SSC Subcommittee Project/Collaborative Project

Towards standardization of Neutrophil Extracellular Trap (NET) measurements in patient samples

Vascular Biology Subcommittee

Person responsible (Chair / Principal Investigator): Kimberly Martinod

Description Abstract

Without a doubt, neutrophils and especially neutrophil extracellular traps (NETs) have led to a paradigm shift in the field of thrombosis and hemostasis[1]. NETs comprise a new mechanism by which neutrophils contribute to the innate immune response by releasing their chromatin lined with granular components, creating fibrous DNA nets with antimicrobial properties[2]. NETs are capable of binding platelets and red blood cells, promote fibrin deposition and bind plasma proteins important for platelet adhesion and thrombus propagation[3]. In the past 8 years, there have been more than 280 publications on NETs in thrombosis, highlighting this as a "hot topic." However, the number of publications using non-specific tools to measure NETs is alarming, and the field suffers from a lack of standardization of methodology. Additionally, it is now becoming clear that there are several distinct pathways that can lead to release of extracellular traps (ETs), and current approaches may underestimate or miss the identification of NETs being formed via distinct signaling pathways (i.e. via histone citrullination or via NADPH oxidase), or of ETs released from cell types other than neutrophils. NETs could very well be predictive of thrombotic events[4,5], and are therefore of great interest to study in a translational and also clinical setting.

The aim of this project is therefore to begin a larger standardization project measurement of NETs in the field of thrombosis and haemostasis. Ideally, this will also be broadly applicable to other fields in which NETs are studied, and thus could also be of broad impact beyond the ISTH. This will be done by first surveying researchers on their current approaches to measuring NETs in research settings. From this, we will select a panel of assays that cover NETs measurement by various approaches, and send a set of consistently prepared samples to 10-15 labs for measurement of NETs using their current approach(es). Based on the results of this study, we will prepare a guideline document bringing together a feasible panel of assays, and ask the same labs to again measure a consistently prepared sample using the reagents/assays that we will provide to them. From this information, we should be able to prepare an official SSC communication on measurement of NETs in patient samples. This will provide an important basis for future application of NETs measurements in clinical settings. This is likely to be of interest in a broad range of pathologies with NETs-involvement, including venous thromboembolism, myocardial ischemia, sepsis, cancer-associated thrombosis, and many others. This project also has the potential to put the field of thrombosis and haemostasis at the forefront of NETs measurement by recommending a well-characterized panel of assays that should conclusively identify a "NET profile" in a given sample.

Design and methodology (Data expected to collect, sample size and statistical analysis):

Aim 1 (Year 1)*: *Survey to identify groups that are currently measuring NETs in either basic research or clinical research settings.

- What do they measure? NETs in vitro/ex vivo? NET biomarkers in plasma? NETs by histology?

- How do they measure this? Approaches: microscopy, flow cytometry, ELISA. Targets: nucleosomes, citrullinated histones, MPO/DNA complexes? Novel approaches?
- How are samples collected/stored? Plasma/serum, anticoagulant, fixation/processing? Neutrophil isolation approach? Pre-processing? i.e. DNase treatment
- In what context? Diseases, in vitro stimuli?

From this we should be able to identify approaches that cover different aspects of NET biology (citrullinated histones, MPO-DNA, neutrophil elastase?). This will be used to design the second aim, where the respondents will be able to show (with data) their methodologies on a standardized test sample.

The survey template has been attached as an appendix to this application. The PI is currently awaiting approval from the KU Leuven ethics board and feedback on GDPR compliance prior to launching the survey (expected September 2019).

Aim 2 (Year 1+2)**: **Sending consistently prepared samples to various labs to test using their current setup
We will send the following plasma samples which have been experimentally produced and added to a biological matrix (from a human plasma pool) to up to 15 labs for them to test their methodologies to quantify/identify NETs. This standardized NET sample is designed to cover multiple pathways of NET formation and will be optimized based on the survey results. This is described in more detail in the general methods section below.

- High NETs, low cell-free DNA/nucleosomes (positive control)
- Low NETs, high cell-free DNA/nucleosomes (false positive)
- Low NETs, low cell-free DNA/nucleosomes (negative control)
- High NETs, high cell-free DNA/nucleosomes (possible interference?)

From this, we expect to have 12-15 groups providing data. As the investigators will be blinded as to the content of the samples, we should be able to feasibly discern between approaches that give a satisfactory measurement of NETs (compared to nucleosome levels).

Aim 3 (Year 2+3)**: **Preparing guidelines for measurement of a "NET profile" using a combined approach
Based on the results from Aim 2, we will select a panel of assays to be run by all groups on the same standardized samples.

Aim 4 (Year 3)**: **Sending the NET standard to test the proposed guidelines in the participating labs
This time, we will provide all of the reagents and ask the investigators to perform the experiment in (potentially) a different manner than they had been performing the assay before. This will give us information as to the reliability of the assays in determining a NET profile that can be accurately measured by different research groups. From this information, we should be ready to prepare an SSC Official Communication on the standardized measurement of NETs in patient samples.

