PTX-3 and zinc as potential markers of disease severity during sickle cell painful crisis

Acronym: ZIP Trial

PROTOCOL version 3, 6 September 2012
CONFIDENTIAL

Study Coordinator: M. Schimmel, MD, Ph-student
Slotervaart Hospital (Amsterdam)
Department of Internal Medicine
Tel. +31-20-5125194
Marein.Schimmel@slz.nl

Principle Investigator: B.J. Biemond, MD, PhD
Academic Medical Center (Amsterdam)
Department of Haematology
Tel.+31-20-5665785
b.j.biemond@amc.uva.nl

Co-Investigators: J.M.M.B. Otten, MD, PhD
Slotervaart Hospital (Amsterdam)
Department of Internal Medicine
Tel. +31-20-5125194
Hans-Martin.Otten@slz.nl

J.J. Schnog, MD, PhD
Sint Elisabeth Hospital (Curacao)
Department of Hematology/Oncology
Tel: +5999-4321055
jbschnog@me.com

Registration: Academic Medical Center
Trial Office of Department of Haematology
hemat.trial@amc.nl
Phone: +31-20-5665950 of pager 59255
Fax: +31-20-6919743

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 ……..

TABLE OF CONTENTS

1. INTRODUCTION AND RATIONALE 7
2. OBJECTIVES 9
3. STUDY DESIGN 10
4. STUDY POPULATION 10
4.1 Population (base) 10
4.2 Inclusion criteria 10
4.3 Exclusion criteria 10
4.4 Sample size calculation 11
5. METHODS 12
5.1 Study procedures 12
5.1.a Urine and blood samples and laboratory determinations 12
5.1.b Questionnaire 13
5.2 Withdrawal of individual subjects 14
5.3 Replacement of individual subjects after withdrawal 14
5.4 Premature termination of the study 14
6. SAFETY REPORTING 14
6.1 Adverse and serious adverse events 14
7. STATISTICAL ANALYSIS 14
7.1 Descriptive statistics 14
8. ETHICAL CONSIDERATIONS 15
8.1 Regulation statement 15
8.2 Recruitment and consent 15
8.3 Benefits and risks assessment, group relatedness 15
8.4 Confidentiality 15
8.5 Compensation for injury 16
9. ADMINISTRATIVE ASPECTS AND PUBLICATION 16
9.1 Handling and storage of data and documents 16
9.2 Data monitoring 16
9.3 Amendments 17
9.4 Annual progress report 17
9.5 End of study report 17
9.6 Study organisation 18
9.7 Ownership of data 18
9.8 Clinical study report 18
10. REFERENCES 19
11. APPENDIX 21

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 Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Biological markers of sickle cell steady state and painful crisis have been studied extensively (1), but more well performed prospective clinical studies are required to test the clinical value of these markers. Recently we showed that plasma levels of pentraxin-3 (PTX3), an acute phase protein, increased significantly during painful crisis and were associated with the duration of subsequent hospital stay (2). In this prospective cohort study we will evaluate the dynamics of PTX3 and its relation with disease severity during sickle cell painful crisis. Another recent study of our group showed significant increases of the crosslinks pyridinoline (PYD) and deoxy-pyridinoline (DPD) as markers of bone degradation during painful crisis. Regular measurements of these and other markers of bone metabolism such as zinc will be performed to evaluate whether they can be used as parameter of disease severity during painful crisis.

Objective:

- Studying PTX3 dynamics during admission for vaso-occlusive painful crisis
- Studying the association of PTX3 levels with duration of painful crisis, degree of inflammation and acute complications during painful crisis
- Evaluating urinary crosslinks and zinc as markers of disease severity during painful crisis.

Study design: Observational prospective cohort study

Study population: 32 HbSS/HbSβ^0- or HbSβ^+-thalassemia or HbSC patients over 18 years old, in steady state and painful (vaso-occlusive) crisis.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: To gain better insight in the pathophysiology of sickle cell disease and vaso-occlusive crisis, studying relevant biological markers in these patients is essential. This knowledge is required to be able to optimize diagnostic- and therapeutic approach of these patients. The expected burden from this protocol is minimized to venipuncture, the collection of a 24 hour urine sample and a short questionnaire and risks are not to be expected.

1. INTRODUCTION AND RATIONALE

Sickle cell disease is the world's most common hemoglobinopathy. It is an inherited genetic disorder which results in the substitution of valine for glutamic acid as the sixth amino acid of the beta globin chain (3). Upon deoxygenation, sickle hemoglobin (HbS) molecules form polymers which after a few cycles become irreversible causing elongated and sometimes the characteristic sickle shaped cells (4). Besides the changes in red blood cell shape the pathophysiology of sickle cell includes activation of and damage to endothelial cells by sickle red blood cells and activated inflammatory cells. The resulting chronic and recurrent state of inflammation, hypercoagulability and oxidative stress result in microvascular dysfunction, vaso-occlusion and multi-organ damage (5).
Biological markers in sickle cell disease have been studied extensively (1). However, the value of most of these biomarkers remains unclear since most of them have not been prospectively studied in a clinical setting. Finding a (set of) biomarkers that would enable the clinician to predict the clinical course of a patient with a painful crisis would provide the opportunity to optimize therapy and may even prevent sickle cell complications.

