

THE ROLE OF NEUTROPHIL EXTRACELLULAR TRAP FORMATION IN SICKLE CELL DISEASE

Acronym: NET, in-vitro experiments

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CONFIDENTIAL

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PRINCIPAL INVESTIGATOR SIGNATURE PAGE

Principal Investigator Name (print)

Principal Investigator Signature Date

PROTOCOL AGREEMENT PAGE

I agree to conduct the Clinical Trial in accordance with the current protocol and comply with its requirements, subject to ethical and safety considerations.

Institute

Local Investigator Name (print)

Local Investigator Signature Date

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)
AE	Adverse Event
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CV	Curriculum Vitae
EU	European Union
GCP	Good Clinical Practice
IB	Investigator's Brochure
IC	Informed Consent
METC	Medical research ethics committee (MREC); in Dutch: medico ethische toetsing commissie (METC)
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgegevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen)

SUMMARY

Rationale: In a recent clinical prospective cohort study we provided indirect evidence that polymorphonuclear neutrophil (PMN) cell death, namely neutrophil extracellular trap (NET) formation, may play a role in the complex process of vaso-occlusive crisis in sickle cell disease and the development of its complications. The exact mechanism of NET formation by PMN of sickle cell patients however remains unknown and deserves further investigation.

Objective: To obtain an in-vitro model of neutrophil extracellular traps (NET) formation and to compare the capacity of NET formation PMNs of patients with sickle cell disease with PMNs of healthy controls.

Study design: Experimental, in vitro study.

Study population: Ten HbSS and HbS β^0 -thalassemia patients over 18 years old in steady state, ten during painful crisis, and ten healthy race matched volunteers with HbAA genotype.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:

The expected burden from this protocol is minimized to venipuncture and risks are not to be expected.

1. INTRODUCTION AND RATIONALE

Sickle cell disease (SCD) is the most common hemoglobinopathy in the world. Due to migration the disease is increasingly diagnosed in Europe which has resulted in the introduction of newborn screening programs and comprehensive care programs in several European countries. Due to newborn screening programs, vaccination, prophylactic antibiotics and risk assessment for stroke by transcranial Doppler, life expectancy has improved significantly for children in the last decade (1). Despite this, life expectancy overall still is remarkably reduced(2;3) and no therapeutic advances have been reported since the introduction of hydroxycarbamide in 1995 underlining the necessity of clinical, experimental and translational research in patients with SCD.

SCD is characterized by a chronic inflammatory response resulting in enhanced endothelial activation, coagulation activation, release of cytokines and microvascular vaso-occlusion resulting in tissue ischemia and cumulating organ damage. These processes are in particular up-regulated during painful vaso-occlusive crises (VOC), which account for the vast majority of sickle cell related hospital admissions (4). VOC and its related complications, like acute chest syndrome and multi-organ failure, are associated with a significant mortality, morbidity and reduction in quality of life (5). Although SCD has been described for the first time a century ago, the exact pathogenesis of these VOCs is still not well understood.

There is an increasing acknowledgement that leukocytes, especially polymorphonuclear neutrophils (PMNs), play an important role in sickle cell related pathology. This is illustrated by the clinical observation that leukocytosis is a risk factor for complications like stroke (6), acute chest syndrome and early death (2) while an increased neutrophil count in steady state has been associated with disease severity (7). Interestingly, the clinical benefit of hydroxycarbamide in prevention of painful crises has partly been attributed to the reduction in neutrophil count following initiation of therapy (8).