General methodology:

Neutrophil isolation.

Peripheral blood neutrophils are isolated from anticoagulated blood using 2 density centrifugation steps: Histopaque followed by a Percoll gradient[6]. These methods result in minimal pre-activation and yield enough cells to perform in vitro NETosis assays by microscopy. Cell purity will be confirmed by flow cytometry and these cells will be used in the NET assays below for generation of the standard sample.

NET formation for standard preparation, and validation.

As this is a standardization study, we aim to prepare a representative set of samples in vitro that will be used to test methodologies in different labs. As such, we will need to prepare samples with a known quantity of NETs arising from different stimulation sources, as well as from different patients, and create a pool that can be divided and distributed to several labs for testing as part of the 2nd and 4th specific aims. This will be done by isolation of peripheral blood neutrophil from 20 healthy volunteers, and stimulation of isolated cells using lipopolysaccharides (potent NET inducer representing bacterial infection), PMA (potent ROS-dependent NET inducer), or HMGB1 (mimicking platelet-induced NETs). This pool will be prepared at KU Leuven in the supervisor-spokesperson's lab. A sample size of 20 should be large enough to account for differences in extent of NET production among different donors, and the inclusion of multiple stimuli will allow for representation of all NETosis pathways in the stock sample. We will then include increasing amounts of non-specific nucleosomes within the samples in order to discern the specificity of the NETs methods being tested vs. non-NETosis cell death identification.

Study population (Inclusion, exclusion, eligibility) (patient population; recruitment of participating institutions/physicians and subjects; minimum number needed; expected number):

As this is a standardization study, we aim to prepare a representative set of samples in vitro that will be used to test methodologies in different labs. As such, we will need to prepare samples with a known quantity of NETs arising from different stimulation sources, as well as from different patients, and create a pool that can be divided and distributed to several labs for testing as part of the 2nd and 4th specific aims. This will be done by isolation of peripheral blood neutrophil from 20 healthy volunteers, and stimulation of isolated cells using lipopolysaccharides (potent NET inducer representing bacterial infection), PMA (potent ROS-dependent NET inducer), or HMGB1 (mimicking platelet-induced NETs). This pool will be prepared at KU Leuven in the Principal Investigator's lab. A sample size of 20 should be large enough to account for differences in extent of NET production among different donors, and the inclusion of multiple stimuli will allow for representation of all NETosis pathways in the stock sample. We will then include increasing amounts of non-specific nucleosomes within the samples in order to discern the specificity of the NETs methods being tested vs. non-NETosis cell death identification.

For recruitment of the participating institutions/groups to be part of the test group, ideally we would like to include as many groups as possible, but as the cost of sending reagents to each lab is high, we will limit this to a maximum of 15 groups. The minimum number of labs needed will be dependent on the answers that we obtain from the initial survey. If there are many distinct methodologies, then we will likely need 15 to obtain meaningful data across several different techniques. If the methodology is consistent among groups (i.e. all groups use commercial ELISAs with low standard deviation), then 7 groups should be sufficient (for an effect size of 2.0

with 90% power). However, we expect there to be different approaches, particularly with respect to the measurement of citrullinated histones.

The list of confirmed labs willing to participate in this study, after an initial recruitment period at the ISTH 2019 Melbourne meeting, is as follows:

Charlotte Thalin, Danderyd Hospital, Sweden
Luise Erpenbeck, University Medical Center Göttingen, Germany
Patricia Liaw, McMaster University, Canada
Johannes Thaler, Medical University of Vienna, Austria
Haruchika Masuda, Tokai University, Japan
Chloé James,University Hospital of Bordeaux, France
Farzaneh Kordbacheh, Australian National University, Australia
Nigel Mackman, University of North Carolina, USA
Yamini Bynagari, Machaon Diagnostics, USA

Expected timeline:

Time	Year 1, Q1	Q2	Q3	Q4	Year 2, Q1	Q2	Q3	Q4	Year 3, Q1	Q2	Q3	Q4
Setup												
Launch												
Duration												
Finalization/ analysis												
Reporting			ISTH 2020				ISTH 2021				ISTH 2022	SSC Commu -nication

Aim 1 (Year 1):
Task 1.1: Design survey to cover important aspects of NET measurements in thrombosis and hemostasis.
Task 1.2: Advertise and send survey to groups that are currently measuring NETs in either basic research or clinical research settings.
Task 1.3: Compile results from the survey and identify additional labs willing to participate in the standardization study
Aim 2 (Years 1+2):
Task 2.1: Preparation and validation of the “NET standard” sample;
Task 2.2: Validation of the different NET inducers used for the standard sample preparation, by fluorescence microscopy
Aim 3A (Year 1+2):
Task 3.1: Sending consistently prepared samples to various labs to test using their current setup
Task 3.2: Collecting and analyzing data from the test labs
Aim 3B (Years 2+3):
Task 3.3: Preparing guidelines for measurement of a “NET profile” using a combined approach
Task 3.4: Communicating guidelines to participating teams to reach consensus
Aim 4 (Year 3):
Task 4.1: Sending standard samples to test the guidelines in the participating labs
Task 4.2: Validating results from NET profile measurements in the test labs
Task 4.3: Preparation of an official SSC communication for the ISTH on measurement guidelines for plasma samples in thrombosis/hemostasis

