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

Nontobeko Thenjiwe Lorraine Ndlovu

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

1. Ndlovu N, Chetty N, Mahlangu J.

Cytokine Analysis in Haemophilia

Poster presented at the 48th Annual Congress of the Federation of South African Societies of Pathology.

Cape Town, 10-21 July 2008

2. Accepted for Paper Presentations.

Ndlovu N, Chetty N, Mahlangu J.

Associations between cytokine profiles, polymorphisms in the IL-10 promoter region and inhibitor development.

PathTech Congress.

ICC Durban, 6-10 September 2009

2nd Cross Faculty Symposium

University of the Witwatersrand, 20-21st October 2009

PAPER IN PREPARATION FOR PUBLICATION

1. JN Mahlangu, N Chetty, **NTL Ndlovu**

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 microsattelite 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.

ACKNOWLEDGEMENTS

Praise the Lord oh my soul and all that is within me praise his Holy Name.

Forget not all his wonderful works.

Prof. Johnny Mahlangu and Prof. Nanthakumarn Chetty

For your guidance, encouragement, inspiration, mentorship, funding, for giving me the space to work and grow independently as a scientist, and always so willingly being available to offer quality advice on all matters, both in and out of the laboratory. A million thanks.

My mother MamRuthana Ndlovu and Mancane Sindy Chikunga

For making me laugh when all I want to do is cry. For your endless support. Many women do noble things, but you surpass them all. You are God's greatest blessing.

The Wits Postgraduate Merit Awards, Dep. of Molecular Medicine & Haematology, NHLS, NRF and MRC -: For the financial support.

The Main Haematology Lab Staff -: Dr Hamakwa Mantina, for passing abundant imperative knowledge. I can never thank you enough. Perry, Andrew, Mercy, Tshidi, Khensani, Mpumi, Mariam etc. for always being available to assist me as I required.

Haemophilia Patients –: The patients at the Johannesburg Hospital Haemophilia Comprehensive Care Centre who agreed to take part in the study.

Johannesburg Hospital Haemophilia Comprehensive Care Staff in particular Sister Bongi Mbele and Sister Ann Gilham -: For helping with the collection of blood.

Tamsanqa Semela -: For believing in me, showing abounding interest in my work and showering me with so much love. I love you very much.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

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

$MgCl_2$ – 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 erythematosus

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