Several cytokines, like IL-8 and IL-1 β , have been reported to be increased in SCD patients as compared with levels in healthy volunteers, and these cytokines can lead to priming of PMNs (9-11). Activation of sickle PMNs leads to the production of toxic oxygen radicals (12), thereby inducing

endothelial damage and cellular adherence. This was confirmed in in-vitro experiments in which isolated PMNs from sickle cell patients showed to have increased adhesion to fibronectin, recombinant intercellular adhesion molecule 1 (ICAM-1) and endothelial monolayers as compared to PMNs from healthy volunteers (13). Furthermore, it has been demonstrated that the initial process of vaso-occlusion starts with tethering and rolling of PMNs to vascular endothelium which is mediated by selectins like E-, and P-selectin (14;15). The subsequent firm adhesion between PMNs and endothelial cells is further mediated by β 2-integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). The expression of Mac-1 on sickle PMNs is increased when compared to the expression of Mac-1 on normal PMNs (10). The processes of rolling, adhesion and activation of PMNs all contribute to the multifaceted mechanism of VOC (14;15) that further comprises oxidative stress (16) and coagulation activation.

Recently, PMNs have been demonstrated to form neutrophil extracellular traps (NET) upon stimulation (17). Following NET formation, DNA and DNA-binding proteins are extruded from the cell exposing a mesh consisting of nucleosomes, histones and neutrophil proteases. Nucleosomes and histones exposed on NET have been shown to be cytotoxic to endothelial cells in-vitro (18). Moreover, nucleosomes exposed on NET have been demonstrated to be strongly pro-coagulant in animal models as well as in patients (19;20). Nucleosomes consist of a core octamer of two copies each holding the histones H2A, H2B, H3 and H4, around which a segment of helical DNA of 146 base pairs is wrapped (21). Circulating nucleosomes detected in sepsis and meningococcal sepsis have been reported to correlate with severity of inflammation and mortality, as well as with markers for coagulation and neutrophil activation (22;23). In baboons and humans, circulating nucleosomes in plasma have been reported to be a suitable marker for NET formation (20;24;25).

Recently, in a prospective cohort study design, we described for the first time elevated plasma levels of nucleosomes in sickle cell patients with painful crises which paralleled neutrophil activation, as evidenced by levels of elastase- α 1-antitrypsin complexes (EA), indicating NET formation in sickle cell patients during painful VOC (26). In addition, a correlation with disease severity was observed given the observation that the highest nucleosome levels were seen in patients with an acute chest

syndrome or a prolonged hospitalization (26). To our knowledge our data are the first providing indirect evidence for NET formation in sickle cell disease patients. However, we still do not know how in sickle cell patients NET are formed and whether PMNs from these patients form NET more easily as compared to PMNs of healthy volunteers. This knowledge will help us to understand how NET play a role in tissue damage during painful crisis. Therefore, clinical as well as experimental in-vitro research are required to further investigate this phenomenon.

In this specific protocol we want to challenge our hypothesis that activated, polymorphonuclear neutrophils of sickle cell disease patients are prone to form NET when compared to PMN of healthy controls. We think this study is an important step in understanding if and how NET formation might play a role in sickle cell disease and sickle cell related complications, like vaso-occlusive crises.

2. OBJECTIVES

1. To investigate if PMN of patients with sickle cell disease are prone to form NET when compared to PMN of healthy controls.
2. To investigate if red blood cells of sickle cell disease patients promote NET formation of PMN in sickle cell patients as well as in PMNs of healthy volunteers.
3. To investigate if plasma of sickle cell disease patients during painful crisis is toxic to endothelial and parenchymal cells, and to explore if this is due to extracellular neutrophil chromatin and proteases present in plasma.

3. STUDY DESIGN

- a. Prospective study, experimental in-vitro design.
- b. Samples will be obtained from SCD patients in steady state as well as from SCD patients during painful crisis. Furthermore, healthy race-matched volunteers will be approached to obtain control samples.
- c. In-vitro experiments will be performed on obtained samples.

4. STUDY POPULATION

All eligible patients have to be registered before start of study. Patients have to meet the criteria as mentioned below.