Two of our recent studies showed increases during painful crisis in the biomarkers pentraxin-3 (PTX3), an acute phase protein, and the urinary levels of the crosslinks pyridinoline (PYD) and deoxypyridinoline (DPD), which are established bone markers (2;6). PTX3 is an acute phase protein which plays a pivotal role in innate immunity. Unlike the short pentraxin C-reactive protein (CRP), which is produced mainly by hepatocytes, PTX3 is produced and released locally at the site of inflammation. While it was originally identified in vascular endothelial cells and fibroblasts (7;8) it is now known to be expressed by many different cell types and myeloid dendritic cells are a major source of PTX3 (9).
The expression of PTX3 is induced by several factors like micro-organisms, microbial components (like LPS) or pro-inflammatory cytokines like TNF-α and IL-1β (10). PTX3 has shown its clinical relevance in several diseases like sepsis, septic shock, auto-immune disorders and ischemic heart disease (11). In myocardial infarction PTX3 appeared to be a more rapid and more appropriate indicator of myocyte irreversible injury caused by ischemia than CRP. In myocardial infarction levels of PTX3 peaked at 7.5 hours, compared to CRP which peaked at 24 hours after admission (12). In another study in critically ill patients with severe infection, ranging from systemic inflammatory response syndrome (SIRS) to septic shock, PTX3 levels were correlated with disease severity (13). Furthermore, in the event of dengue fever where PTX3 levels were taken in longitudinal series, it was shown that PTX3 levels were elevated early in the course of disease and that higher levels were also associated with disease severity (14).

PTX3 also appears to be a useful marker to predict disease outcome. In patients with myocardial infarction with ST-elevation PTX3 was shown to be the strongest independent predictor of all-cause 3 month-mortality compared to other biological markers such as CRP, creatinine kinase (CK), troponin T(TnT) and N-terminal pro-brain natriuretic peptide (NT-proBNP) (15). Persisting high levels of PTX3 in the first days after onset of severe sepsis or septic shock were found to be an early marker associated with poorer outcome in these patients (16).

The clinical relevance of PTX3 in sickle cell disease, a disease characterized by ischemic inflammation due to recurrent vaso-occlusions, was shown by our above mentioned study. We showed that PTX3 levels in asymptomatic state were comparable with those in healthy controls but increased significantly upon presentation with a painful crisis and were related to the duration of hospital stay (2). While these results suggest PTX3 to be a useful marker of painful crisis severity, a critical remark on this study is that plasma levels of PTX3 were taken only on admission and we were not informed about the onset and duration of complaints.
In this new protocol our aim is to perform serial measurements of PTX3 levels in sickle cell patients admitted with a painful crisis. Thorough information about the duration of pain prior to presentation to the emergency department, information about the location of pain and other subjective complaints will be acquired, using a questionnaire (see appendix 1). Pain severity will be evaluated using a visual analogue pain scale. A relation between PTX3 levels, duration of complaints and possible acute complications during painful crises will be evaluated. Throat swab for PCR on respiratory pathogens will be performed within the first 24 hours of admission to exclude airway infection as the cause of increase in PTX3 levels. Other information regarding infections (blood cultures, imaging and clinical observations) during the painful crisis will be drawn from patient records.
Through serial measurements we will be able to determine how many hours after the onset of painful crisis PTX3 will reach its peak level and whether PTX3 peaks earlier than CRP. Furthermore, the serial PTX3 levels will be correlated with markers of coagulation and sVCAM-1 as a marker of endothelial activation.

Painful crisis is a manifestation of muskulo-skeletal and visceral ischemic damage due to vaso-occlusion. Since vaso-occlusion can cause (reversible) ischemic and reperfusion tissue- and bone damage, an increase in excretion of urinary bone markers during painful crisis is likely to occur. Our above mentioned study showed indeed higher levels of urinary crosslinks in sickle cell patients compared to healthy controls with further increments during painful crisis (6). However, in 4 out of 19 patients with paired data, the urinary crosslinks to creatinine ratios decreased during painful crisis. Also, PYD and DPD after the first night of

