<0.001	60085 (62037)	44624 (32194)	0.12
	TNF-α	458 (1851)	99 (211)	<0.001	318 (13432)	816 (3283)	0.91
Chemokines	IL-8	5863 (5643)	27 (85)	<0.001	7464 (42437)	7414 (17687)	0.51
	RANTES	12751 (18851)	33212 (15767)	<0.001	86 (730)	199 (358)	0.40
Anti-inflammatory	IL-1ra	86436 (62842)	4436 (11506)	<0.001	5750 (15913)	91290 (153540)	0.001
	IL-10	5413 (14438)	183 (418)	<0.001	160 (1123)	1246 (3485)	0.032
Ratios (95% CI)	IL-10: TNF-α	6.9 (1.6-26.4)	1.9 (1.5-3.1)	-	0.6 (0.01-2.5)	1.8 (0.7-3.0)	-
	IL-10: IL-1β	19.0 (10.7-45.9)	39.3 (32.0-74.0)	-	0.7 (0.02-2.5)	0.7 (0.5-1.6)	-
	IL-1ra: IL-1β	254.4 (60.8-3834.1)	1283.0 (992.8-2158.7)	-	17.9 (3.2-68.2)	55.8 (28.4-73.9)	-

IQR= Interquartile range. CI = Confidence interval. * Mann Whitney U test used to compare medians between survivors and non-survivors.









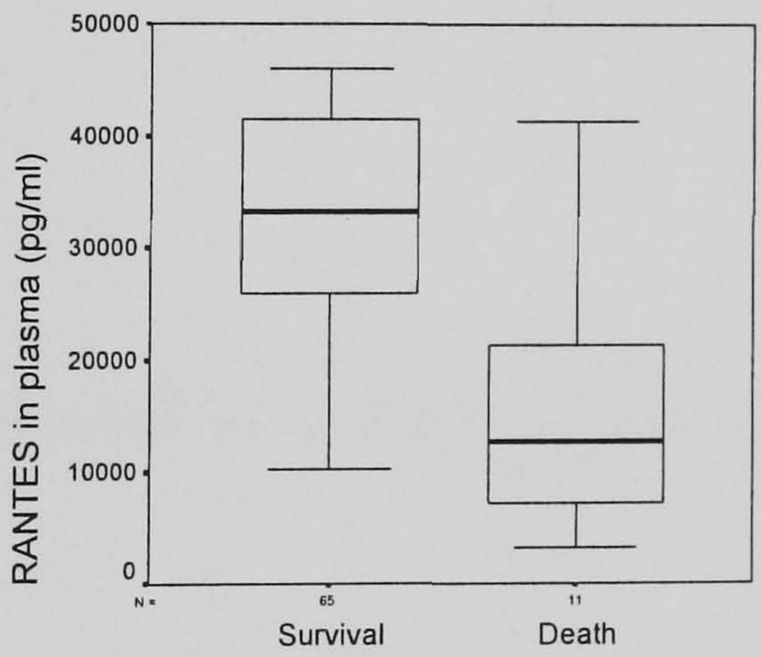
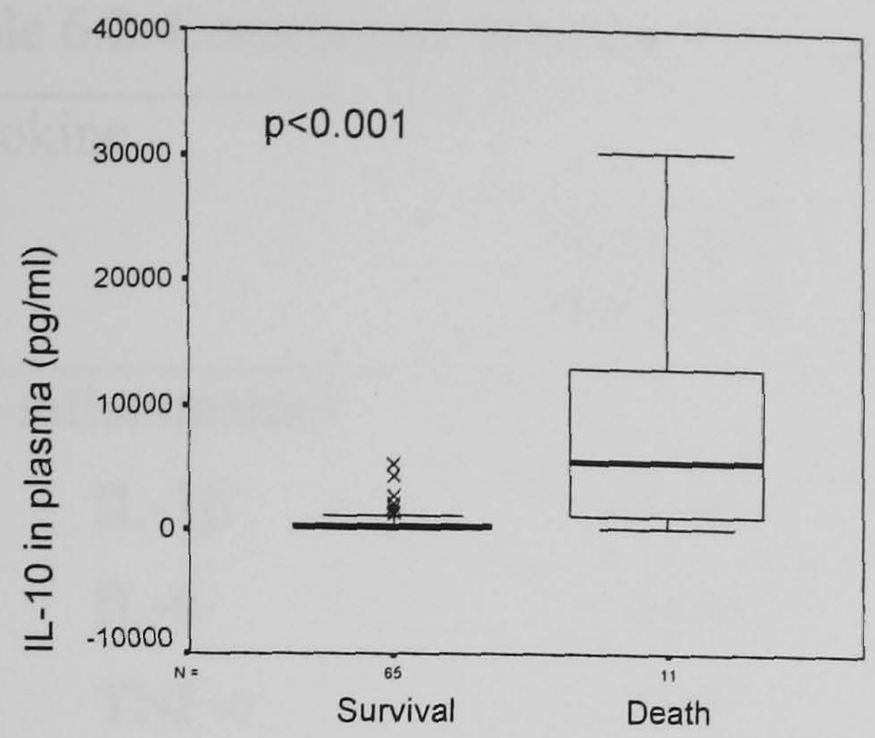
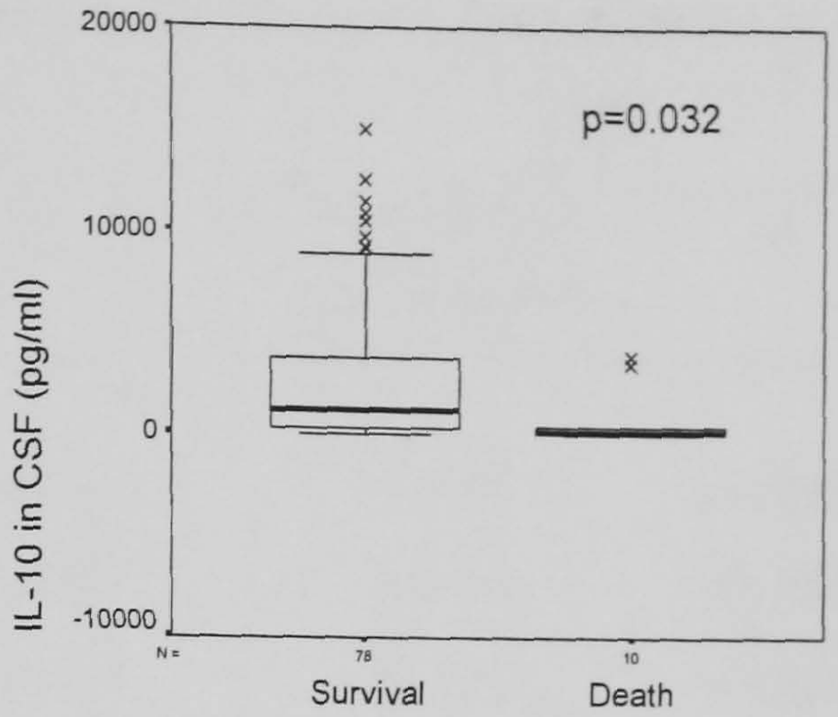
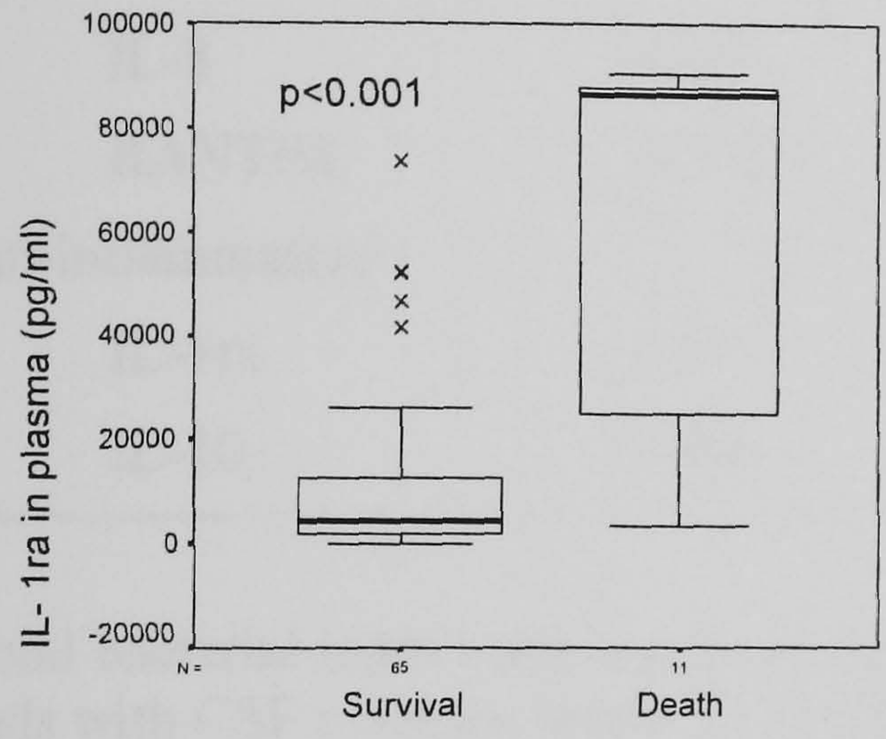


Fig. 6.1. Levels of plasma pro-inflammatory cytokines and chemokines in **Brazilian** patients by outcome. In all comparisons, $p<0.001$ (Mann-Whitney). IL-1 = IL-1β.







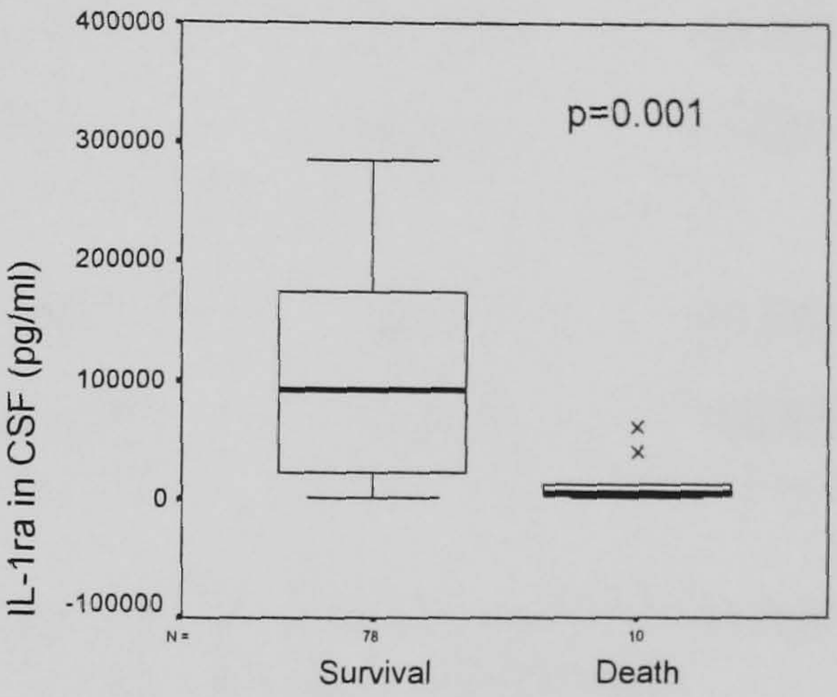


Fig. 6.2. Levels of anti-inflammatory cytokines in plasma and in CSF of **Brazilian** patients.

Table 6.2. Correlations between cytokines and bacterial load in **Brazilian** cases.

Cytokine	Blood		CSF	
	Correlation coefficient [a]	p	Correlation coefficient [a]	p
Pro-inflammatory				
IL-1β	0.408	<0.001	0.605	<0.001
IL-6	0.676	<0.001	0.474	<0.001
TNF-α	0.405	<0.001	0.346	<0.001
Chemokines				
IL-8	0.642	<0.001	0.374	<0.001
RANTES	- 0.258	0.025	0.368	0.001
Anti-inflammatory				
IL-1ra	0.515	<0.001	0.592	<0.001
IL-10	0.454	<0.001	0.473	<0.001

Blood bacterial loads were compared to plasma cytokine levels and CSF bacterial loads with CSF cytokine levels. a) Spearman's rho correlation coefficient.

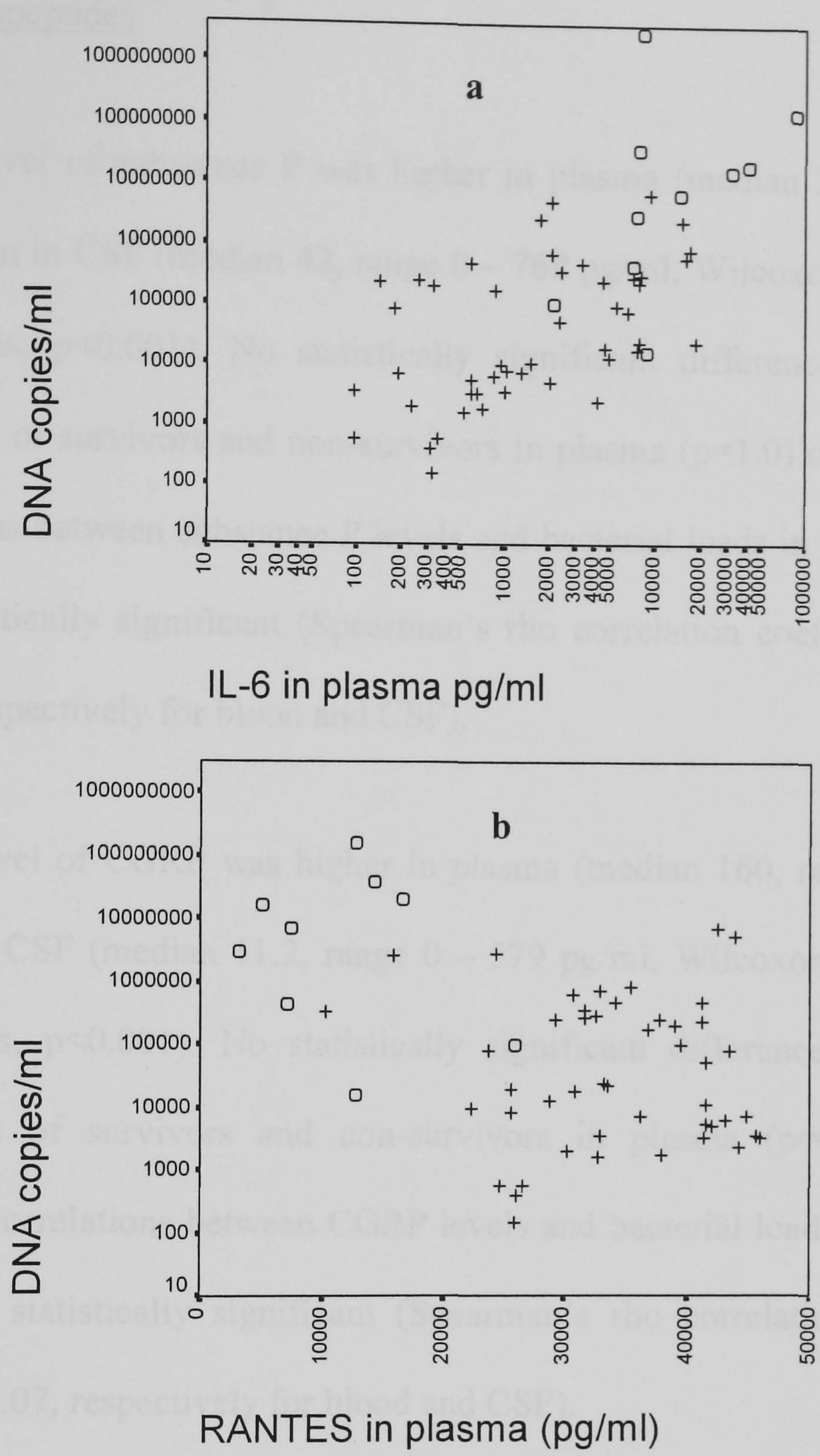


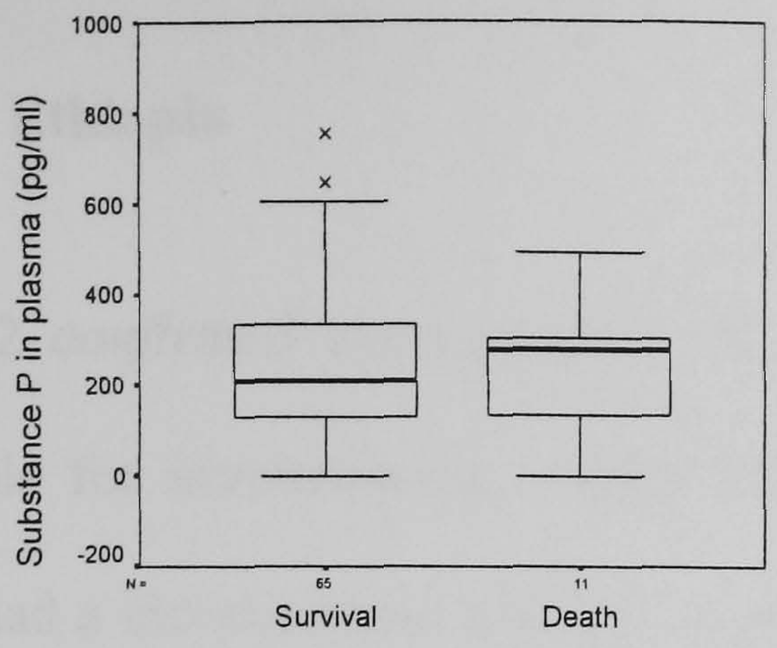
Fig. 6.3. Correlations between plasma levels of IL-6 and RANTES with blood bacterial load in **Brazilian** patients. (+) = survivors; (□) = non-survivors. a) positive correlation (Spearman's rho correlation coefficient 0.676, $p<0.001$); b) negative correlation (coefficient – 0.368, $p=0.001$).