4.1 Population (base)

Consecutive HbSS and HbS β^0 - thalassemia patients, of 18 years or older, both male and female, visiting the outpatient clinic for their regular ambulant visit, or admitted for an acute painful crisis, at the Academic Medical Center and the Slotervaart Hospital in Amsterdam will be asked to participate in this study. A painful crisis is defined as musculo-skeletal pain not otherwise explained and recognized as such by the patient and requiring medical treatment. Since phenotypically sickle cell disease patients with the HbSC or HbS β^+ - thalassemia genotypes differ from patients with the clinically more severe affected HbSS or HbS β^0 - thalassemia genotypes (27;28), we choose to include only patients with the HbSS or HbS β^0 - thalassemia genotype.

As a control we will ask patients to bring a healthy race-matched family member or acquaintance who is known not to carry a sickle cell gene.

4.2 Inclusion criteria patient

1. High performance liquid chromatography (HPLC) confirmed diagnosis of HbSS or HbS β^0 - thalassemia genotype in SCD patients.
2. ≥ 18 years
3. Written informed consent by the patient.

4.3 Exclusion criteria patient

1. HbSC or HbS β^+ - thalassemia genotype ; 2. Pregnancy ; 3. Active cancer ; 4. Chronic HIV infection; 5. Recent vaso-occlusive crisis (<1 month); 6. Chronic transfusion therapy; 7. Recent blood transfusion (< 3 months)

4.4 Inclusion criteria – healthy volunteers

- Age ≥ 18 years

- WHO performance score 0
- healthy race-matched family member or acquaintance

4.5 Exclusion criteria – healthy volunteers

- Carrier of sickle cell gene or other hemoglobinopathy

4.4 Sample size calculation

No sample size calculation was performed since this study design follows an experimental design.

The aim is to include 10 sickle cell patients in steady state, 10 sickle cell patients during painful crisis as well as 10 controls.

5 METHODS

5.1 Study procedures. Blood samples and laboratory determinations.

After informed consent is obtained, blood will be drawn via venipuncture according to the local protocol.

Two citrate tubes, 3.2%, (both 9 mL) will be drawn from study patients to answer **objective 1**. One other citrate tube, 3.2% (9 mL) will subsequently be drawn subsequently to answer **objective 2**.

To be able to relate obtained experimental results to basic clinical patient information 3 extra tubes will be drawn. To this purpose 2 small EDTA tubes (2x 3,0 ml) will be drawn to perform a full blood exam with differential (Hb, Ht, MCV, MCH, MCHC, RDW, RBCs, reticulocytes, WBCs, WBCs differentiation, platelets) and one heparin tube (4.5 ml) will be drawn to determine parameters of hemolysis and inflammation (bilirubin, LDH and CRP).

The tubes will be held on room temperature during transportation to the laboratory. Further analysis will be performed as soon as possible after venipuncture.

From control patients a 3,0 mL EDTA tube will be drawn to confirm a normal hemoglobin genotype using HPLC.

Processing

Objective 1:

Are PMNs of patients with sickle cell disease prone to form NET as compared to PMNs from healthy controls? Is this different for PMN of sickle cell disease patients during painful crisis?

To answer this question the following procedures will be followed.

1. Fresh blood, citrated sample, will be used to isolate PMN.
2. Isolation of PMN's will be done based on density centrifugation using Ficoll/percoll.

Contaminating erythrocytes will be lysed hypotonically by means of an ammonium chloride buffer on ice.
3. After seeding PMNs in a 96-well microtiterplate NET formation is induced by different stimuli; eg. phorbol myristate acetate (PMA), LPS, or IL-8.
 - The ability of sickle cell erythrocytes to induce NET formation will furthermore be tested.
4. NET formation in supernatant of stimulated PMN is assessed and quantified by visualization (confocal microscopy and fluorescence microscopy) and measurement of cell-free DNA as well as neutrophilic proteins released after NET degradation by DNase/MNase. Cell free DNA will be measured in form of nucleosomes (ELISA), by means of a picogreen assay as well as by a RT-PCR specific for cell-free neutrophilic DNA. Moreover, histones and neutrophilic proteases (e.g. elastase, MPO) will be measured by ELISA. All these assays are operational. The total amount of extracellular DNA and sensitivity of PMN to external stimuli to form NET will be compared between groups.
6. In another approach we will investigate the capacity of plasma of SCD patients to digest NET as compared with plasma of healthy volunteers. For this purpose NET formation will be induced in PMNs of sickle cell patients as well as healthy volunteers. Thereafter formed NET will be incubated with (pooled) plasma from either healthy volunteers or sickle cell patients. Release of DNA (measured with the assays described above) from NET after