hospital admission were not related to the duration of hospital stay or to other parameters of disease severity (17). It is plausible to expect that it takes some time for the crosslinks to appear in urine after the onset of a painful crisis and that therefore the urinary crosslinks might not have reached their peak levels upon presentation at the emergency department. Furthermore in that study we were not informed about the duration of the painful crises prior to the hospital admission. Serial sampling and a detailed questionnaire concerning the onset of complaints, as described above, will give us more insight in dynamics of markers of bone-damage during sickle cell painful crisis.
Urinary zinc concentration is an established marker of bone-turnover and increased levels indicate a higher degree of bone degradation (18-21). In a pilot study we found a highly significant increase in urinary zinc excretion in sickle cell patients during painful crisis compared to patients in steady state (unpublished data). This is remarkable and needs further investigation, since zinc concentrations in plasma and urine have been studied in sickle cell disease previously (22-24), but without consistency in reported data. By performing serial measurements of zinc and correlating these results with the urinary crosslink excretion and clinical parameters of disease severity in this study, we will evaluate whether measurement of urinary zinc can serve as a potential marker of disease severity during painful crisis.

2. OBJECTIVES

Primary Objective:

- To determine when during a painful crisis PTX3, the urinary crosslinks and zinc reach their peak levels.
- To evaluate the relation between the above mentioned markers during painful crisis and clinical parameters of disease severity (pain score, duration of hospitalization, time to next crisis/readmission and other acute complications of painful crisis such as acute chest syndrome).
- To evaluate which of the serial measurements is the best predictor of disease severity.
- To correlate PTX3 levels with markers of coagulation, endothelial activation and inflammation.
- To correlate zinc excretion with the excretion of the urinary crosslinks and clinical parameters of disease severity.

3. STUDY DESIGN

a. Prospective cohort study.
b. Comparison of biomarkers between steady state and painful vaso-occlusive events (within the same patients) in sickle cell disease. In this way patients will provide their own control samples.
c. Performing serial measurements during admission for vaso-occlusive crisis.
d. Documentation of VAS score, requirement of pain medication, the occurrence of complications, hospitalisation duration and, if applicable, time to relapse.

(Time to relapse is defined as readmission for painful crisis.)

4. STUDY POPULATION

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

4.1 Population (base)

Consecutive HbSS, HbSβ^0- thalassemia, HbSβ^+-thalassemia and HbSC patients 18 years or older, both male and female, visiting the emergency room (acute vaso-occlusive painful crisis) of the Academic Medical Center, and 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. Included patients will be re-evaluated >4 weeks after the initial admission at the outpatient clinic in steady-state.

4.2 Inclusion criteria

1. High performance liquid chromatography confirmed diagnosis of HbSS, HbSC, HbSβ^0- or HbSβ^+-thalassemia genotype in SCD patients.
2. ≥ 18 years
3. Written informed consent by the patient.

4.3 Exclusion criteria

1. Pregnancy
2. Active cancer
3. Chronic HIV infection
4. Recent vaso-occlusive crisis (< 3 months)
5. Chronic transfusion therapy
6. Recent blood transfusion (< 3 months)

4.4 Sample size calculation

Data from our previous study concerning PTX3 levels in sickle cell patients were used. (2) Since we are interested in how PTX3 levels correlate with disease severity, our calculation is based on two independent groups whereby the distinction was made between patients who developed a severe complication (acute chest syndrome) during admission (group 1) versus patients who did not develop this complication (group 2).

We used the software from power analysis program G*Power 3.1.3. The following input parameters were chosen:

Power:	80 %
Significance:	0.05
Test family:	t-test
Tail(s):	two
Mean group 1:	4.67
SD group 1:	2.64
Mean group 2:	2.36
SD group 2:	1.67

When entering the above numbers in the program a sample size of **32 patients** was calculated, creating an actual power of 82%.

Weekly approximately 3-4 patients are admitted with a painful crisis in the 2 clinics combined. Based on this number and with the calculated sample size of 32 patients the expected study duration will be approximately 8 to 11 weeks. However, since a small group of sickle cell patients with recurrent crisis accounts for a vast percentage of total admissions for painful crisis (25) and we choose not to include the same patients more than twice in this study (to limit the influence of measurements of a single patient on study results), the expected study duration will exceed this 11 weeks.

5.0 METHODS

5.1 Study procedures

After informed consent is obtained blood will be drawn via venipuncture according to the protocol. Samples will be processed at 4.0 degree Celsius and stored in aliquots at -80 degrees Celsius for further investigations.

5.1.a Urine and blood samples and laboratory determinations.

5.1.a1 Urine

A 24-hour urine collection and a spot urine will be collected at home in steady state. At admission the 24 hour urine collection will be performed the first day. For practical reasons the other days a urine sample will be collected only. Since a circadian rhythm has been described in zinc excretion the first fasting morning void will be collected in admitted patients (26-29). Daily protein excretion will be determined according to local protocols and in urine samples corrected for urinary creatinin excretion.

- Urinary zinc concentration will be determined using an atomic absorption spectrophotometer.
- Urinary crosslinks will be determined by high performance liquid chromatography using commercial agents.