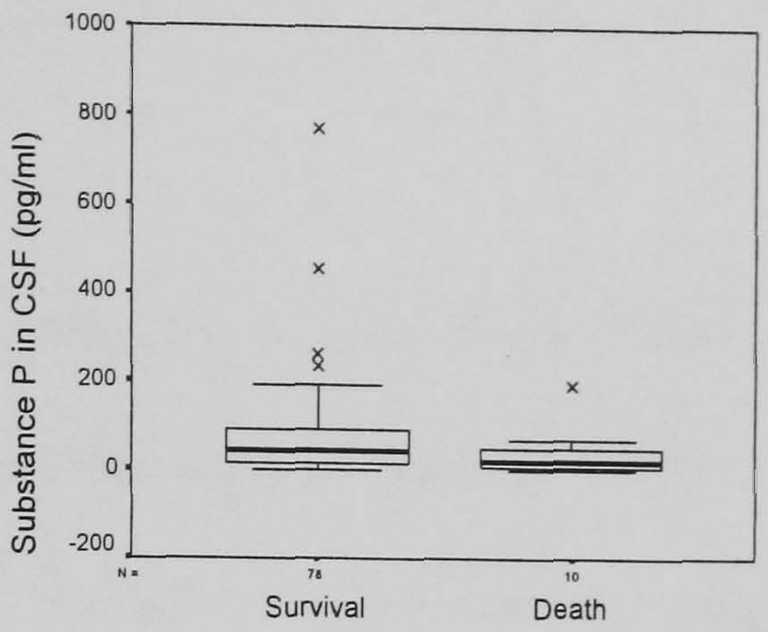
6.3.1.2 Neuropeptides

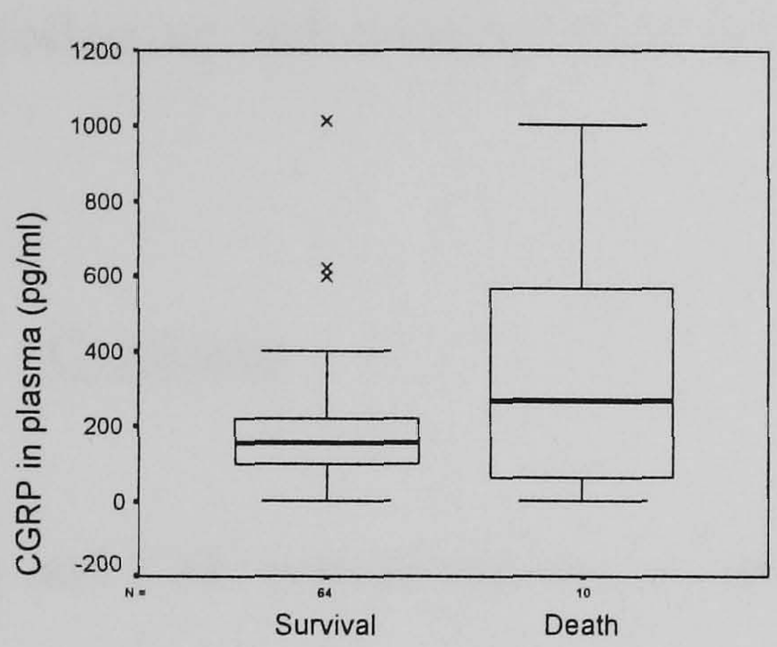
The median level of substance P was higher in plasma (median 215, range 0 to 755 pg/ml) than in CSF (median 42, range 0 – 768 pg/ml, Wilcoxon ranks test for related samples, $p<0.001$). No statistically significant differences were found between levels of survivors and non-survivors in plasma ($p=1.0$) or CSF ($p=0.2$). The correlations between substance P levels and bacterial loads in blood and CSF were not statistically significant (Spearman's rho correlation coefficient, $p=0.57$ and $p=0.94$, respectively for blood and CSF).

The median level of CGRP was higher in plasma (median 160, range 0 to 1012 pg/ml) than in CSF (median 11.2, range 0 – 579 pg/ml, Wilcoxon ranks test for related samples, $p<0.001$). No statistically significant differences were found between levels of survivors and non-survivors in plasma ($p=0.32$) or CSF ($p=0.16$). The correlations between CGRP levels and bacterial loads in blood and CSF were not statistically significant (Spearman's rho correlation coefficient, $p=0.14$ and $p=0.07$, respectively for blood and CSF).

Levels of substance P and CGRP in plasma and CSF of Brazilian patients are shown in figure 6.4.







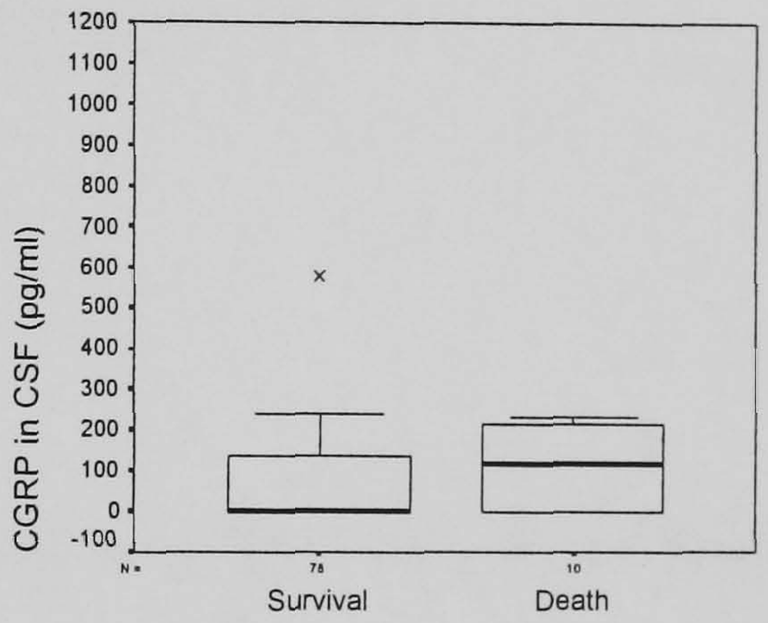


Fig. 6.4. Levels of neuropeptides in plasma and CSF of **Brazilian** patients. None of the comparisons by outcome was statistically significant at the 5% level.

6.3.2 Ethiopia

All 132 *confirmed* cases enrolled in Ethiopia had at least one clinical sample available for immunoassay testing. One hundred and seventeen (89%) of these cases had a blood sample and 123 cases (93%) had a CSF sample. Six cases were lost to follow up and excluded from the analysis by outcome.

6.3.2.1 Cytokines

Plasma and CSF concentrations for the various cytokines are shown in table 6.3. There were no statistically significant differences in plasma concentrations of IL-1β, IL-6, TNF-α, IL-8, IL-1ra and IL-10 between survivors and non-survivors. Most cases had undetectable levels of IL-10 in plasma (median concentration 0 pg/ml). Levels of RANTES were high (medians above 24,000 pg/ml) both in survivors and in non-survivors, but the comparison by outcome did not yield a statistically significant difference (p=0.313).

In addition, there were no statistically significant differences in CSF concentrations of IL-1β, IL-6, TNF-α, IL-1ra, IL-10 and RANTES between survivors and non-survivors. The only exception to this was IL-8, which was found to be approximately 2-fold higher in the CSF of those who died compared to those who survived (p=0.044).

The anti: pro inflammatory ratios of IL-10: TNF-α, IL-10: IL-1β and IL-1ra: IL-1β are presented in the bottom of table 6.3. No statistically significant differences

were observed when the ratios were compared between survivors and non-survivors.

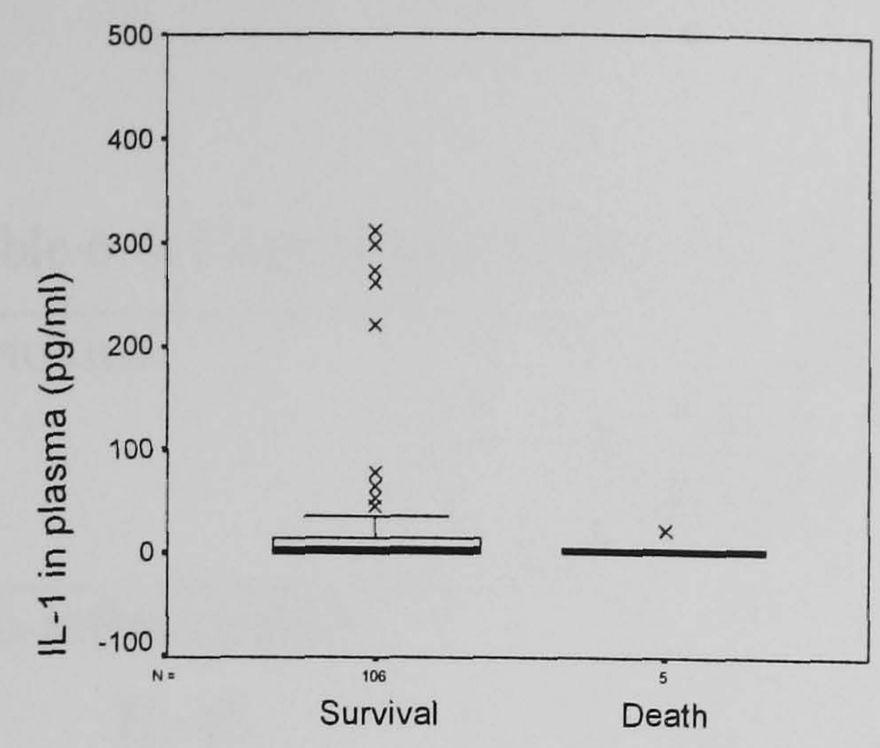
Figure 6.5 shows box-and-whisker plots for pro-inflammatory cytokines and chemokines by outcome. Correlations between concentrations of cytokines and levels of bacterial loads in each of the body compartments (peripheral blood and CSF) are shown in table 6.4. Plasma levels of IL-1β, IL-6, IL-8, RANTES and IL-1ra were found to be positively correlated with blood bacterial load at the 5% significance level, whereas TNF-α and IL-10 had weaker, non-statistically significant correlations (Spearman's rho coefficient of correlation 0.170 and 0.177 respectively for TNF-α and IL-10). In the CSF compartment, only levels of IL-1β, IL-8, IL-1ra and IL-10 were found to be statistically significantly correlated with CSF bacterial load (all correlations were positive, please see table 6.4 for respective rho coefficients and values of p).

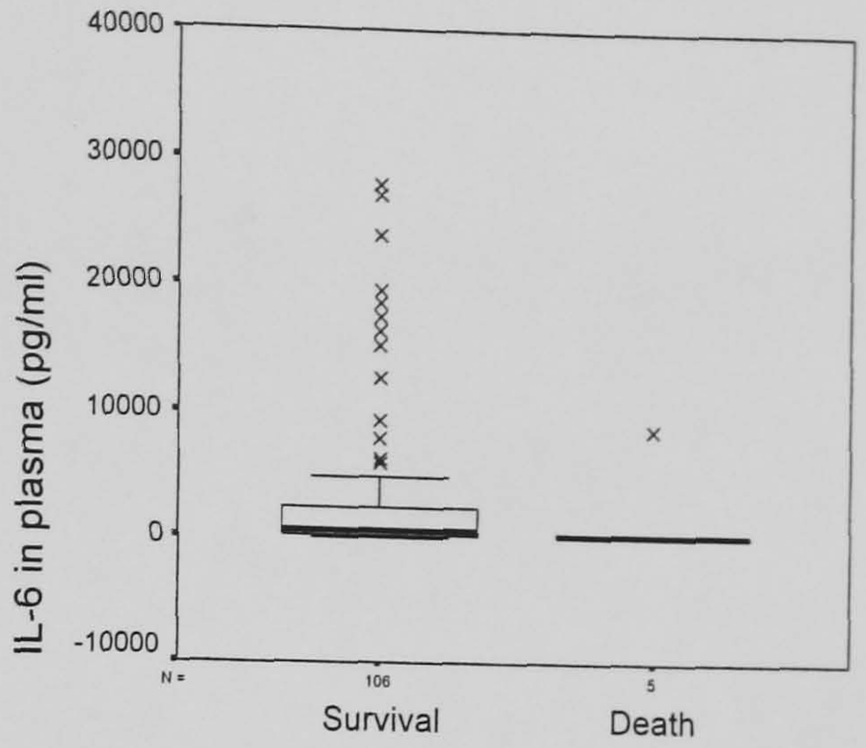
When Ethiopian cases were categorised according to levels of bacterial load in blood (table 6.5), the levels of IL-1β, IL-6, IL-8 and IL-1ra were all found to be higher in those cases categorised as 'high blood bacterial load' when compared to the remaining 'not-high bacterial load' cases (Mann Whitney, $p<0.001$ in each case).

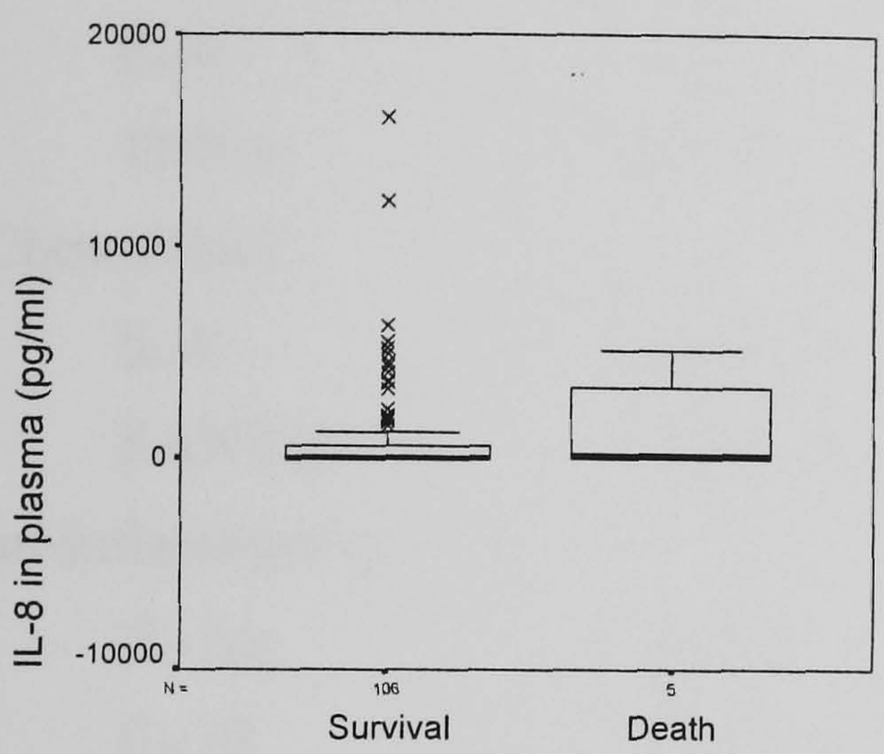
Table 6.3 Levels of pro–inflammatory cytokines and chemokines, anti-inflammatory cytokines and anti: pro inflammatory cytokine ratios in **Ethiopian** patients by **outcome**.