incubation with plasma indirectly correlate with the capacity of plasma to digest NET and hence to “detoxify” NET.

Objective 2:

Do DNA and DNA-binding proteins exposed and released by NET of PMNs from sickle cell patients induce (endothelial) cell damage?

To answer this question the following procedures will be followed.

1. Human pulmonary artery endothelial cells (HPAEC), human umbilical vein endothelial cells (HUVEC) or murine alveolar type II (AT-II) will be seeded into plates.
2. Cells will be incubated with
 - a) plasma of controls and patients
 - b) the supernatant of PMNs, from SCD patients and controls, which formed NET.
3. As positive controls, we will use apoptosis inducers (e.g. staurosporine), isolated histones and nucleosomes.
4. Cell death of the endothelial and epithelial cells will be assessed by LDH release into the supernatant by cytotoxicity detection kit (Roche Applied Science, Germany).
5. In this set up we will furthermore test the effects of interventions, such as neutralizing antibodies to histones and DNA.
6. Differences between groups will be compared and visualized graphically.

5.2 Premature termination of the study

No premature termination of the study is expected.

6. SAFETY REPORTING

6.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

6.2 Adverse and serious adverse events

No (serious) adverse events are expected from this study.

7. STATISTICAL ANALYSIS

7.1 Descriptive statistics

All data will be entered in IBM SPSS Statistics 20.0 for statistical analysis. Descriptive statistical methods will be used to investigate if data follows a parametric distribution.

For between group comparisons either Two sample T-test or Wilcoxon signed rank test will be used.

Correlation analyses will be performed using either Pearson or Spearman rank correlation tests. Two-tailed p values ≤ 0.05 are considered statistically significant.

8. ETHICAL CONSIDERATIONS

8.1 Regulation statement

This protocol is in accordance with the principles laid down by the 18th world medical assembly (Helsinki 1964) and amendments laid down by the 29th (Tokyo 1975), the 35th (Venice, 1983), the 41st (Hong Kong 1989) and 64th ([versie Fortalezee, 2013](#)) World Medical Assemblies.

This protocol is in accordance with laws and regulations of the country in which the study is performed.

8.2 Recruitment and consent

The informed consent document will be used to explain in simple terms, before persons are entered into this study, the nature, scope and possible consequences of the study. The participant will give consent in writing. The signature of the physician and participant must confirm the participant's consent. The investigator is responsible to see that informed consent is obtained from the participant and for obtaining the appropriate signatures and dates on the informed consent document prior to the performance of any protocol procedure. The signed informed consent forms remain with the investigator.

8.3 Benefits and risks assessment, group relatedness

For the subjects included in this study no direct positive effect can be expected from participation in this study. The burden from participants will be limited to venipuncture and risks are not to be expected.

When the study is closed, data is collected and analyzed we expect to have gained more insight in the pathophysiology of sickle cell disease and sickle cell disease related painful crisis. In the future we can use this knowledge to optimize diagnostics and therapeutic approach of patients with sickle cell disease.

8.4 Confidentiality

Personal information on the patients will be treated confidentially and anonymously according to the 'Wet Bescherming Persoonsgegevens'. All patient names will be kept secret to anyone other than the investigator. Participants will be numbered consecutively in the order in which they are included in the study, the next participant receiving the next available number. The number allotted to them during the study will identify patients throughout documentation and evaluation. The participants will be told that

all study findings will be stored on computer and handled in strictest confidence formulated in the 'Wet op de geneeskundige behandelovereenkomst' (WGBO).