Creatinine excretion will be determined according to standard procedures. Zinc and crosslinks excretions will be expressed as zinc to creatinine and PYD DPD to creatinine ratios respectively, in order to adjust for the degree of urinary concentration. Zinc measurements in the 24 hour urine will be used to exclude potential confounding effects of the adjustment for creatinine excretion in the spot urine.

5.1.a2 Blood

One EDTA tube (4.5 mL), one serum tube (5 mL) and one citrate tube (4.5 mL) will be drawn for this study at day 1, 2 and 3 and before discharge. The total daily amount drawn is 14 mL with a maximum amount of blood drawn of 56 mL. The expected duration of hospitalisation in the case of a painful crisis will normally not exceed 3-4 days. However, in the case of an unexpected extended hospitalisation, for example when complications occur, the same amount of blood will be drawn maximally one day extra, preferably on day 5.

At the control visit at the ambulant clinic with the haematologist/internal medicine physician (at least 4 weeks after the admission for painful crisis) again one EDTA tube (4.5 mL), one serum tube (5 mL) and one citrate tube (4.5 mL) will be drawn

from the patients. The venipuncture will be combined with the regular venipuncture required for the routine ambulant medical check-up.
The serum tube will be allowed to clot for 30 minutes whereupon blood samples will be centrifuged at 3000 g at 4 ºC for 15 minutes.

Processing

EDTA blood (1 x 4.5 cc)

- The prototypic long pentraxin3 (PTX3) will be determined with a non-commercial Enzyme Linked Immunosorbent Assay (ELISA) based on the monoclonal antibody MNB10 and rabbit anti-serum as described by others (13;15;30).

Serum (1 x 5 cc)

- Serum levels of high sensitive CRP (hCRP) will be determined with ELISA according to local protocols.
- Zinc concentration will be determined using an atomic absorption spectrophotometer.
- sVCAM-1 will be determined with ELISA methods.

Citrated plasma (1x 4.5 cc)

- Pro-thrombin fragments (F1.2), D-dimer levels, protein S (free and total) and C activity, vWF-Ag activity will be determined as described previously.

5.1.b

After informed consent, from all patients a questionnaire will be obtained as soon as possible after admission for painful crisis by attending nurse, physician or investigator.
At each moment of sampling during admission patients will be asked to quantify their pain on a visual analog pain scale (appendix 1).

5.2 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

5.3 Replacement of individual subjects after withdrawal

To achieve the number of patients required as calculated new patients will be included in the study after subject withdrawal until the required number of subjects is obtained.

5.4 Premature termination of the study

No premature termination of the study is expected.

6. SAFETY REPORTING

6.1 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 SPSS 18.0 for statistical analysis.

The non-parametric Mann-Whitney U (or when necessary Kruskal-Wallis) test will be applied for between group comparisons. Spearman rank correlation will be used to study the relationship between different laboratory results. 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, revision of 2008) and amendments laid down by the 29th (Tokyo 1975), the 35th (Venice, 1983) and the 41st (Hong Kong 1989) 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, the 24 hour collection of urine and the cooperation in a questionnaire and risks are not to be expected.

When the study is closed, data is collected and analyzed we expect we have gained more insight in the pathophysiology of sickle cell disease and 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 Persoongegevens'. 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, rapports 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.

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.

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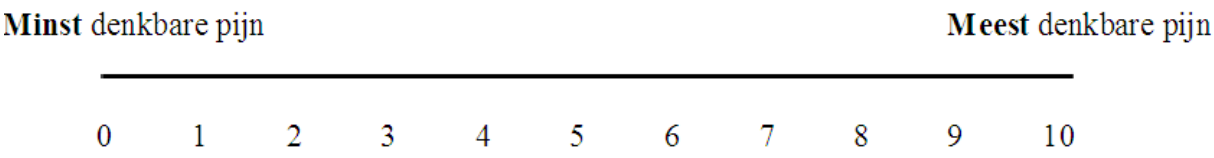
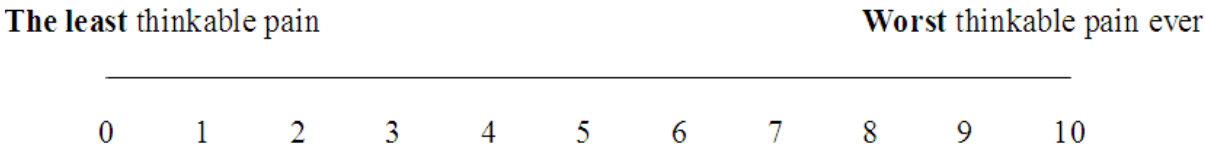
Appendix 1. Visual analogue pain scale (VAS score).



Zet een kruisje op die plek op de lijn zoals u pijn ervaart.



Mark the position on the line which corresponds to the pain you experience.