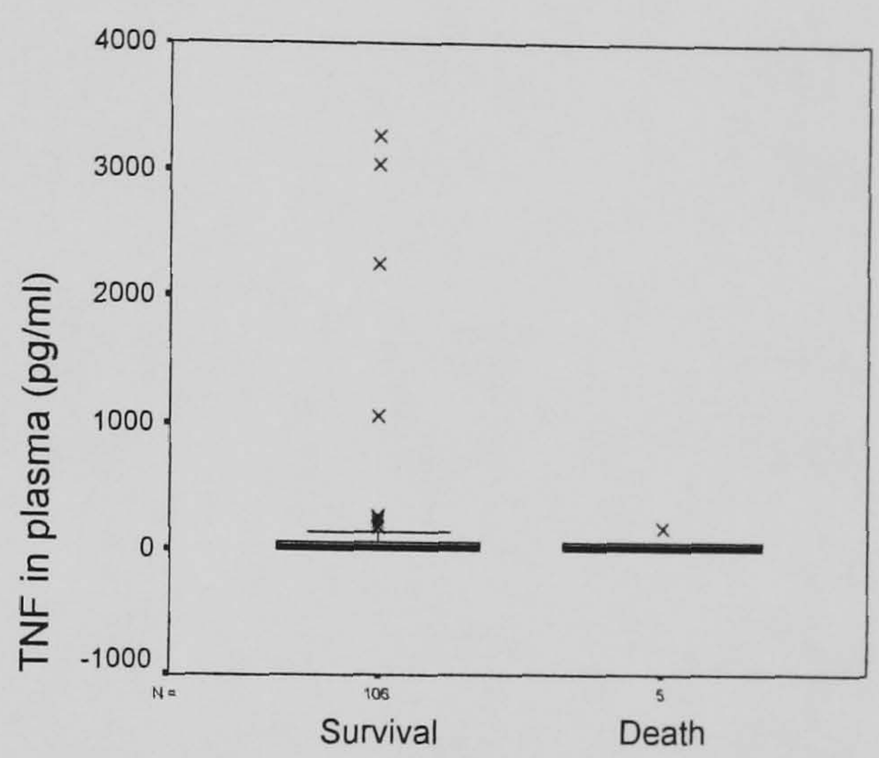
Cytokine		Median concentration in pg/ml (IQR)					
		Blood			CSF		
		Dead (n=5)	Alive (n=106)	p*	Dead (n=7)	Alive (n=110)	p*
Pro-inflammatory	IL-1β	4 (14)	4 (16)	0.613	2874 (6204)	1321 (3464)	0.138
	IL-6	118 (4332)	420 (2320)	0.203	18518 (20940)	14491 (27928)	0.569
	TNF-α	15 (110)	20 (61)	0.873	1213 (1365)	130 (665)	0.094
Chemokines	IL-8	127 (4195)	82 (540)	0.386	29972 (9998)	16535 (20326)	0.044 †
	RANTES	30154 (25837)	24956 (30152)	0.313	47 (642)	45 (109)	0.312
Anti-inflammatory	IL-1ra	189 (47374)	638 (4514)	0.737	167936 (122903)	90860 (127031)	0.103
	IL-10	0 (678)	0 (143)	0.802	2114 (3411)	836 (4441)	0.254
Ratios (95% CI)	IL-10: TNF-α	3.3 (0-51.0)	0.04 (0-0.903)	-	2.2 (1.2-60.1)	5.7 (2.9-8.9)	-
	IL-10: IL-1β	0.0 (0-26.9)	0.0 (0-6.0)	-	1.5 (0-9.3)	0.7 (0.3-1.5)	-
	IL-1ra: IL-1β	35.3 (0-4084.3)	140.2 (81.8-271.7)	-	62.5 (11.3-142.4)	46.5 (32.3-84.9)	-

IQR= Interquartile range. CI = Confidence interval. * Mann Whitney U test used to compare medians between survivors and non-survivors.
†Significant at 5% level.









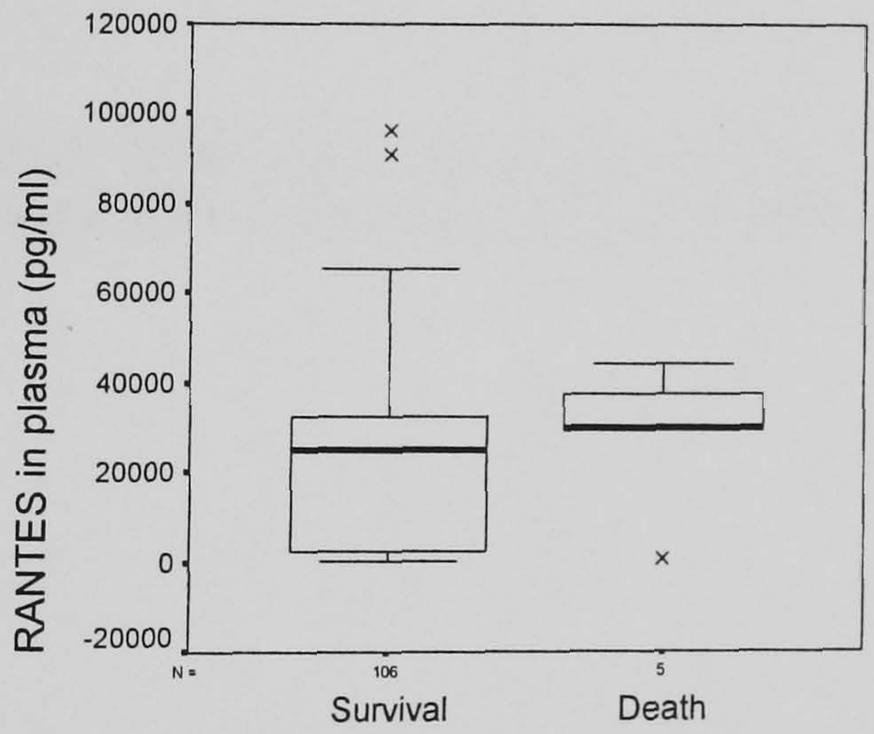


Fig. 6.5. Levels of plasma pro-inflammatory cytokines and chemokines in **Ethiopian** patients by outcome. In all comparisons, $p>0.05$ (Mann-Whitney). IL-1 = IL-1β.

Table 6.4. Correlations between cytokines and bacterial load in **Ethiopian** cases.

Cytokine	Blood		CSF	
	Correlation coefficient [a]	p	Correlation coefficient [a]	p
Pro-inflammatory				
IL-1β	0.518	<0.001	0.394	<0.001
IL-6	0.411	<0.001	0.158	0.089
TNF-α	0.170	0.077	0.161	0.082
Chemokines				
IL-8	0.605	<0.001	0.298	0.001
RANTES	0.241	0.012	0.016	0.864
Anti-inflammatory				
IL-1ra	0.581	<0.001	0.430	<0.001
IL-10	0.177	0.065	0.211	0.025

Blood bacterial loads were compared to plasma cytokine levels and CSF bacterial loads with CSF cytokine levels. a) Spearman's rho correlation coefficient.

Table 6.5. Levels of cytokines in blood of **Ethiopian** patients by degree of bacterial load.

Cytokine	Median pg/ml (IQR)		Statistic †
	High blood bacterial load [a] (n=29)	Not-high blood bacterial load (n=86)	p
Pro-inflammatory			
IL-1β	22 (40)	1.4 (6.5)	<0.001
IL-6	2669 (17075)	341 (1247)	<0.001
TNF-α	36 (78)	18 (59)	0.075
Chemokines			
IL-8	1967 (3760)	18 (135)	<0.001
RANTES	28105 (20903)	22548 (31258)	0.250
Anti-inflammatory			
IL-1ra	10444 (47942)	86 (2415)	<0.001
IL-10	0 (703)	0 (115)	0.332

IQR = Interquartile range. (a) Cases with bacterial load above the 75th percentile (i.e. levels comparable with Brazilian septicaemic cases). (b) Cases with bacterial load equal to or below the 75th percentile. †Mann Whitney U test.

6.3.2.2 Neuropeptides

The median level of substance P was higher in plasma (median 231, range 0 to 1731 pg/ml) than in CSF (median 30, range 0 –3192 pg/ml, Wilcoxon ranks test for related samples, $p<0.001$). No statistically significant differences were found between levels for survivors and non-survivors in plasma ($p=0.20$) or CSF ($p=0.34$). The correlations between substance P levels and bacterial loads in blood and CSF were not statistically significant (Spearman's rho correlation coefficient, $p=0.83$ and $p=0.85$, respectively for blood and CSF).

Levels of substance P in plasma and CSF of Ethiopia patients are shown in figure 6.6. Levels of CGRP were not measured in Ethiopian patients.

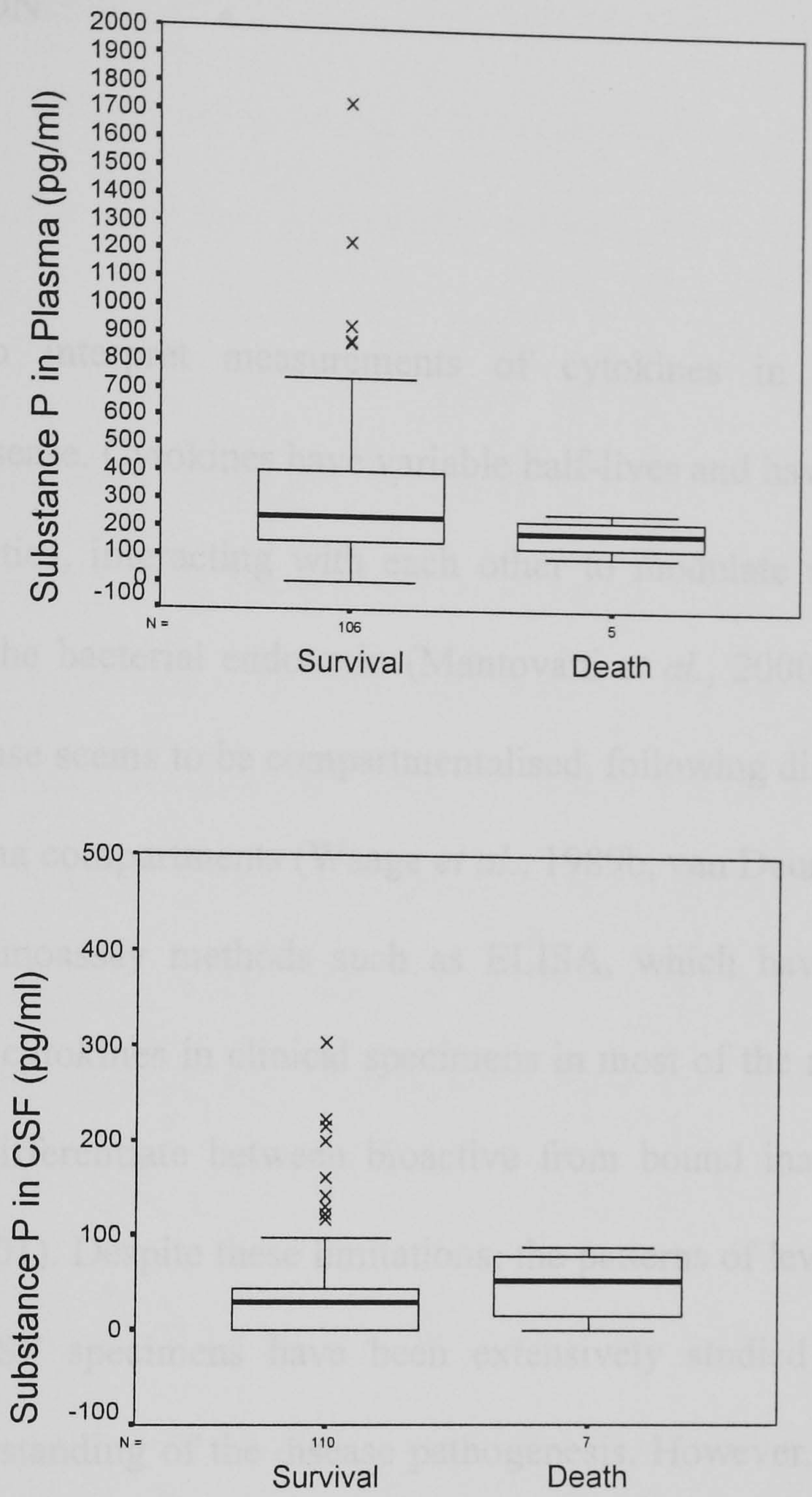


Fig. 6.6. Levels of substance P in plasma and CSF of **Ethiopian** patients. None of the comparisons by outcome was statistically significant at the 5% level.

6.4 DISCUSSION

6.4.1 Cytokines

It is difficult to interpret measurements of cytokines in the context of meningococcal disease. Cytokines have variable half-lives and have redundant and pleiotropic properties, interacting with each other to modulate and produce the host response to the bacterial endotoxin (Mantovani *et al.*, 2000, Hackett *et al.*, 2001). This response seems to be compartmentalised, following distinct patterns in the CSF and plasma compartments (Waage *et al.*, 1989b, van Deuren *et al.*, 1997). In addition, immunoassay methods such as ELISA, which have been used to measure levels of cytokines in clinical specimens in most of the recent studies in humans, cannot differentiate between bioactive from bound inactive molecules (Hackett *et al.*, 2001). Despite these limitations, the patterns of levels of cytokines in plasma and CSF specimens have been extensively studied and helped to increase our understanding of the disease pathogenesis. However, scarce data are available from patients from settings other than Western developed countries.

This study illustrates that patients from Brazil and Ethiopia have a wide range of pro and anti-inflammatory cytokine levels in plasma and CSF and appear to have distinct cytokine profiles at the time of admission to hospital.

6.4.1.1 Pro-inflammatory cytokines and chemokines

In agreement with the literature, high plasma levels of TNF-α, IL-1β, IL-6 and IL-8 were associated with fatal outcome in the Brazilian study site, where only

patients with septicaemia (with or without meningitis) died (Waage *et al.*, 1989a, van Deuren *et al.*, 1995, Hackett *et al.*, 2001). As expected, but not previously shown, each of these cytokines correlated positively with the levels of blood bacterial load.

In Ethiopian cases, plasma levels of IL-1β, IL-6, IL-8 and TNF-α were shown to be positively correlated with bacterial loads in blood, though with TNF-α the correlation did not quite reach statistical significance. This suggests that, despite not having signs of septicaemia, high bacterial loads do elicit a higher systemic pro-inflammatory response. Conversely, none of these cytokines was found to be associated with a fatal outcome and this reinforces the assumption that the mechanisms causing death in cases of meningitis differ from those of septicaemia (Nugent *et al.*, 1979).

Exceptionally in Brazil, RANTES, a secondary pro-inflammatory cytokine (chemokine), was found to be negatively correlated with blood bacterial load and to be present in significantly lower levels in those who died. This finding confirms what had been previously described in children with meningococcal septicaemia from the UK (Carrol *et al.*, 2000a). However, in Ethiopian patients, RANTES levels in plasma correlated positively with bacterial load in blood and did not show a statistically significant difference according to outcome. This apparently divergent profile of RANTES in cases of septicaemia versus meningitis needs to be further studied and reinforces the proposed role of RANTES in the pathogenesis of MD.

6.4.1.2 Anti-inflammatory cytokines

In Brazil, both plasma IL-10 and IL-1ra were found to be higher in non-survivors, in accordance with previous reports (Riordan *et al.*, 1996, Lehmann *et al.*, 1995). In Ethiopia, conversely, most cases had undetectable plasma levels of IL-10 on admission, regardless of outcome.

The CSF levels of both IL-10 and IL-1ra, however, were found to be significantly lower in those who died in Brazil, but not in Ethiopia.

IL-1ra has been shown to modulate the bioactivity of IL-1, particularly in the earlier stages of meningococcal infections, but this regulation follow different patterns in the plasma and in the CSF (van Deuren *et al.*, 1997). The role of IL-10 in the modulation of inflammation is far more complex. Even though its main functions are to inhibit inflammation, IL-10 also has pro-inflammatory properties (Mocellin *et al.*, 2003). Moreover, some studies have shown that a predominant IL-10 mediated anti-inflammatory response is associated with fatal outcome in MD (Riordan *et al.*, 1996, Westendorp *et al.*, 1997).

It has been proposed that an innate deficiency in cytokine release during acute severe infections leads to a rapid multiplication of the invading micro organism, leading to a secondary overwhelming host inflammatory reaction, which could ultimately cause death (Netea *et al.*, 2003).

Despite the fact that the number of enrolled cases in both study sites exceeded the minimum sample size required (see section 2.4), a relatively low number of cases died in the Ethiopian cohort. This meant that the ability to detect significant

differences might have been reduced for that arm of the study, with an increased risk of type II error (i.e. failing to reject a false null hypothesis) (Kirkwood & Sterne, 2003). However, patterns of cytokine profiles do appear to differ between Brazilian and Ethiopian patients.

An increasing number of host genetic determinants have been associated with susceptibility to and severity of *N. meningitidis* infections, but knowledge about the prevalence of these genetic markers in populations from developing countries in scarce (Emonts *et al.*, 2003). Further studies should address this problem.