8.5 Compensation for injury

As participating in this trial is considered to be without risk, no insurance policy will be obtained to cover the risks of participating in this trial.

9. ADMINISTRATIVE ASPECTS AND PUBLICATION

9.1 Handling and storage of data and documents

All patient material will be anonymized. Any excess material will be stored to a maximum of 15 years. Only investigators mentioned in this protocol will have access to these samples. The handling of personal data will comply with the Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens, Wbp) and the privacy regulations of the Academic Medical Center. Personal data will be stored anonymously by means of a code number. Only this number will be used in study documentation, reports and publications. Only the person who possesses the code key will know the identity of the persons behind the code numbers. Other persons/authorities that are allowed access to the code key are the members of the Independent Ethics Committee of the Academic Medical Center, members of the research team and the Health Inspection. This might be necessary to inspect the accuracy and quality of the study.

9.2 Data monitoring

Data monitoring will be performed by a certified clinical research associate of our institute. The monitor will compare the data entered into the database with the hospital or clinic records (source documents). The nature and location of all source documents will be identified to ensure that all sources of original data required to complete the database are known to the investigational staff and are accessible for verification. At a minimum, source documentation must be available to substantiate: subject

identification, eligibility and participation; proper informed consent procedures; dates of visits; adherence to protocol procedures; records of safety and efficacy parameters; adequate reporting and follow-up of adverse events; date of subject completion, discontinuation from treatment, or withdrawal from the study, and the reason if appropriate. Direct access to source documentation (medical records) must be allowed for the purpose of verifying that the data recorded in the database are consistent with the original source data.

9.3 Amendments

Amendments are changes made to the research after a favorable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favorable opinion.

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority. Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

9.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of

subjects included and numbers of subjects that have completed the trial, serious adverse events/serious adverse reactions, other problems, and amendments.

9.5 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 90 days. The end of the study is defined as the last patient's inclusion.

In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

9.6 Study organisation

This project will be carried out by the CURAMA programme, which is a collaborative effort between the Department of Hematology, Academic Medical Center (Amsterdam, the Netherlands), the Department of Internal Medicine Slotervaart Hospital (Amsterdam, the Netherlands), the Department of Internal Medicine, Saint Elisabeth Hospital (Curacao, Netherlands Antilles), Red Cross Blood bank Foundation Curacao (Curacao, Netherlands Antilles), the Laboratory of Clinical Thrombosis and Hemostasis in the Department of Internal Medicine, Academic Hospital Maastricht (Maastricht, the Netherlands), the Department of Clinical Chemistry (Groningen, the Netherlands) and the department of Hematology, Erasmus Medical Center (Rotterdam, the Netherlands). CURAMA is embedded in the Antillean Institute of Health Research. For this specific study CURAMA joins forces with the Sanquin Landsteiner Laboratory of the University of Amsterdam, the Netherlands. This laboratory has a long track record in setting up assays to assess inflammation. The laboratory produced a series of monoclonal antibodies to neutrophil proteases, histones, nucleosomes and double stranded DNA. By means of these antibodies a series of assays for extracellular DNA and neutrophil activation have been set up. These assays are operational.

9.7 Ownership of data

CURAMA has the ownership of all data and results collected during this study. In consequence, CURAMA reserves the right to use these data, either in the form of case record forms, or the form of a report, with or without comments or with or without analysis, in order to submit them to health authorities.

9.8 Public disclosure and publication policy

It is our intention that the findings of the study be published in scientific journals and presented at scientific meetings. The responsibility for presentations and/or publications belongs to the CURAMA. Furthermore, a PhD thesis will be written and defended. No restriction regarding the public disclosure and publication of the research data have been, or will be made by the funding agency.

10. REFERENCES

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