Associations between Coagulation Factors, Clinical Phenotypes, Cytokine Profiles and Polymorphisms in Immune Response Genes of Haemophilia A and B Patients With and Without Inhibitors.

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science in Medicine.

Johannesburg 2009
DECLARATION

I, Nontobeko Thenjiwe Lorraine Ndlovu declare that this research report is my own unaided work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at this or any other University.

……………………………………..
Candidate signature
……………………………………day of …………………………………2009.
DEDICATION

In loving memory of my father

Jabulani Chris `Over` Ndlovu

1956-2003
PRESENTATIONS ARISING FROM THIS STUDY

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Associations between coagulation factors, clinical phenotypes, cytokine profiles, inhibitor development and IL-10 gene promoter polymorphisms in haemophilia.
ABSTRACT

The underlying mechanism and determinants of inhibitor formation in approximately 30% haemophilia A and 5% haemophilia B patients are not fully understood. A large amount of the data on immune responses against FVIII and FIX is from animal models. Studies investigating cytokines in haemophilia are very limited and fragmentary, and the classification of hemophilia patients according to their factor activity levels has been observed to be inconsistent. The current study aims to find the associations between factor levels, clinical phenotype, cytokine profiles and polymorphisms in the IL-10 gene promoter of haemophilia A and B patients with and without inhibitors. This may give more insight into the pathophysiology of haemophilia, help improve the understanding of the pathogenic mechanisms that underlie inhibitor development, and facilitate new diagnostic and therapeutic strategies for haemophilia.

Haemophilia A and B patients with and without inhibitors were enrolled in the current study. Forty (40) patients from the Charlotte Maxeke Johannesburg Academic Hospital Haemophilia Comprehensive Care Centre (CMJAH-HCCC) were randomly selected. An equal number of frequent bleeders and infrequent bleeders were recruited. Frequent bleeders were defined as those patients with 2 or more bleeding episodes per month on three consecutive months. Bleeding frequency was evaluated on the patient’s bleeding charts.
FVIII and FIX activity levels of all patients were measured using the Dade Behring Sysmex CA-7000 coagulation analyzer, and information on each patient’s bleeding episodes was obtained from the haemophilia bleeding charts. The inhibitor status of all patients was evaluated using the Bethesda inhibitor assay. IL-1β, IL-6 and TNF-α were analyzed using an ELISA kit method. IL-2, IL-4, IL-10 and IFN-γ were analyzed using the CBA Human TH1/TH2 Cytokine Kit. DNA was extracted using the Nucleon BACC3 from Amersham Biosciences. Polymorphisms in the IL-10 gene promoter region were analyzed using PCR. The Statistica Release 8 statistics package was used for statistical analysis.

The present study population showed significant discrepancies in the theoretic classification of haemophilia patients. Severe haemophilia patients had significantly higher levels of IL-6 than the mild/moderate group and biochemical classification correlated positively with IL-6. IL-6 was also the only significant predictor of biochemical classification. IL-1β and IL-4 was found to be significantly higher in the mild/moderate group than in the severe group. There were no significant differences in the levels of IL-2, IL-10, and IFNγ between the mild/moderate and severe groups and between patients with inhibitors and without inhibitors. There were also no differences in the cytokine profiles of low and high responders.

No significant differences were found between cytokine profiles of frequent and infrequent bleeders. IL-6 and TNF-α were found to be significantly higher in patients with inhibitors than in haemophilia patients without inhibitors. IL-6 and IL-1β were
the only significant predictors of the inhibitor status of haemophilia patients. Haemophilia severity and race were found to be significant risk factors for inhibitor development. A 150 bp allele of the IL-10 promoter region with the microsatellite marker was observed in patients with and without inhibitors as well as the healthy controls. The 150 bp allele was also observed in both black and white subjects.

Large phenotypic heterogeneity exists in haemophilia patients. The pro-inflammatory cytokines IL-6 and IL-1β together with IL-4 may be involved in determining the biochemical severity of haemophilia. IL-6 was the only cytokine in this study found to be a significant predictor of bleeding frequency. The study results also suggest that IL-6 and IL-1β may be involved in the production of antibodies against infused factor in patients with inhibitors.

The presence of a 150 bp allele of the highly polymorphic IL-10 promoter region in patients with and without inhibitors as well as the healthy controls suggests that, polymorphisms in this gene do not influence inhibitor development in this population.
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LIST OF ABBREVIATIONS

ATIII - anti-thrombin 3
bp – base pairs
CBA – cytometric bead array
CMJAH – Charlotte Maxeke Johannesburg Academic Hospital
DNA – deoxyribonucleic acid
EDTA – ethylenediaminetetra-acetic acid
EGF – epidermal growth factor
ELISA – Enzyme linked immuno-sorbent assay
FIX – factor nine
FV – factor five
FVII – factor seven
FVIII – factor eight
FX - factor ten
FXa – active factor ten
FXIII – factor thirteen
Gla – glutamic acid
HCCC – Haemophilia Comprehensive Care Centre
HIV – human immunodeficiency virus
HRP - horseradish peroxidase
IFN-γ – interferon gamma
IgG – immunoglobulin G
IL-10 – interleukin ten
IL-1β - interleukin one beta
IL-2 – interleukin two
IL-4 – interleukin four
IL-6 – interleukin six
IU – international unit
MAbs - monoclonal antibodies
MgCl₂ – magnesium chloride
MHC – major histocompatibility complex
MIBS - Malmo International Brother Study
ml – milli litre
nm – nano metre
PBMCs - peripheral blood mononuclear cells
PCR – polymerase chain reaction
PE - phycoerythrin
SA – South Africa
SLE - systemic lupus erythematous
SPSS - Statistical Package for Social Sciences
TF – tissue factor
TFPI - tissue factor pathway inhibitor
TH1 and TH2 – T cell helper 1 and T cell helper 2
TNF-α- tumour necrosis factor alpha

µl – micro litre

VWD - von Willebrand disease