6.4.2 Neuropeptides

Confirmed cases of MD had significantly higher levels of substance P and CGRP in plasma compared to CSF.

There were no statistically significance differences between levels of substance P in survivors and non-survivors, and no correlations with bacterial loads. Despite the fact that the release of substance P has been shown to be triggered by bacterial LPS, previous studies in humans with sepsis failed to show correlation with disease severity or outcome when substance P was measured at the onset of the disease (Beer *et al.*, 2002, Arnalich *et al.*, 1996). However, in one of these studies, higher levels of substance P were seen at later stages of the hospital stay (Beer *et al.*, 2002).

In our study, there were no statistically significance differences between levels of CGRP in survivors and non-survivors, and no correlations with bacterial loads.

Arnalich et al (1996) prospectively enrolled 31 patients with bacterial sepsis and found no correlation between CGRP and levels of endotoxin, but found higher levels of CGRP in those with septic shock when compared with those cases without shock. However, none of the cases in the study had MD.

Our findings suggest that confirmed cases of MD may have high levels of substance P and CGRP in plasma, but these do not appear to correlate with outcome when measured at the time of admission to hospital. Further studies should address the role of these neuropeptides in the pathogenesis of MD, as well as their value as late markers of death and sequelae.

Chapter Seven

7 GENERAL DISCUSSION

7.1 INTRODUCTION

In this general discussion, the contribution of this work to the body of knowledge on meningococcal disease in developing countries is reviewed, according to the aims set in chapter one. Summaries of the main findings and conclusions are presented, study limitations are acknowledged and questions for further research are proposed.

7.2 CONTRASTING FEATURES OF MENINGOCOCCAL DISEASE IN BRAZIL AND ETHIOPIA

In our study, clinical and laboratory features of meningococcal disease were described in two disparate developing countries. While the Brazilian study site illustrated an urban setting from a middle-income Latin American country, where meningococcal disease is endemic and most cases have group B *N. meningitidis* (Sacchi *et al.*, 2001), the Ethiopian study site exemplified the situation in the highly endemic, epidemic-prone Sub-Saharan African meningitis belt, where most cases present with group A *N. meningitidis* (Greenwood, 1999).

In addition, the background health indicators show that life expectancy in Ethiopia is 20 years shorter than in Brazil and Ethiopian children are 4 and a half times

more likely to die before the age of 5 years. The per capita annual health expenditure in Ethiopia is 40 times lower than in Brazil (WHO, 2003) and this situation is aggravated by higher prevalence of malaria, malnutrition and HIV infection in the African country. Interpretation and potential applicability of the findings of this study should consider these variables.

7.2.1 Clinical features

This study confirmed that *N. meningitidis* remains an important cause of invasive disease both in Brazil and Ethiopia. The degree of variation in meningococcal phenotype reflected the endemic nature of MD in Brazil and the epidemic – or hyper endemic – circumstances of Ethiopia, as epidemics tend to be due to single clonal strains, as observed in Ethiopia (Crowe *et al.*, 1989, Norheim *et al.*, 2004).

Interestingly, the age distribution was similar between the two sites, with a median age at around 10 years. While this finding was expected for Ethiopia, the age distribution in Brazil contradicted studies from the UK that have shown that endemic disease due to serogroup B *N. meningitidis* affects young children in a proportion greater than 50% (Thomson *et al.*, 1990, Riordan *et al.*, 1995a).

The expected differences in clinical presentation between Brazil and Ethiopia were confirmed. While two thirds of Brazilian cases presented with septicaemia (with or without meningitis), all Ethiopian cases presented with meningitis alone and these findings are in accordance with observations in similar settings (Salih *et al.*, 1990, Donalisio *et al.*, 2000).

Case-fatality rates seen in Brazil were high (14% overall), but comparable to what has been reported in other parts of Latin America (Donalisio *et al.*, 2000) and elsewhere serogroup B is predominant (Riordan *et al.*, 1995a). The case-fatality rate seen in the Ethiopian cohort (5.6%) was relatively low by sub-Saharan African standards (Hart & Cuevas, 1997, Salih *et al.*, 1990). This might be due to the phase in the epidemic in which the study was conducted, as it has been suggested that case-fatality rates decrease with time during the course of an epidemic (Greenwood *et al.*, 1979b). This relatively low mortality in Ethiopia affected the number of cases in the category of non-survivors and became a limitation of our study.

Probably due to differences in care-seeking behaviour and access to health services, on average, Ethiopian cases were seen in hospital one and a half days later than Brazilian cases of meningitis. Ethiopian cases presented more often with impaired consciousness, which was identified as a marker of poor prognosis in both sites, in conformity with the literature (Kahn & Blum, 1978, Sinclair *et al.*, 1987, Castellanos-Ortega *et al.*, 2002). Conversely, the presence of decreased peripheral perfusion on admission was associated with a poor outcome in Brazil, where most cases had septicaemia, but not in Ethiopia, where cases had meningitis alone. This reinforces the idea that, contrary to the sustained shock seen in fulminant meningococcaemia, early manifestations of compensated shock (i.e. signs of decreased perfusion without hypotension) may indicate the systemic response to infection, and might be easily reversible with the initiation of antibiotic and fluid therapy (Stiehm & Damrosch, 1966).

The results of this clinical study served as the basis for categorisation of cases in the remaining chapters of this thesis.

7.2.2 Polymerase chain reaction as a diagnostic tool in meningococcal disease

This was the first study to assess the performance of meningococcal PCR with a *ctrA* gene target in the developing world and the first time the *ctrA* PCR test was assessed in relation to disease presentation.

While acknowledging the difficulty of assessing the performance of diagnostic tests in the context of meningococcal disease, this study provides figures for sensitivity, specificity, PPV and NPV for two complementary approaches: by comparing *ctrA* PCR with culture alone and by using a combination of clinical assessment and confirmatory laboratory results as a 'gold standard'.

The relatively low specificities found by comparing with culture alone in our study (67% in Brazil and 29% in Ethiopia) are likely to reflect a high proportion of true cases missed by culture. This was reinforced by the fact that both clinical characteristics and complementary laboratory evidence such as microscopy (Gram staining) would have categorised between 86 and 96% of these 'false positives' as 'probable' cases. On the other hand, the discrepancy between Brazilian and Ethiopian figures might reflect the fact the standard microbiological diagnostic facilities were superior in the Brazilian study site.

Therefore, it seemed logical to assess the performance of the *ctrA* PCR with a defined 'gold standard' that also included clinical criteria as well as laboratory

data. The weakness of this approach was that, because 'possible' cases were included as likely meningococcal disease (thus in the gold standard group), the sensitivity and NPV figures were underestimated. Nevertheless, by using a combination of clinical assessment and confirmatory laboratory results as a 'gold standard', the *ctrA* PCR was shown to be highly sensitive and specific, both in Brazil and in Ethiopia, where cases were due to serogroup A *N. meningitidis* and there had been concerns about the performance of the test in such circumstances (Guiver *et al.*, 2000). When assessed by clinical presentation (i.e. meningitis versus septicaemia), which are usual diagnostic scenarios in clinical practice and affect pre-test probabilities, the *ctrA* PCR test performed well, contributing to an overall increase between 31 to 50% in the number of confirmed cases. The overall figures for meningococcal PCR sensitivity observed in Brazil (88%, 95%CI 80-95) and in Ethiopia (89%, 95%CI 83-95) were comparable to those previously described in the UK (Carrol *et al.*, 2000b, Hackett *et al.*, 2002a) and Australia (Bryant *et al.*, 2004). In accordance with previous reports (Hackett *et al.*, 2002a), the whole blood PCR tended to perform better than plasma. In cases presenting as bacterial meningitis alone, meningococcal PCR in CSF samples consistently outperformed blood PCR in terms of sensitivity. This is a reminder that in cases of meningitis a lumbar puncture is an essential diagnostic procedure (Kneen *et al.*, 2002).

The findings of our study reinforce the recommendation of inclusion of the *ctrA* PCR in the gold standard definition to which other future tests might be compared.

It is unrealistic to propose that expensive PCR techniques could have any immediate applicability in the management of cases in Sub-Saharan Africa.

However, PCR-based centralised surveillance systems have a potential role in monitoring epidemics (Sidikou *et al.*, 2003). Conversely, the uses of PCR as a diagnostic tool is on the increase in Brazil and operational costs have decreased considerably.

7.2.3 Bacterial load, disease presentation and outcome

This was the first study to describe levels of meningococcal bacterial loads in patients from developing countries and is the largest study to date to describe bacterial loads in the CSF compartment. The study has shown that levels of bacterial loads were associated with fatal outcome, but the significance of these bacterial loads as markers of prognosis varied according to the clinical presentation.

Blood bacterial loads in patients with septicaemia with or without meningitis were associated with outcome, age and duration of symptoms. In accordance with previous reports, bacterial loads were higher in the blood samples of patients who died compared to the loads of patients who survived (Hackett *et al.*, 2002b, Ovstebo *et al.*, 2004). This finding reinforces the assumption that bacterial loads are proportional to the amount of bacterial lipooligossacharides and support the potential of blood quantitative PCR as a prognostic marker in cases of septicaemia.

The association between blood bacterial load and age and duration of symptoms in septicaemic patients had not been previously described. In our study, children with less than 5 years of age had higher blood bacterial loads than patients above this

age. Younger children may be less able to prevent the multiplication of *N. meningitidis* in the bloodstream, thus yielding higher bacterial loads. Patients presenting within 24 hours since the appearance of the first symptoms had higher blood bacterial loads than those presenting later and this may denote differences in disease severity (Ansari *et al.*, 1979).

In patients who had meningitis alone, blood and CSF bacterial loads were not associated with outcome. This might be explained by the fact that while endotoxin-induced shock is the major cause of death in septicaemic cases, the inflammatory response is compartmentalised to the brain in the case of meningitis and most deaths are due to cerebral oedema with raised intracranial pressure and its consequences (Nugent *et al.*, 1979).

The study has identified that a number of Ethiopian patients had high bacterial loads in blood (levels comparable to Brazilian septicaemic cases) despite not having clinical signs of septicaemia.

7.2.4 Cytokine profile and neuropeptides in meningococcal disease

7.2.4.1 Cytokines

To our knowledge, this was the largest study on cytokine profiles in cases of meningococcal disease from developing countries and the first to describe levels of IL-1β, IL-1ra, IL-6, IL-8, IL-10, TNF-α and RANTES in paired blood and CSF samples from the same cohorts of patients. This was also the first description of

correlations between DNA bacterial loads in blood and CSF and the cytokine profile in the respective compartment.

This study has shown evidence of variable cytokine profiles in blood and CSF and this seems to reinforce the assumption that the inflammatory response in MD is compartmentalised (Waage *et al.*, 1989b, van Deuren *et al.*, 1997). This study also illustrates that patients from Brazil and Ethiopia do appear to have divergent cytokine profiles at the time of admission to hospital.

As expected, but not previously shown, plasma levels of TNF-α, IL-1β, IL-6 and IL-8 in Brazilian patients correlated positively with the levels of blood bacterial load and, in agreement with the literature, with fatal outcome (Waage *et al.*, 1989a, van Deuren *et al.*, 1995, Hackett *et al.*, 2001). In Ethiopian cases, pro-inflammatory cytokines were also positively correlated with bacterial load, suggesting that despite not having signs of septicaemia, high bacterial loads do elicit a higher systemic pro-inflammatory response. However, none of these cytokines was found to be associated with a fatal outcome, reinforcing the assumption that the mechanisms causing death in cases of meningitis differ from those of septicaemia (Nugent *et al.*, 1979).

This study has shown that, in Brazilian cases, RANTES was negatively correlated with blood bacterial load and lower levels of RANTES were found in the plasma of those who died. However, in Ethiopian patients, RANTES levels in plasma correlated positively with bacterial load in blood and did not show a statistically significant difference according to outcome. This apparently divergent profile of RANTES in cases of septicaemia versus meningitis needs to be further studied and

reinforces the proposed role of RANTES in the pathogenesis of MD (Carrol *et al.*, 2000a).

In Brazil, both plasma IL-10 and IL-1ra were found to be higher in non-survivors, in accordance with previous reports (Riordan *et al.*, 1996, Lehmann *et al.*, 1995). In Ethiopia, conversely, most cases had undetectable plasma levels of IL-10 on admission, regardless of outcome. The CSF levels of both IL-10 and IL-1ra, however, were found to be significantly lower in those who died in Brazil, but not in Ethiopia.

Patterns of cytokine profiles do appear to differ between Brazilian and Ethiopian patients.

7.2.4.2 Neuropeptides

To our knowledge, this was the first report of levels of neuropeptides in blood and CSF of cases of meningococcal disease.

Confirmed cases of MD had significantly higher levels of substance P and CGRP in plasma compared to CSF at the time of admission to hospital, but here were no statistically significance differences between levels in survivors and non-survivors, and no correlations with bacterial loads. The same pattern was observed in Brazil and Ethiopia, though CGRP was only available in cases from Brazil.

7.3 LIMITATIONS OF THE STUDY

The inclusion criteria described in section 2.3.1 have resulted in the collection of cases with meningitis and/or rash only, thus patients with meningococcal bacteraemia without a rash or meningeal signs were not included. This was therefore a study of meningitis and septicaemia with rash caused by *N. meningitidis*.

Despite the fact that the number of enrolled cases in both study sites exceeded the minimum sample size required (see section 2.4), a relatively low number of cases died in the Ethiopian cohort and this might have decreased our ability to detect significant differences in levels of cytokines and neuropeptides for that arm of the study.

We were not able to follow our patients for longer than the time they were in hospital. Even so, because of the high demand for hospital beds, they often were discharged early. The adverse conditions in the field limited the amount of clinical information made available to the research team. It was therefore not possible to record data on subtler outcomes, such as neurological sequelae (including hearing impairment), or detailed treatment variables, which could have been confounders in the analysis of data. However, each of the sites followed a standardised treatment guideline and this could have reduced the potential for biases.

We were not able to control for malnutrition, micronutrient deficiencies and HIV co-morbidity, which could have played a role in the cytokine response. However, unlike other bacterial diseases such as pneumococcal infections, HIV and malnutrition seem to interfere less with the course of MD.

7.4 RECOMMENDATIONS FOR FUTURE RESEARCH

Studies addressing the care-seeking behaviour in cases of suspected meningitis. especially in rural areas that are prone to epidemics, are desirable. A better understanding of the care-seeking behaviour might help to design locally relevant educational interventions that could contribute to reducing the interval between the first symptom and the admission to hospital, perhaps with an impact on mortality.

Affordable alternatives to the automated *ctrA* PCR need to be developed and cost effectiveness studies to assess the feasibility of their introduction in reference laboratories of areas with fewer resources should be encouraged.

Further studies on cytokine profile and bacterial loads in developing countries should include longer-term follow up, a more detailed description of disease severity and data on subtler outcomes such as hearing impairment. Ethical and logistic constraints allowing, should also control for malnutrition, micronutrient deficiencies and HIV co-morbidity.

Further studies should look at the compartmentalised inflammation in the sub-arachnoid space, in comparative perspective with other causes of bacterial meningitis, such as pneumococcus and *H. influenzae*.

Further studies should look at host genetic determinants of susceptibility to and of severity of *N. meningitidis* infections in populations from the most affected areas.

Simpler, yet accurate clinical prognostic scores need to be validated in the context of meningitis in Africa.

Further studies should look at differences in LOS and lipid A structure between the serogroups and phenotypes causing septicaemia and serogroups and phenotypes associated with meningitis.

References

Adu-Bobie, J., Capecchi, B., Serruto, D., Rappuoli, R. and Pizza, M. (2003) Two years into reverse vaccinology. *Vaccine,* 21, 605-10.

Altman, D. G. (1991) *Practical statistics for medical research,* Chapman & Hall, London.

Ansari, B. M., Davies, D. B. and Boyce, J. M. (1979) A comparative study of adverse factors in meningococcaemia and meningococcal meningitis. *Postgrad Med J,* 55, 780-3.

Arnalich, F., Hernanz, A., Jimenez, M., Lopez, J., Tato, E., Vazquez, J. J. and Montiel, C. (1996) Relationship between circulating levels of calcitonin gene-related peptide, nitric oxide metabolites and hemodynamic changes in human septic shock. *Regul Pept,* 65, 115-21.

Barquet, N., Domingo, P., Cayla, J. A., Gonzalez, J., Rodrigo, C., Fernandez-Viladrich, P., Moraga-Llop, F. A., Marco, F., Vazquez, J., Saez-Nieto, J. A., Casal, J., Canela, J. and Foz, M. (1999) Meningococcal disease in a large urban population (Barcelona, 1987-1992): predictors of dismal prognosis. Barcelona Meningococcal Disease Surveillance Group. *Arch Intern Med,* 159, 2329-40.

Beer, S., Weighardt, H., Emmanuilidis, K., Harzenetter, M. D., Matevossian, E., Heidecke, C. D., Bartels, H., Siewert, J. R. and Holzmann, B. (2002) Systemic

neuropeptide levels as predictive indicators for lethal outcome in patients with postoperative sepsis. *Crit Care Med,* 30, 1794-8.

Beutler, B. and Cerami, A. (1986) Cachectin/tumor necrosis factor: an endogenous mediator of shock and inflammation. *Immunol Res,* 5, 281-93.

Bland, M. (2000) *An introduction to medical statistics,* Oxford University Press, Oxford.

Bone, R. C., Balk, R. A., Cerra, F. B., Dellinger, R. P., Fein, A. M., Knaus, W. A., Schein, R. M. and Sibbald, W. J. (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest,* 101, 1644-55.

Borrow, R., Claus, H., Guiver, M., Smart, L., Jones, D. M., Kaczmarski, E. B., Frosch, M. and Fox, A. J. (1997) Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA. *Epidemiol Infect,* 118, 111-7.

Brandtzaeg, P., Kierulf, P., Gaustad, P., Skulberg, A., Bruun, J. N., Halvorsen, S. and Sorensen, E. (1989) Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. *J Infect Dis,* 159, 195-204.

Brandtzaeg, P., Ovsteboo, R. and Kierulf, P. (1992) Compartmentalization of lipopolysaccharide production correlates with clinical presentation in meningococcal disease. *J Infect Dis,* 166, 650-2.

Brogan, P. A. and Raffles, A. (2000) The management of fever and petechiae: making sense of rash decisions. *Arch Dis Child,* 83, 506-7.

Bryan, J. P., de Silva, H. R., Tavares, A., Rocha, H. and Scheld, W. M. (1990) Etiology and mortality of bacterial meningitis in northeastern Brazil. *Rev Infect Dis,* 12, 128-35.

Bryant, P. A., Li, H. Y., Zaia, A., Griffith, J., Hogg, G., Curtis, N. and Carapetis, J. R. (2004) Prospective study of a real-time PCR that is highly sensitive, specific, and clinically useful for diagnosis of meningococcal disease in children. *J Clin Microbiol,* 42, 2919-25.

Carrol, E. D., Baines, P., Thomson, A. P. and Hart, C. A. (1999) Pathophysiology of meningococcal sepsis in children. *Eur J Pediatr,* 158, 766.

Carrol, E. D., Thomson, A. P. and Hart, C. A. (2001) New therapies and vaccines for meningococcal disease. *Expert Opin Investig Drugs,* 10, 1487-500.

Carrol, E. D., Thomson, A. P., Mobbs, K. J., Fraser, W. D., Sills, J. A. and Hart, C. A. (2002) Myositis in children with meningococcal disease: a role for tumour necrosis factor-alpha and interleukin-8? *J Infect,* 44, 17-21.

Carrol, E. D., Thomson, A. P., Mobbs, K. J. and Hart, C. A. (2000a) The role of RANTES in meningococcal disease. *J Infect Dis,* 182, 363-6.

Carrol, E. D., Thomson, A. P., Shears, P., Gray, S. J., Kaczmarski, E. B. and Hart, C. A. (2000b) Performance characteristics of the polymerase chain reaction assay to confirm clinical meningococcal disease. *Arch Dis Child,* 83, 271-3.

Cartwright, K. (1995) In *Meningococcal Disease*(Ed, Cartwright, K.) John Wiley & Sons, Chichester, pp. 1-19.

Castellanos-Ortega, A., Delgado-Rodriguez, M., Llorca, J., Sanchez Buron, P., Mencia Bartolome, S., Soult Rubio, A., Milano Manso, G., Dominguez Sampedro, P., Blanco Montero, R., Rodriguez Nunez, A., Zambrano Perez, E., Rey Galan, C., Lopez Negueruela, N. and Reig Saenz, R. (2002) A new prognostic scoring system for meningococcal septic shock in children. Comparison with three other scoring systems. *Intensive Care Med,* 28, 341-51.

CDC (1998) *Laboratory Methods for the Diagnosis of Meningitis Caused by Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae,* Centers for Disease Control and Prevention, Atlanta.

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J. and Kaczmarski, E. B. (2001) Simultaneous detection of Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol,* 39, 1553-8.

Crowe, B. A., Wall, R. A., Kusecek, B., Neumann, B., Olyhoek, T., Abdillahi, H., Hassan-King, M., Greenwood, B. M., Poolman, J. T. and Achtman, M. (1989) Clonal and variable properties of Neisseria meningitidis isolated from cases and carriers during and after an epidemic in The Gambia, West Africa. *J Infect Dis,* 159, 686-700.

Davison, K. L., Crowcroft, N. S., Ramsay, M. E., Begg, N. T., Kaczmarski, E. B., Stuart, J. M., White, J. M. and Orr, H. (2002) Enhanced surveillance scheme for suspected meningococcal disease in five regional health authorities in England: 1998. *Commun Dis Public Health,* 5, 205-12.

De Wals, P., Hertoghe, L., Borlee-Grimee, I., De Maeyer-Cleempoel, S., Reginster-Haneuse, G., Dachy, A., Bouckaert, A. and Lechat, M. F. (1981) Meningococcal disease in Belgium. Secondary attack rate among household, day-care nursery and pre-elementary school contacts. *J Infect,* 3, 53-61.

Decosas, J. and Koama, J. B. (2002) Chronicle of an outbreak foretold: meningococcal meningitis W135 in Burkina Faso. *Lancet Infect Dis,* 2, 763-5.

Donalisio, M. R., Kemp, B., Rocha, M. M. and Ramalheira, R. M. (2000) [Fatality rate in the epidemiology of meningococcal disease: study in the region of Campinas, SP, Brazil, 1993 to 1998]. *Rev Saude Publica,* 34, 589-95.

Downs, N. J., Hodges, G. R. and Taylor, S. A. (1987) Mixed bacterial meningitis. *Rev Infect Dis,* 9, 693-703.

Emonts, M., Hazelzet, J. A., de Groot, R. and Hermans, P. W. (2003) Host genetic determinants of Neisseria meningitidis infections. *Lancet Infect Dis,* 3, 565-77.

Frieling, J. T., van Deuren, M., Wijdenes, J., van Dalen, R., Bartelink, A. K., van der Linden, C. J. and Sauerwein, R. W. (1996) Interleukin-6 and its soluble receptor during acute meningococcal infections: effect of plasma or whole blood exchange. *Crit Care Med,* 24, 1801-5.

Gogos, C. A., Drosou, E., Bassaris, H. P. and Skoutelis, A. (2000) Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis,* 181, 176-80.

Greenwood, B. (1999) Manson Lecture. Meningococcal meningitis in Africa. *Trans R Soc Trop Med Hyg,* 93, 341-53.

Greenwood, B. M., Bradley, A. K., Cleland, P. G., Haggie, M. H., Hassan-King, M., Lewis, L. S., Macfarlane, J. T., Taqi, A., Whittle, H. C., Bradley-Moore, A. M. and Ansari, Q. (1979a) An epidemic of meningococcal infection at Zaria, Northern Nigeria. 1. General epidemiological features. *Trans R Soc Trop Med Hyg,* 73, 557-62.

Greenwood, B. M., Cleland, P. G., Haggie, M. H., Lewis, L. S., Macfarlane, J. T., Taqi, A. and Whittle, H. C. (1979b) An epidemic of meningococcal infection at Zaria, Northern Nigeria. 2. The changing clinical pattern. *Trans R Soc Trop Med Hyg,* 73, 563-6.

Guiver, M., Borrow, R., Marsh, J., Gray, S. J., Kaczmarski, E. B., Howells, D., Boseley, P. and Fox, A. J. (2000) Evaluation of the Applied Biosystems automated Taqman polymerase chain reaction system for the detection of meningococcal DNA. *FEMS Immunol Med Microbiol,* 28, 173-9.

Haberberger, R. L., Jr., Fox, E., Asselin, P., Said-Salah, Y., Martinez, S. and Abbatte, E. A. (1990) Is Djibouti too hot and too humid for meningococci? *Trans R Soc Trop Med Hyg,* 84, 588.

Hackett, S. J., Carrol, E. D., Guiver, M., Marsh, J., Sills, J. A., Thomson, A. P., Kaczmarski, E. B. and Hart, C. A. (2002a) Improved case confirmation in meningococcal disease with whole blood Taqman PCR. *Arch Dis Child,* 86, 449-52.

Hackett, S. J., Guiver, M., Marsh, J., Sills, J. A., Thomson, A. P., Kaczmarski, E. B. and Hart, C. A. (2002b) Meningococcal bacterial DNA load at presentation correlates with disease severity. *Arch Dis Child,* 86, 44-6.

Hackett, S. J., Thomson, A. P. and Hart, C. A. (2001) Cytokines, chemokines and other effector molecules involved in meningococcal disease. *J Med Microbiol,* 50, 847-59.

Halstensen, A., Ceska, M., Brandtzaeg, P., Redl, H., Naess, A. and Waage, A. (1993) Interleukin-8 in serum and cerebrospinal fluid from patients with meningococcal disease. *J Infect Dis,* 167, 471-5.

Harrison, L. H., Pass, M. A., Mendelsohn, A. B., Egri, M., Rosenstein, N. E., Bustamante, A., Razeq, J. and Roche, J. C. (2001) Invasive meningococcal disease in adolescents and young adults. *Jama,* 286, 694-9.

Harrison, S. and Geppetti, P. (2001) Substance P. *Int J Biochem Cell Biol,* 33, 555-76.

Hart, C. A. and Cuevas, L. E. (1997) Meningococcal disease in Africa. *Ann Trop Med Parasitol,* 91, 777-85.

Hart, C. A. and Rogers, T. R. (1993) Meningococcal disease. *J Med Microbiol,* 39, 3-25.

Hashim, I. A., Walsh, A., Hart, C. A. and Shenkin, A. (1995) Cerebrospinal fluid interleukin-6 and its diagnostic value in the investigation of meningitis. *Ann Clin Biochem,* 32 (Pt 3), 289-96.

Hubert, B. and Caugant, D. A. (1997) Recent changes in meningococcal disease in Europe. *Euro Surveill,* 2, 69-71.

IBGE (2004), Vol. 2004 Instituto Brasileiro de Geografia e Estatística.

Iversen, B. G. and Aavitsland, P. (1996) Meningococcal disease in Norway 1992-1995. Epidemiology and fatality. *Scand J Infect Dis,* 28, 253-9.

Jensen, E. S., Schonheyder, H. C., Lind, I., Berthelsen, L., Norgard, B. and Sorensen, H. T. (2003) Neisseria meningitidis phenotypic markers and septicaemia, disease progress and case-fatality rate of meningococcal disease: a 20-year population-based historical follow-up study in a Danish county. *J Med Microbiol,* 52, 173-9.

Kaczmarski, E. B., Ragunathan, P. L., Marsh, J., Gray, S. J. and Guiver, M. (1998) Creating a national service for the diagnosis of meningococcal disease by polymerase chain reaction. *Commun Dis Public Health,* 1, 54-6.

Kahn, A. and Blum, D. (1978) Factors for poor prognosis in fulminating meningococcemia. Conclusions from observations of 67 childhood cases. *Clin Pediatr (Phila),* 17, 680-2, 7.

Kaiser, A. B., Hennekens, C. H., Saslaw, M. S., Hayes, P. S. and Bennett, J. V. (1974) Seroepidemiology and chemoprophylaxis disease due to sulfonamide-resistant Neisseria meningitidis in a civillian population. *J Infect Dis,* 130, 217-24.

Katz, W. H. (1999) *Multivariable analysis: a practical guide for clinicians,* Cambridge University Press, Cambridge.

Kirkwood, B. R. and Sterne, J. A. C. (2003) *Essential medical statistics,* Blackwell Science, Oxford.

Klein, D. (2002) Quantification using real-time PCR technology: applications and limitations. *Trends Mol Med,* 8, 257-60.

Kneen, R., Solomon, T. and Appleton, R. (2002) The role of lumbar puncture in children with suspected central nervous system infection. *BMC Pediatr,* 2, 8.

Kornelisse, R. F., Hazelzet, J. A., Hop, W. C., Spanjaard, L., Suur, M. H., van der Voort, E. and de Groot, R. (1997) Meningococcal septic shock in children: clinical and laboratory features, outcome, and development of a prognostic score. *Clin Infect Dis,* 25, 640-6.

Kristiansen, B. E., Ask, E., Jenkins, A., Fermer, C., Radstrom, P. and Skold, O. (1991) Rapid diagnosis of meningococcal meningitis by polymerase chain reaction. *Lancet,* 337, 1568-9.

Kuipers, B., Dobbelsteen, G. v. d., Wedege, E. and Alphen, L. v. (2001) In *Meningococcal Disease: Methods and Protocols*, Vol. 67 (Eds, Pollard, A. J. and Maiden, M. C. J.) Humana Press Inc., Totowa, pp. 131-45.

Lapeyssonnie, L. (1963) La méningite cérébro-spinale en Áfrique [summary in English]. *Bull World Health Organ,* 28 (supplement), 98-100.

Lehmann, A. K., Halstensen, A., Sornes, S., Rokke, O. and Waage, A. (1995) High levels of interleukin 10 in serum are associated with fatality in meningococcal disease. *Infect Immun,* 63, 2109-12.

Mandl, K. D., Stack, A. M. and Fleisher, G. R. (1997) Incidence of bacteremia in infants and children with fever and petechiae. *J Pediatr,* 131, 398-404.

Mantovani, A., Dinarello, C. A. and Ghezzi, P. (2000) *Pharmacology of cytokines,* Oxford University Press, Oxford.

Mocellin, S., Panelli, M. C., Wang, E., Nagorsen, D. and Marincola, F. M. (2003) The dual role of IL-10. *Trends Immunol,* 24, 36-43.

Molesworth, A. M., Cuevas, L. E., Connor, S. J., Morse, A. P. and Thomson, M. C. (2003) Environmental risk and meningitis epidemics in Africa. *Emerg Infect Dis,* 9, 1287-93.

Molesworth, A. M., Thomson, M. C., Connor, S. J., Cresswell, M. P., Morse, A. P., Shears, P., Hart, C. A. and Cuevas, L. E. (2002) Where is the meningitis belt? Defining an area at risk of epidemic meningitis in Africa. *Trans R Soc Trop Med Hyg,* 96, 242-9.

Nadel, S., Newport, M. J., Booy, R. and Levin, M. (1996) Variation in the tumor necrosis factor-alpha gene promoter region may be associated with death from meningococcal disease. *J Infect Dis,* 174, 878-80.

Netea, M. G., van der Meer, J. W., van Deuren, M. and Kullberg, B. J. (2003) Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol,* 24, 254-8.

Newcombe, J., Cartwright, K., Palmer, W. H. and McFadden, J. (1996) PCR of peripheral blood for diagnosis of meningococcal disease. *J Clin Microbiol,* 34, 1637-40.

Ni, H., Knight, A. I., Cartwright, K., Palmer, W. H. and McFadden, J. (1992) Polymerase chain reaction for diagnosis of meningococcal meningitis. *Lancet,* 340, 1432-4.

Norheim, G., Rosenqvist, E., Aseffa, A., Yassin, M., Mengistu, G., Kassu, A., Fikremariam, D., Tamire, W., Fritzsonn, E., Merid, Y., Hoiby, E. A., Harboe, M., Abebe, D., Alabel, T., Tangen, T. and Caugant, D. (2004) In *14th International Pathogenic Neisseria Conference*Milwaukee, USA.

Nugent, S. K., Bausher, J. A., Moxon, E. R. and Rogers, M. C. (1979) Raised intracranial pressure: its management in Neisseria meningitidis meningoencephalitis. *Am J Dis Child,* 133, 260-2.

Olcen, P., Kjellander, J., Danielsson, D. and Lindquist, B. L. (1981) Epidemiology of Neisseria meningitidis; prevalence and symptoms from the upper respiratory tract in family members to patients with meningococcal disease. *Scand J Infect Dis,* 13, 105-9.

Opal, S. M., Wherry, J. C. and Grint, P. (1998) Interleukin-10: potential benefits and possible risks in clinical infectious diseases. *Clin Infect Dis,* 27, 1497-507.

Ovstebo, R., Brandtzaeg, P., Brusletto, B., Haug, K. B., Lande, K., Hoiby, E. A. and Kierulf, P. (2004) Use of robotized DNA isolation and real-time PCR to quantify and identify close correlation between levels of Neisseria meningitidis DNA and lipopolysaccharides in plasma and cerebrospinal fluid from patients with systemic meningococcal disease. *J Clin Microbiol,* 42, 2980-7.

Pathan, N., Hemingway, C. A., Alizadeh, A. A., Stephens, A. C., Boldrick, J. C., Oragui, E. E., McCabe, C., Welch, S. B., Whitney, A., O'Gara, P., Nadel, S., Relman, D. A., Harding, S. E. and Levin, M. (2004) Role of interleukin 6 in myocardial dysfunction of meningococcal septic shock. *Lancet,* 363, 203-9.

Peltola, H. (2001) Burden of meningitis and other severe bacterial infections of children in africa: implications for prevention. *Clin Infect Dis,* 32, 64-75.

PHLSMF (2002) Guidelines for public health management of meningococcal disease in the UK. *Commun Dis Public Health,* 5, 187-204.

Raeymaekers, L. (1999) In *Quantitative PCR protocols*, Vol. 26 (Eds, Kochanowski, B. and Reischl, U.) Humana Press, Totowa, pp. 31-41.

Ramsay, M., Kaczmarski, E., Rush, M., Mallard, R., Farrington, P. and White, J. (1997) Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. *Commun Dis Rep CDR Rev,* 7, R49-54.

Read, R. C. (1998) Experimental therapies for sepsis directed against tumour necrosis factor. *J Antimicrob Chemother,* 41 Suppl A, 65-9.

Read, R. C., Camp, N. J., di Giovine, F. S., Borrow, R., Kaczmarski, E. B., Chaudhary, A. G., Fox, A. J. and Duff, G. W. (2000) An interleukin-1 genotype is associated with fatal outcome of meningococcal disease. *J Infect Dis,* 182, 1557-60.

Read, R. C., Cannings, C., Naylor, S. C., Timms, J. M., Maheswaran, R., Borrow, R., Kaczmarski, E. B. and Duff, G. W. (2003) Variation within genes encoding interleukin-1 and the interleukin-1 receptor antagonist influence the severity of meningococcal disease. *Ann Intern Med,* 138, 534-41.

Reischl, U. and Kochanowski, B. (1999) In *Quantitative PCR protocols*, Vol. 26 (Eds, Kochanowski, B. and Reischl, U.) Humana Press, Totowa, pp. 3-30.

Riordan, F. A., Marzouk, O., Thomson, A. P., Sills, J. A. and Hart, C. A. (1995a) The changing presentations of meningococcal disease. *Eur J Pediatr,* 154, 472-4.

Riordan, F. A., Marzouk, O., Thomson, A. P., Sills, J. A. and Hart, C. A. (1996) Proinflammatory and anti-inflammatory cytokines in meningococcal disease. *Arch Dis Child,* 75, 453-4.

Riordan, F. A., Marzouk, O., Thomson, A. P., Sills, J. A. and Hart, C. A. (2002) Prospective validation of the Glasgow Meningococcal Septicaemia Prognostic Score. Comparison with other scoring methods. *Eur J Pediatr,* 161, 531-7.

Riordan, F. A., Thomson, A. P., Sills, J. A. and Hart, C. A. (1995b) Bacterial meningitis in the first three months of life. *Postgrad Med J,* 71, 36-8.

Ryder, C. S., Beatty, D. W. and Heese, H. D. (1987) Group B meningococcal infection in children during an epidemic in Cape Town, South Africa. *Ann Trop Paediatr,* 7, 47-53.

Sacchi, C. T., Lemos, A. P., Popovic, T., De Morais, J. C., Whitney, A. M., Melles, C. E., Brondi, L. M., Monteiro, L. M., Paiva, M. V., Solari, C. A. and Mayer, L. W. (2001) Serosubtypes and PorA types of Neisseria meningitidis serogroup B isolated in Brazil during 1997--1998: overview and implications for vaccine development. *J Clin Microbiol,* 39, 2897-903.

Sackett, D. L., Haynes, R. B., Guyatt, G. H. and Tugwell, P. (1991) *Clinical epidemiology: a basic science for clinical medicine,* Little, Brown and Company, Boston.

Salih, M. A., Ahmed, H. S., Karrar, Z. A., Kamil, I., Osman, K. A., Palmgren, H., Hofvander, Y. and Olcen, P. (1990) Features of a large epidemic of group A meningococcal meningitis in Khartoum, Sudan in 1988. *Scand J Infect Dis,* 22, 161-70.

Saunders, N. B., Zollinger, W. D. and Rao, V. B. (1993) A rapid and sensitive PCR strategy employed for amplification and sequencing of porA from a single colony-forming unit of Neisseria meningitidis. *Gene,* 137, 153-62.

Sidikou, F., Djibo, S., Taha, M. K., Alonso, J. M., Djibo, A., Kairo, K. K., Chanteau, S. and Boisier, P. (2003) Polymerase chain reaction assay and bacterial meningitis surveillance in remote areas, Niger. *Emerg Infect Dis,* 9, 1486-8.

Sinclair, J. F., Skeoch, C. H. and Hallworth, D. (1987) Prognosis of meningococcal septicaemia. *Lancet,* 2, 38.

Spanjaard, L., Bol, P., de Marie, S. and Zanen, H. C. (1987) Association of meningococcal serotypes with the course of disease: serotypes 2a and 2b in the Netherlands, 1959-1981. *J Infect Dis,* 155, 277-82.

Stanwell-Smith, R. E., Stuart, J. M., Hughes, A. O., Robinson, P., Griffin, M. B. and Cartwright, K. (1994) Smoking, the environment and meningococcal disease: a case control study. *Epidemiol Infect,* 112, 315-28.

Stiehm, E. R. and Damrosch, D. S. (1966) Factors in the prognosis of meningococcal infection. Review of 63 cases with emphasis on recognition and management of the severely ill patient. *J Pediatr,* 68, 457-67.

Sullivan, T. D. and LaScolea, L. J., Jr. (1987) Neisseria meningitidis bacteremia in children: quantitation of bacteremia and spontaneous clinical recovery without antibiotic therapy. *Pediatrics,* 80, 63-7.

Thomson, A. P., Hart, C. A. and Sills, J. A. (1990) Meningococcal disease in Liverpool children 1977 - 1987: mode of presentation. *Pediatric Rev Commun,* 5, 109-16.

Thorburn, K., Baines, P., Thomson, A. and Hart, C. A. (2001) Mortality in severe meningococcal disease. *Arch Dis Child,* 85, 382-5.

Tikhomirov, E., Santamaria, M. and Esteves, K. (1997) Meningococcal disease: public health burden and control. *World Health Stat Q,* 50, 170-7.

Trotter, C. L., Andrews, N. J., Kaczmarski, E. B., Miller, E. and Ramsay, M. E. (2004) Effectiveness of meningococcal serogroup C conjugate vaccine 4 years after introduction. *Lancet,* 364, 365-7.

Trotter, C. L., Fox, A. J., Ramsay, M. E., Sadler, F., Gray, S. J., Mallard, R. and Kaczmarski, E. B. (2002) Fatal outcome from meningococcal disease--an association with meningococcal phenotype but not with reduced susceptibility to benzylpenicillin. *J Med Microbiol,* 51, 855-60.

Urwin, R., Kaczmarski, E. B., Guiver, M., Fox, A. J. and Maiden, M. C. (1998) Amplification of the meningococcal porB gene for non-culture serotype characterization. *Epidemiol Infect,* 120, 257-62.

van Deuren, M., Brandtzaeg, P. and van der Meer, J. W. (2000) Update on meningococcal disease with emphasis on pathogenesis and clinical management. *Clin Microbiol Rev,* 13, 144-66, table of contents.

van Deuren, M., Netea, M. G., Hijmans, A., Demacker, P. N., Neeleman, C., Sauerwein, R. W., Bartelink, A. K. and van der Meer, J. W. (1998) Posttranscriptional down-regulation of tumor necrosis factor-alpha and interleukin-1beta production in acute meningococcal infections. *J Infect Dis,* 177, 1401-5.

van Deuren, M., van der Ven-Jongekrijg, J., Bartelink, A. K., van Dalen, R., Sauerwein, R. W. and van der Meer, J. W. (1995) Correlation between

proinflammatory cytokines and antiinflammatory mediators and the severity of disease in meningococcal infections. *J Infect Dis,* 172, 433-9.

van Deuren, M., van der Ven-Jongekrijg, J., Vannier, E., van Dalen, R., Pesman, G., Bartelink, A. K., Dinarello, C. A. and van der Meer, J. W. (1997) The pattern of interleukin-1beta (IL-1beta) and its modulating agents IL-1 receptor antagonist and IL-1 soluble receptor type II in acute meningococcal infections. *Blood,* 90, 1101-8.

Vilcek, J. (1998) In *The Cytokine Handbook*(Ed, Thomson, A. W.) Academic Press, London, pp. 1-20.

Waage, A., Brandtzaeg, P., Halstensen, A., Kierulf, P. and Espevik, T. (1989a) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. *J Exp Med,* 169, 333-8.

Waage, A., Halstensen, A. and Espevik, T. (1987) Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet,* 1, 355-7.

Waage, A., Halstensen, A., Shalaby, R., Brandtzaeg, P., Kierulf, P. and Espevik, T. (1989b) Local production of tumor necrosis factor alpha, interleukin 1, and interleukin 6 in meningococcal meningitis. Relation to the inflammatory response. *J Exp Med,* 170, 1859-67.

Ward, J., Hanna, J. N., Bates, J. R. and Selvey, L. A. (2000) Enhanced surveillance for meningococcal disease in Queensland in 1999. *Commun Dis Intell,* 24, 332-5.

Westendorp, R. G., Langermans, J. A., Huizinga, T. W., Verweij, C. L. and Sturk, A. (1997) Genetic influence on cytokine production in meningococcal disease. *Lancet,* 349, 1912-3.

Whittle, H. C. and Greenwood, B. M. (1976) Meningococcal meningitis in the northern savanna of Africa. *Trop Doct,* 6, 99-104.

WHO (1998) *Control of Epidemic Meningococcal Disease: WHO practical guidelines,* World Health Organization, Geneva.

WHO (2003) *The World health report: 2003: shaping the future,* World Health Organisation, Geneva.

Woerden, H. C. v., Howard, A., Lyons, M., Westmoreland, D. and Thomas, M. (2004) Use of pre- and post-test probability of disease to interpret meningococcal PCR results. *Eur J Clin Microbiol Inf Dis,* 23, 658-60.

Zangwill, K. M., Schuchat, A., Riedo, F. X., Pinner, R. W., Koo, D. T., Reeves, M. W. and Wenger, J. D. (1997) School-based clusters of meningococcal disease in the United States. Descriptive epidemiology and a case-control analysis. *Jama,* 277, 389-95.

Zwahlen, A. and Waldvogel, F. A. (1984) Magnitude of bacteremia and complement activation during Neisseria meningitidis infection: study of two co-primary cases with different clinical presentations. *Eur J Clin Microbiol,* 3, 439-41.

Appendix

1 CONSENT FORM: ENGLISH

Instituto Materno Infantil de Pernambuco - Correia Picanço Hospital - LACEN – University of Liverpool

INFORMED CONSENT FORM

Dear Mr(s) *(name)*.

My name is Dr Jailson Correia. I am a research student from the University of Liverpool.

The doctor in charge of your case (your child's case) suspects you have (he/she has) meningitis / septicaemia. That is why you were (he/she was) admitted. To be sure of the diagnosis and decide the better treatment, the doctors will need to take blood samples and some spinal fluid as well.

At the University of Liverpool, we are studying ways of better understanding how the disease occurs. So, in the future, it could be possible to provide better treatment to other people. This is why we are asking your consent to participate in this study. But I would like to clarify first:

- The hospital staff will take care of you (he/she). The treatment you (he/she) will receive will be the same if you participate or not.
- We would ask you some questions such as: age, address, symptoms, and housing conditions.
- From the blood and spinal fluid samples the doctors will take, a small amount would be used for the research laboratory tests. No samples will be taken without your doctors' request.
- We will make sure your (his/her) name will be kept confidentially.
- You are free to withdraw from the study at any time and without any disadvantage or prejudice.

Thanks for your attention.

_____

Patient / guardian signature

2 CONSENT FORM: PORTUGUESE

Instituto Materno Infantil de Pernambuco - Hospital Correia Picanço - LACEN - Universidade de Liverpool

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Caro Sr(a): *(nome)*.

Meu nome é Dr. Jailosn Correia. Eu sou estudante de doutorado da Universidade de Liverpool. O médico de plantão suspeita que você (seu filho(a)) tem uma doença chamada meningite / septicemia. Por isso você (ele(a)) ficou internado(a). Para ter certeza e poder escolher o melhor tratamento ele precisou fazer alguns exames, como tirar sangue e líquor (água da espinha).

Na Universidade de Liverpool, estamos fazendo uma pesquisa para tentar entender melhor sobre estas doenças, para que possamos no futuro poder melhorar o tratamento dado a outras pessoas. Por isso estou pedindo a sua autorização para participar da pesquisa. Gostaria de deixar claro que:

- Ele(a) será acompanhado(a) pela equipe do Hospital e o tratamento dado será o mesmo, não importa se participe da pesquisa ou não.
- Seriam feitas algumas perguntas sobre os sintomas da criança, sua idade e se tem outras doenças.
- Dos exames de sangue e líquor (água da espinha) já colhidos, seria retirada uma pequena quantidade a mais para estudos no laboratório. Além disso, no segundo dia do internamento e antes da alta seriam colhidas amostras de sangue. Não seria colhido líquor se o médico acompanhante não achasse necessário;
- Em nenhum momento seria divulgado o nome de seu filho(a) nem a sua doença;
- Caso não aceite ou queira deixar de participar no meio do tratamento, não tem nenhum problema. O tratamento dado seria o mesmo.

Nós agradecemos sua atenção.

_____

Assinatura do paciente ou seu responsável

3 CONSENT FORM: AMHARIC

የስምምነት ቅፅ

ስሜ ዶ/ር ዴይልሰን ይባላል; በእንግሊዝ ሀገር ሊቨርፑል ዩኒቨርስቲ ተመራማሪ ነኝ። የርስዎን (የልጅዎን) ጉዳይ የያዘው ዶክተር የያዘዎት (የያዘው/ት) በሽታ የማጅራት ገትር ሊሆን ይችላል ብሎ ጠርጥሯል። ለዚህም ነው እዚህ የተኙት (ልጅዎን ያስተኙት) ስለበሽታው ርግጠኛ ለመሆንና የተሻለ ሕክምና ለመስጠት ዶክተሩ የደምና የሕብለሰርሰር ፈሳሽ ወስዶ ይመረምራል። እኛ ሊቨርፑል ዩኒቨርስቲ ውስጥ በሽታው እንዴት ሊከሰት እንደሚችል የተሻለ ግንዛቤ እንዲኖር እያጠናን ነው። ስለዚህ ወደፊት ለሌሎች ሰዎች የተሻለ ህክምና ሊኖር ይችላል።
ለዚህም ነው በጥናቱ ለመሣተፍ ፍቃደኝነትዎን የምንጠይቀው። ነገር ግን ግልፅ ላደርግልዎት የምፈልገው ፤

ሀ. ለርስዎ (ልጅዎ) እንክብካቤ የሚያደርጉት የሆስፒታሉ ሰራተኛ ናቸው። የሚሰጥዎት ህክምና በዚህ ጥናት ቢሳተፉም ባይሳተፉም ለውጥ አይኖረውም።

ለ. አንዳንድ ጥያቄዎችን እንጠይቅዎታለን። ዕድሜ፤ አድራሻ፤ የበሽታው ምልክቶችን ስለቤትዎ የመሣሰሉትን

ሐ. ሐኪሙ ከሚወስደው ደምና የህብለሰረሰር ፈሳሽ ትንሽ ለጥናቱ የላቦራቶሪ ምርመራ እንወስዳለን። ከሐኪሙ ፍቃድ ውጪ ምንም ናሙና አይወሰድም።

መ. ስምዎ (የልጅዎ ስም) በምስጢር እንደሚጠበቅ አረጋግጥልዎታለሁ።

ሠ. በጥናቱ ላለመሣተፍ ከፈለጉ በፈለጉት ጊዜ ያለምንም ጉዳት ሊያቋርጡ ይችላሉ።

እናመሰግናለን

ፊርማ _____

ቀን _____

4 GUIDELINES FOR THE MANAGEMENT OF MENINGOCOCCAL DISEASE (RECIFE)

Guidelines for MENINGOCOCCAL DISEASE were prepared by the staff from Correia Picanço Hospital and distributed by the local health authorities.

A **suspected case** is any patient, of any age, who is unwell and present with fever of sudden onset, with or without meningeal signs (severe headache, neck stiffness, drowsiness or seizures), with or without a petechial or ecchymotic skin rash. In children less than 1 year of age, cases might present only with fever, irritability and a bulging fontanelle.

a) Initial treatment at referring health centre / hospital

1) Health care workers should use a facial mask for self protection
2) Patients must be immediately evaluated for: level of consciousness, respiratory pattern, clinical signs of shock (long capillary refill time, cold extremities, feeble pulse, blood pressure might still be normal).
3) Give oxygen
   - Through a nasal prong (2 l/min)
   - Intubate / ventilate if necessary.
4) Obtain a good calibre peripheral IV line.
5) Start antibiotics
   - Penicillin + chloramphenicol or Ceftriaxone (IV)
   - START AS EARLY AS POSSIBLE – THIS IS IMPORTANT FOR A BETTER OUTCOME.
6) If SHOCK is evident, start fluid resuscitation with:
   - Normal saline 0.9% 20 ml/Kg (repeat if necessary up to 60 ml/Kg)

- Plasma 20ml/Kg in 2 h

7) Provide adequate warming.

8) Check blood pressure every 15 minutes.

9) In case of acidosis:

- Guided by blood gases: base excess x 0.3 x weight (Kg) = mEq of Sodium Bicarbonate to be given over 2h.
- Without blood gases: 3 mEq/Kg of Sodium Bicarbonate intravenously over 3h.

10) In case of seizures:

- Diazepam or Midazolam: 0.2 mg/Kg IV
- If seizure persists: Phenytoin: 20 mg/Kg infused at 50 mg/min

11) Corticosteroids:

- If meningitis, start Dexamethasone 0.15 mg/Kg (1g in adults) every 6h, first dose 20 minutes before antibiotics.

12) Obtain samples for laboratory tests

- Blood and CSF

13) Arrange transport in ambulance with an oxygen source, resuscitation equipment and a doctor or nurse.

14) Notification of the case to the public health authority (phone or Fax)

b) Chemoprophylaxis of contacts

Give chemoprophylaxis to:

- People living in the same house as the case
- People who had contact with oral secretions of the case

- People sleeping in crèches or military camps who shared the same room with the case
- Children under 5 years of age who shared the same classroom as the case

The drug of choice is rifampicin:

- Adults 600 mg 2x daily for 2 days
- Children 10 mg/Kg 2x daily for 2 days

c) Antibiotic guidelines

For children between 1 and 6 months of age:

- Ceftriaxone 100 mg/Kg/d or Cefotaxime 200 mg/Kg/d

For older children:

- Penicillin (crystalline) 400,000 UI/kg/d plus chloramphenicol 100 mg/kg/d
- Alternatively, Ceftriaxone or Cefotaxime in the doses above

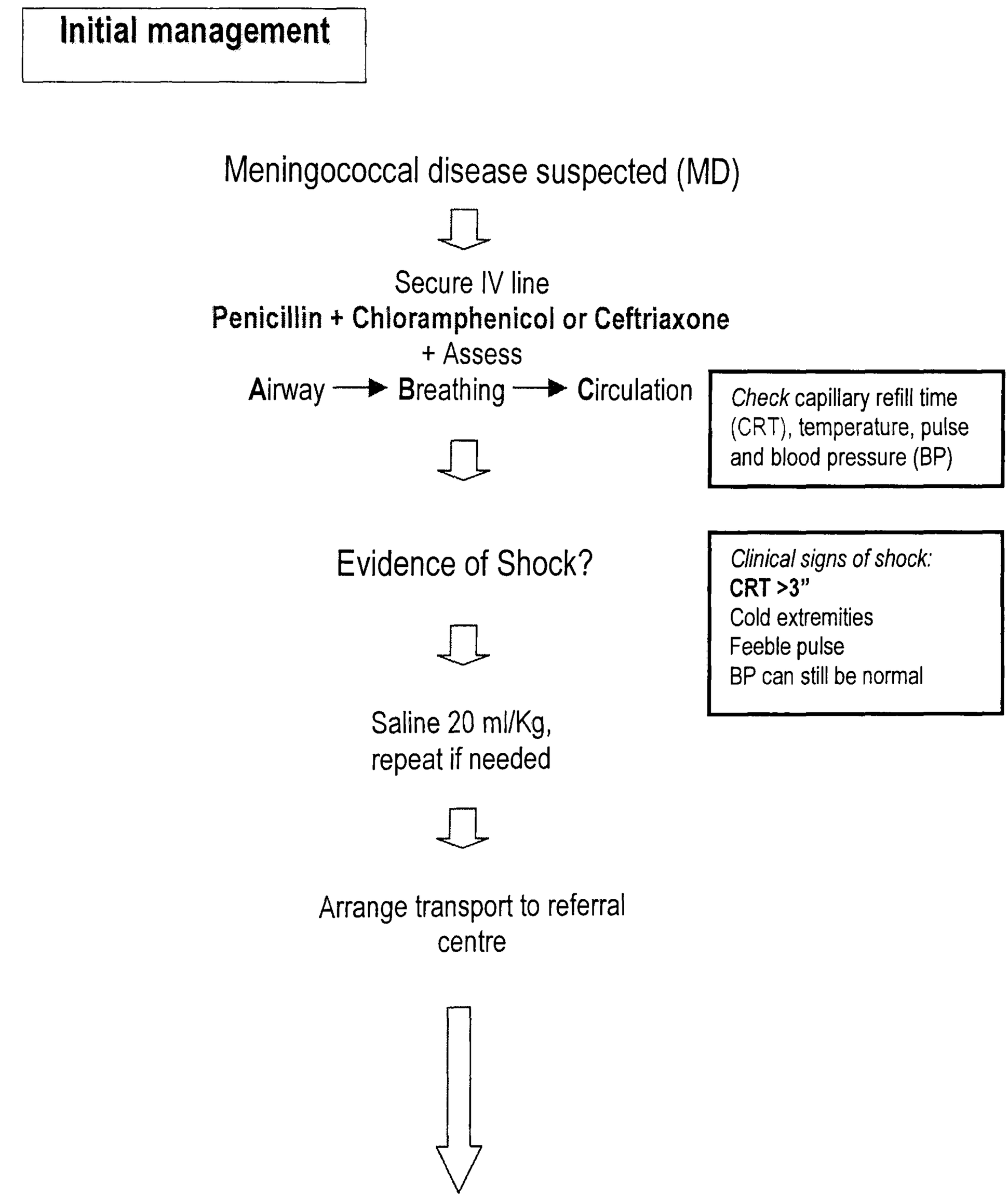
For adults and adolescents:

- Penicillin (crystalline) 5,000,000 UI every 4 h plus chloramphenicol 1g every 6 h
- Alternatively, Ceftriaxone 2g 2x daily or Cefotaxime 2g 4x daily

For pregnant women:

- Ceftriaxone 2g 2x daily or ampicillin 2g 6x daily.

5 GUIDELINES FOR MENINGOCOCCAL DISEASE IN CHILDREN (IMIP)



Referral Centre

Reassess:
Evidence of Shock?

Yes ← | → No

PICU

Ward
+ reassess

- Secure airway / give O_2
- Two IV lines (1 central for CVP)
- Saline 20 ml/Kg, repeat if needed
- Obtain blood culture + CSF →
- Begin dexamethasone 0,6 mg/Kg/d if meningitis
- Assess blood gases and electrolytes
- Assess severity score (GMSPS)

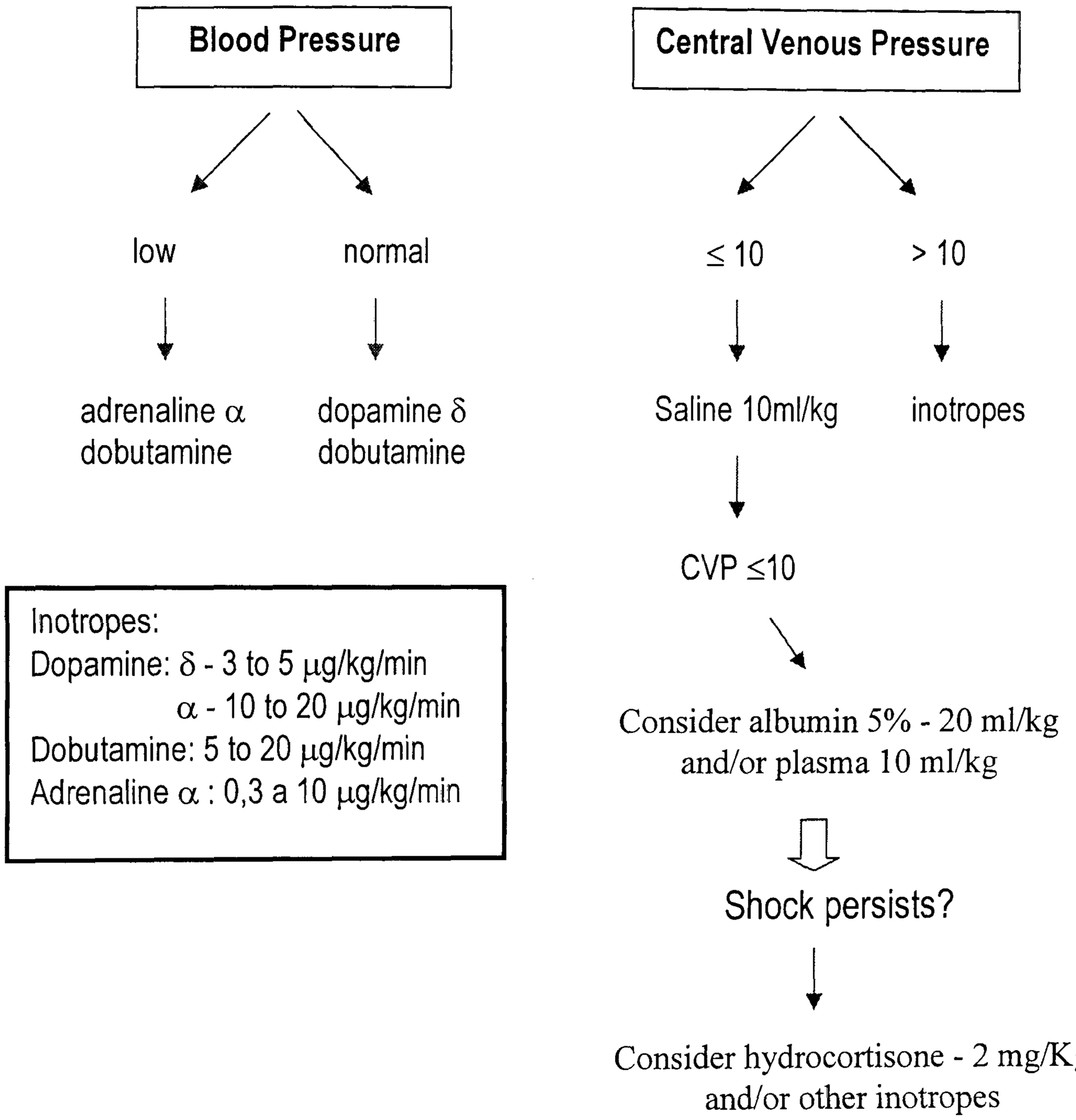
** Do not LP if:*

Low blood pressure or focal neurological signs

Shock persists?

Intubate, ventilate if in PICU
Repeat 20ml/Kg saline
Inotropes

Management according to BP and CVP


Blood Pressure
low
normal
adrenaline α
dobutamine
dopamine δ
dobutamine
Central Venous Pressure
≤ 10
> 10
Saline 10ml/kg
inotropes
CVP ≤10
Consider albumin 5% - 20 ml/kg
and/or plasma 10 ml/kg
Shock persists?
Consider hydrocortisone - 2 mg/Kg
and/or other inotropes
Inotropes:
Dopamine: δ - 3 to 5 μg/kg/min
α - 10 to 20 μg/kg/min
Dobutamine: 5 to 20 μg/kg/min
Adrenaline α : 0,3 a 10 μg/kg/min

6 ALGORITHM FOR ENROLMENT OF CASES

BACTERIAL MENINGITIS SUSPECTED

In the presence of fever, headache, vomiting, stiff neck – or bulging fontanel in infants AND ***CSF is NOT CRISTAL CLEAR.***

OR

FEVER + PETECHIAE + PATIENT UNWELL

*Possible MENINGOCOCCAEMIA with or without meningitis, regardless of CSF appearance**

*** Do not LP if patient is in shock, has focal neurological signs or any other contra-indication**

Please:

- Report admission to a member of the research team, who will obtain informed consent
- Make sure when clinical specimens are taken, the research samples are stored in the fridge immediately after collection
- Make sure the clinical assessment is properly completed. Thanks.

Reminder: will not be included:

- Newborn babies (0 to 28 days)
- Known cases of HIV-AIDS
- People with ventriculo-peritoneal shunts

7 PROFORMA FOR THE BRAZILIAN STUDY SITE

Study number: _____

Admission: Date: ____/____/_____ Time: _____ h

Hospital number: _____

Name: _____

Sex: ( ) Male ( ) Female Date of birth: ____/____/____

Parent's names: _____

Address: _____

First symptoms of current illness: Date: ____/____/_____ Time: _____ h

Chief complaint and history: _____

_____

Symptom checklist:

( ) Fever ( ) Rash ( ) Vomiting ( ) Dyspnoea

( ) Headache ( ) Cyanosis ( ) Convulsion ( ) Lethargy

( ) Disorientation ( ) Drowsiness ( ) Irritability ( ) Any other

Specify: _____

Physical examination:

Temperature (axillary): ____ C Blood pressure: _____x_____ mmHg

Weight: _____ Kg

( ) Nuchal rigidity ( ) Kernig sign ( ) Convulsion

( ) Respiratory distress ( ) Cold extremities ( ) Cyanosis

( ) Focal neurological signs ( ) Anisocoria ( ) Rash (see below)

( ) Petechiae in conjunctiva ( ) Bulging fontanel (infants only)

Any further relevant information / observation / comment / finding:

_____

_____

Study number: _____

Level of consciousness: ( )

(1) **A**lert (2) Respond to **V**erbal command (3) Respond to **P**ainful stimuli

(4) Does **N**ot respond

In case a RASH is present:

Time since noticed: _____ h

Type: ( ) (1) Macular/maculopapular (2) Localised petechiae / ecchymosis

(3) Disseminated or expanding petechiae / ecchymosis

Heart rate: _____ bpm Respiratory rate: _____ ipm

Capillary refill time: _____ s Skin temperature: _____ °C

Did the patient condition get worse in the last hour? ( )

(1)Yes (2) No (3) Not possible to evaluate

Glasgow coma scale: on admission ( ) 1h after first score: ( )

(Refer to wall charts for parameters according to age and enter total score)

Diagnosis on admission (suspected):

( ) Bacterial meningitis ( ) Viral meningitis ( ) Fungal meningitis

( ) TB meningitis ( ) Meningococcal septicaemia

( ) Other: _____

Final diagnosis:

Meningococcal ( ) meningitis ( ) septicaemia ( ) both

( ) Other bacterial meningitis: _____

( ) Viral meningitis ( ) Fungal meningitis ( ) TB meningitis

( ) Other: _____

Outcome: ( ) improved ( ) transferred ( ) dead

Sequelae: ( ) Decribe: _____

Study number: _____

Follow up during hospital stay: (use extra sheet if needed)

__/__/___ Remarks: _____

__/__/___ Remarks: _____

__/__/___ Remarks: _____

__/__/___ Remarks: _____

__/__/___ Remarks: _____

__/__/___ Remarks: _____

Outpatient visits: (use extra sheet if needed)

__/__/___ Remarks: _____

__/__/___ Remarks: _____

Last fever: ___/___/____ ___ h Date/time left hospital: ___/___/____ __ h

Patient data:

Occupation: _____ Schooling: _____ Family income: _____

Housing type: _____ No. of rooms: _____ No. of people: _____

Anti-meningococcal vaccine? ( ) [1-Yes 2-No] Date: ____/____/_____

Treatment:

Fluids given in first 6 h ( )

[1-mantainance 2-crystalloid expansion 3-colloid expansion 4-both crystalloid and colloid expansions 5-no intravenous fluids prescribed]

Corticosteroids: ( )

[1-dexamethasone 2-hydrocortisone 3-other _____
5-not prescribed]

1st dose __/__/___ ____h Last dose: __/__/___ ____h

Initial antibiotics: ( )

[1-penicillin + chloramphenicol 2-ceftriaxone 3-other _____]

1st dose __/__/___ ____h Last dose: __/__/__ _____h

Antibiotics prior to admission? ( ) [1-Yes __/__/___ _____h 2-No]

Study number: _____

Subsequent antibiotics: ( )

[1-penicillin only 2-chloramphenicol only 3-ceftriaxone

4-other _____ 5-no changes to initial course]

Blood products: Plasma: ( ) Packed RBC: ( ) Other: ( ) _____

[1-Yes 2-No]

Tracheal intubation: ( ) [1-Yes 2-No] Time (duration) _____ h

Oxygen: ( ) [1-Yes 2-No] Time (duration) _____ h

Most invasive support: ( )

[1-mask/nasal prong 2-CPAP 3-Mechanical ventilation]

Inotropic agents: ( ) [1-Yes 2-No] ___ h Inotr1: ( ) Inotr2: ( ) Inotr3: ( )

[1-dopamine 2-dobutamine 3-adrenaline 4-other _____]

Referral letter: ( ) [1-Yes 2-No] remarks: _____

Any other remarks:

Study number: _____

Laboratory tests: (continue on extra sheet, if needed)

Blood

Date	Hct (%)	Hb (g/dL)	WBC /mm^3	Neutrophils: immature (%)	Neutrophils: mature (%)	Eosinophils (%)	Lymphocytes: typical (%)	Lymphocytes: atypical (%)	Monocytes (%)	Platelets /mm^3

Date	Glucose mg/dL	Urea nitrogen mg/dL	Creatinine mg/dL	Sodium mEq/L	Potassium mEq/L	Calcium mg/dL	pH	pO_2	pCO_2	Base excess	Bicarbonate mEq/L	Prothrombin time

Blood culture: __/__/____ ( ) other: _____ Sensitivity: ( ) if resistant, to which? _____

Culture: [1-Men. A 2-Men. B 3-Men. C 4-HIB 5-pneumo 6-staph 7-other 8-negative 9-not available]

Resistance: [1-sensitive to all tested 2-resistant to any tested 9-not available]

Study number: _____

Laboratory tests: (continue on extra sheet, if needed)

CSF

Date	Appearance	WBC /mm^3	Polymorphs (%)	Mononuclear cells (%)	RBC /mm^3	Glucose mg/dL	Proteins mg/dL	Latex agglutination	Gram	CIE

Appearance: [1-clear 2-opalescent 3-turbid 4-blood stained 5-other _____]

Latex agglutination: [1-Men. A 2-Men. B 3-Men. C 4-HIB 5-pneumo 6-other 7-negative 9-not available]

Gram staining: [1-Gram neg. diplococci 2-Gram pos. diplococci 3-Gram neg. rods 4-Gram pos. rods 5-Gram pos. cocci 6-other 7-no bacteria seen 9-not available]

Contra-immune-electrophoresis (CIE): [1-Men. A 2-Men. B 3-Men. C 4-HIB 5-pneumo 7-other 8-negative 9-not available]

CSF culture: __/__/____ ( ) other: _____ Sensitivity: ( ) if resistant, to which? _____

Culture: [1-Men. A 2-Men. B 3-Men. C 4-HIB 5-pneumo 6-staph 7-other 8-negative 9-not available]

Resistance: [1-sensitive to all tested 2-resistant to any tested 9-not available]

7 PROFORMA FOR THE ETHIOPIAN STUDY SITE

Study number: _____

I – To be completed on **admission**:

Patient's name: _____ Sex: [1-Male 2-Female] ( )

Origin: _____ Hospital No/bed: _____

Age: ( ) months / years Weight: ( ) Kg Height: ( ) m

Current admission: ___/___/____ ___ h

Collection of CSF: ___/___/____ ___ h

For how long has the patient being ill? (current symptoms) ( ) h / d / w

Ask patient / guardian for each of the following symptoms:

( ) Fever ( ) Rash ( ) Vomiting ( ) Dyspnoea

( ) Headache ( ) Cyanosis ( ) Convulsion ( ) Lethargy

( ) Disorientation ( ) Drowsiness ( ) Irritability ( ) Any other

Specify: _____

Physical examination:

Heart rate: _____ bpm Respiratory rate: _____ ipm

Capillary refill time: _____ s Temperature (axillary): ____ °C

Blood pressure: _____x_____ mmHg

( ) Nuchal rigidity ( ) Kernig sign ( ) Convulsion

( ) Respiratory distress ( ) Cold extremities ( ) Cyanosis

( ) Focal neurological signs ( ) Anisocoria ( ) Rash (see below)

( ) Petechiae in conjunctiva ( ) Bulging fontanel (infants only)

Study number: _____

Level of consciousness: ( )

(1) **A**lert (2) Respond to **V**erbal command (3) Respond to **P**ainful stimuli (4) Does **N**ot respond

In case a RASH is present:

Time since noticed: _____ h

Type: ( ) (1) Macular/maculopapular (2) Localised petechiae / ecchymosis

(3) Disseminated or expanding petechiae / ecchymosis

II – To be completed in the **ward**:

First dose of antibiotic: ___/___/____ ___ h

First dose of steroids (if given): ___/___/____ ___ h

Ask patient / guardian the following questions:

Where appropriate: [1-Yes, 2-No, 3-Don't know]

How many people live in the house? ( )

How many rooms are there in the house? ( )

Contact with a case of meningitis (last month)? ( )

Did the patient have meningitis before? ( )

Any previous vaccine for meningitis A? ( )

Is the patient pregnant? ( )

Was the patient on antibiotics before admission? ( )

Does the patient have another health condition? ( )

Which? _____

Study number: _____

III – To be completed on **discharge**:

Date and time of outcome ___/___/____ ____ h

Outcome (1) Discharge -alive (2) Death (3) Left against medical advice ( )

If death, specify cause: _____

Sequelae (1) Yes (2) No ( )

If yes, specify in detail: _____

List all final diagnosis: _____

Number of days on antibiotics ( ) Number of days on steroids ( )

Duration of fever during hospital stay (days) ( )

Did the patient use any of these treatments? (1) Yes (2) No

Oxygen ( ) Anticonvulsant ( ) Inotropes ( )

Blood products ( )

Did the patient develop any of these complications? (1) Yes (2) No

Brain abscess ( ) Convulsions ( ) Late skin rash ( )

Arthritis ( ) Carditis ( ) Amputation ( )

Brain damage ( ) Secondary (hospital) bacterial infection ( )

Any other ( ) Specify: _____

Any further relevant information / observation / comment / finding:

_____

_____

Name of doctor:_____ Signature: _____

Study number: _____

Laboratory tests: (continue on extra sheet, if needed)

Blood

Date	Hct (%)	Hb (g/dL)	WBC /mm^3	Neutrophils: immature (%)	Neutrophils: mature (%)	Eosinophils (%)	Lymphocytes: typical (%)	Lymphocytes: atypical (%)	Monocytes (%)	Platelets /mm^3

CSF

Date	Appearance	WBC /mm^3	Polymorphs (%)	Mononuclear cells (%)	RBC /mm^3	Glucose mg/dL	Proteins mg/dL	Latex agglutination	Gram	Remarks

Appearance: [1-clear 2-opalescent 3-turbid 4-blood stained 5-other _____]

Latex agglutination: [1-Men. A 2-Men. B 3-Men. C 4-HIB 5-pneumo 6-other 7-negative 9-not available]

Gram staining: [1-Gram neg. diplococci 2-Gram pos. diplococci 3-Gram neg. rods 4-Gram pos. rods 5-Gram pos. cocci 6-other 7-no bacteria seen 9-not available]

Other tests: Malaria Blood Film: Date: __/__/____ Result: _____

Other tests and remarks: _____

8 MATERIALS FOR DUOSET ELISAS

Solutions required and preparation instructions

Phosphate buffered saline (PBS): (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2P0_4, pH 7.2 - 7.4, 0.2 µm filtered). Dissolve one tablet of PBS per 100µL of distilled water in a magnetic stirrer. Store at room temperature.

Wash Buffer: (0.05% Tween 20 in PBS, pH 7.2 - 7.4). Dilute 500µL of Tween 20 in 1 L of PBS in a magnetic stirrer and keep at room temperature. Renew wash buffer in the auto washer on a weekly basis.

Block Buffer: (1% bovine serum albumin (BSA), 5% Sucrose in PBS with 0.05% NaN_3). Prepare a 10% stock solution of BSA (store at +4°C) and mix 100mL with 900 mL of PBS, 50g of sucrose and 0.5g of NaN_3 in a magnetic stirrer. Store at +4°C.

Reagent diluent type A: (1% BSA in PBS). Dilute 100mL of BSA (10% solution) into 900mL of PBS), adjust the pH to 7.2 – 7.4 and filter with a 0.2µm bell-shaped filter. Store at +4°C.

Reagent diluent type B: (0.1 % BSA, 0.05% Tween 20 in Tris-buffered saline (20mM Trizma Base, 150 mM NaCl)). Prepare a 2M stock solution of Trizma base (24g in 100 mL of distilled water). Using a magnetic stirrer, mix 10 mL of 2M Trizma base, 10 mL of BSA (10% solution), 500µL of Tween 20, 8.76g of NaCl and 980mL of distilled water. Adjust the pH to 7.2 - 7.4. Filter with a 0.2µm bell-shaped filter. Store at +4°C.

Substrate Solution: Five minutes before use, prepare a 1:1 mixture of hydrogen peroxide (H_20_2) and Tetramethylbenzidine. Keep in a bottle covered with an aluminium foil until use.

Stop Solution: Carefully dilute the available concentrated stock solution to prepare a 2N solution of H_2S0_4. Store at room temperature.

9 MATERIALS FOR SUBSTANCE P IMMUNOASSAY

Reagents provided

Microplate: 96 well microplate coated with a goat anti-rabbit polyclonal antibody.

Substance P Conjugate: 6 mL of Substance P conjugated to alkaline phosphatase, with blue dye and preservative.

Substance P Standard: 0.5 mL of Substance P (100,000 pg/mL) in buffer, with preservative.

Substance P Antibody Solution: 6 mL of rabbit polyclonal antibody to Substance P, with yellow dye and preservative.

Assay Buffer ED1: 30 mL of a buffered protein base with preservative.

Wash Buffer Concentrate: 30 mL of a 10-fold concentrated solution of a buffered surfactant with preservative.

pNPP Substrate: 20 mL of p-nitrophenyl phosphate in a buffered solution.

Stop Solution: 6 mL of a trisodium phosphate (TSP) solution.

Plate Cover: 1 adhesive strip

Other supplies required:

- Microplate reader capable of measuring absorbance at 405 nm.
- Pipettes and pipette tips
- 500 mL graduated cylinder for preparation of Wash Buffer

- Deionised or distilled water
- Multi-channel pipette, squirt bottle, manifold dispenser, or auto washer
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 +/- 50 rpm.

10 MATERIALS FOR CGRP IMMUNOASSAY

Reagent preparation

EIA buffer: reconstitute one vial with 50 ml of distilled or deionised water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Store at +4°C.

CGRP standard: reconstitute the vial with 1 ml of EIA buffer (or CGRP-free-plasma). Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 1000 pg/ml. Store at +4°C for up to 24 hours.

Quality control: reconstitute one vial with 1 ml of EIA buffer (or CGRP-free-plasma). Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Store at +4°C for up to 24 hours.

Anti-CGRP-AChE tracer: reconstitute one vial with 10 ml of EIA buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Store at +4°C.

Wash buffer: dilute 1 ml of concentrated wash buffer to 400 ml with distilled or deionised water. Add 200 µL of Tween 20 (Use a magnetic stirrer to mix the contents). Store at +4°C.

Ellman's Reagent: five minutes before use, reconstitute with 49 ml of distilled water and 1 ml of concentrated wash buffer. The tube contents should be

thoroughly mixed. Store at +4°C in a bottle covered with aluminium foil for up to 4 days.

Preparation of CGRP-free plasma

For assaying plasma or serum samples, CGRP standard and quality control need to be reconstituted with plasma or serum that is free of human CGRP instead of the EIA buffer.

- Pour the CGRP Affinity Sorbent gel (Spi-Bio, Massy, France) in an empty column (around 10 x 1 cm).
- Let the storage solution elute entirely (the gel should become dry but not for a long time).
- Add around 20 ml of plasma on top of the column with the outlet of the column closed.
- Let it incubate overnight at + 4°C with gentle agitation.
- Elute the plasma that is now free of CGRP and ready to be used to dilute standards and samples in the assay.
- So as to regenerate the gel (eliminate the bound CGRP), wash the column with around 20 ml of 0.1 ML HCI pH 1.5 an then with around 50 ml of 0.1 M Phosphate buffer pH 7.4.
- Store the sorbent in 3 to 4 ml of EIA buffer (buffer of the kit) at + 4°C till the next use. It is stable at + 4°C for many months.

11 PCR METHODS: DNA EXTRACTION AND PREPARATION OF MASTER MIX

a) DNA Extraction

Extraction of CSF, serum and plasma

Prepare an appropriate amount of extraction solution by pipetting 1ml of DNAzol solution (Gibco BRL) for every sample to be extracted into a sterile universal. Add 7µl of polyacryl carrier for every 1ml of DNAzol solution and mix thoroughly. Add 1ml extraction solution to a labelled 1.5ml tube. A meningococcal PCR negative and PCR positive plasma sample should be included in every batch. Water negative controls are included every fifth sample tested.

Pipette 100µl CSF, plasma or serum into appropriate tube containing DNAzol extraction solution. Add 100µl sterile injectable water to the negative control tube. Add 100µl of negative control and positive control plasma to appropriate tubes. Close caps, vortex thoroughly and leave at room temperature for 10 minutes. Add 500µl 95% ethanol into each of the extracted samples and controls. Vortex and leave at room temperature for 10 minutes. Spin at 12,000 rpm for 10minutes at 4°C.

Remove supernatant using a fine tipped sterile pastette being careful not to disturb the pellet and discard into a universal. Discard waste DNAzol extraction solution properly. Add 1ml of 95% ethanol to each tube, close cap and vortex. Spin tubes for 5 minutes at 12,00rpm at 4°C. Remove supernatant and discard. "Pulse" spin tubes (10 seconds at 12,000rpm) and carefully remove residual ethanol. Add 50µl

of sterile injectable water and leave at 50°C in a dry heating block for 10 minutes to re-dissolve DNA pellets.

EDTA Whole Blood Extraction Using Gentra Columns

This method uses Gentra whole blood extraction kits (Flowgen D5-0410). Label a column supplied within a blue collection tube for each sample to be tested.

Vortex EDTA blood sample and add 200 µl to the centre of the column. Leave at room temperature for 10 minutes. Add 400 µl of wash buffer (Solution 1) to each column. Take care not to touch the tops of the columns with the pipette tip. Leave for 2 minute and then centrifuge at 6000 rpm for 1 minute. Transfer columns to a new blue collection tube and add 400 µl of Solution 1, leave for 1 minute and centrifuge as before.

Add 200 µl of elution buffer (Solution 2) to each column and centrifuge immediately at 6000 rpm for 1 minute. Transfer column to the clear collection tube and add 200 µl of Solution 2. Transfer to one of the Grant heating blocks set at 99^0 C and incubate for 10 minutes.

At the end of the 10 minutes incubation immediately transfer columns to a microfuge and spin at 6000 rpm for 1 minute. The sample is now ready for PCR amplification.

b) PCR Reagent Preparation (master mix)

Preparation of master mix must be carried out in a PCR clean room using a PCR laminar flow cabinet. Primers and probes must be stored frozen. Universal mix must be stored at 4^0C.

TaqMan™ PCR reaction mix is prepared by mixing together the appropriate primers and probes and Universal master mix. Use appropriate *ctrA*, *siaD* B or *siaD* C primer and probe sets accordingly.

Below are the volumes required for one reaction. Prepare reaction mix as multiples of these volumes for the relevant number of samples and controls. Prepare enough reactions plus an additional two to allow for pipetting errors.

- Universal mix	1	2.5µl
- Forward Primer (2µM)		2.5µl
- Reverse primer (2µM)		2.5µl
- FAM labelled probe (1µM)		2.5µl
- Injectable water		3.0µl

c) Primer and probe sequences

ctrA gene (5'-3'):

- Forward primer: TTGTGTGGAAGTTTAATTGTAGGATGC (185-211)
- Reverse primer: TCAGATTGTTGCCCTAAAGAGACA (273-250)
- Probe: TCCTTCATCAGGCCCCAGCG (220-239)

siaD B (5'-3'):

- Forward primer; TGCATGTCCCCTTTCCTGA (3708-3726)
- Reverse primer: AATGGGGTAGCGTTGACTAACAA (3877-3855)
- Probe: TGCTTATTCCTCCAGCATGCGCAAA (3794-3770)

siaD C (5'-3'):

- Forward primer: GATAAATTTGATATTTTGCATGTAGCTTTC (562-591)
- Reverse primer: TGAGATATGCGGTATTTGTCTTGAAT (710-685)
- Probe: TTGGCTTGTGCTAATCCCGCCTGA (672-649)