Expected outcomes (ie. publications):

Publication type (SSC Communication, Guidance document or original article):

1. ISTH RedCAP survey in first year to recruit participating labs– aiming for 40-50 labs to respond to the survey, and 15 labs to participate in the initial comparative study. Presentation of survey results at ISTH 2020 in SSC Vascular Biology session.
2. Presentation of recommended parameters at ISTH 2021 on identification/characterization of NETs for establishing a "NET profile," in year 2 based on survey results and results of Aim 2
3. SSC Official Communication on measurement of NETs in plasma samples expected as a result of Aim 4; preparation for submission by the end of year 3

Description of project set/up and management, needed infrastructure and resources (summary):

Project setup/management:

This project aims to define a standard panel of assays to be used for identification of NETs within patient samples. It has become clear that one test is not sufficient; particularly not tests that only measure cell-free DNA or nucleosomes. Approaches such as measuring MPO-DNA complexes or citrullinated histones may yield nuanced information as to the signaling pathways that are activated to induce NETosis, and thus it will be likely necessary to include a combination of assays to provide the full "NET profile." The first two aims of this proposal are to survey the current researchers in the ISTH and to use a standardized sample prepared by the PI's lab to identify the most promising candidates for this NET profile. For the third and fourth aims, the management structure will broaden to include one "expert" lab from each assay being run. These expert labs will then have the task of training the rest of the groups in their particular assay, under the management of the PI. These respective labs will also be involved in the data analysis from the collected data and thus also in the preparation of the SSC Official Communication publication in the Journal of Thrombosis and Haemostasis.

Recruitment of study participants via online survey:

We will publish an open call via the ISTH for participation in the online survey, seeking groups that are currently measuring NETs in their research. This approach is preferred to hand-selecting labs by the supervisor-spokesperson, as this would introduce experience bias and would be counter to the idea of a standardization study, which should be open to all those willing to participate. The survey will include questions about the types of measurements and types of samples that the groups are measuring. It will also ask if they would be willing to participate in the larger standardization study, and to commit to participation for the entire study period. For recruitment of the participating institutions/groups to be part of the test groups, ideally we would like as many groups as possible, but as the reagents to be sent to each lab are expensive, we will limit this to a maximum of 15 groups. The minimum number of labs needed will be dependent on the answers that we obtain from the initial survey. If there are many distinct methodologies, then we will likely need 15 to obtain meaningful data across several different techniques. If the methodology is consistent among groups (i.e. all groups use commercial ELISAs with low standard deviation), then 7 groups should be sufficient (for an effect size of 2.0 with 90% power). However, we expect there to be different approaches, particularly with respect to the measurement of citrullinated histones.

Infrastructure:

The labs involved in this study will need to have capability to measure NETs in either ELISA or fluorescence conditions. This is done using a standard fluorescence/ absorbance multiwell-plate reader that should be commonly found in most research lab settings, but specific filters may be needed. While some labs may be able to participate in Aim 2 of the study, they may not be able to complete all assays in Aim 4. This will be taken into consideration when choosing the expert labs. With the equipment requested in this project, the PI's lab will have the infrastructure in place to perform all potential NET measurements.

Resources:

The potential to advertise and recruit participants in the initial survey will be needed. The survey will be conducted using the ISTH RedCAP platform and communicated on the Vascular Biology SSC website and in the ISTH newsletter. In addition, the PI will reach out to research groups with Journal of Thrombosis and Haemostasis publications on NETs in thrombosis, or in person in the SSC session at the ISTH2020 and ISTH 2021 congresses. We will also use social media to disseminate the survey, recruit additional labs to participate in the study, and communicate the results and progress of the study to the scientific community.

References:

1. Martinod K and Wagner DD. Thrombosis: tangled up in NETs. *Blood*. 2014;**123**:2768-76.
2. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y and Zychlinsky A. Neutrophil extracellular traps kill bacteria. *Science*. 2004;**303**:1532-5.
3. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr., Wrobleski SK, Wakefield TW, Hartwig JH and Wagner DD. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A*. 2010;**107**:15880-5.
4. Mauracher LM, Posch F, Martinod K, Grilz E, Daullary T, Hell L, Brostjan C, Zielinski C, Ay C, Wagner DD, Pabinger I and Thaler J. Citrullinated histone H3, a biomarker of neutrophil extracellular trap formation, predicts the risk of venous thromboembolism in cancer patients. *J Thromb Haemost*. 2018;**16**:508-18.
5. Wolach O, Sellar RS, Martinod K, Cherpokova D, McConkey M, Chappell RJ, Silver AJ, Adams D, Castellano CA, Schneider RK, Padera RF, DeAngelo DJ, Wadleigh M, Steensma DP, Galinsky I, Stone RM, Genovese G, McCarroll SA, Iliadou B, Hultman C, Neuberg D, Mullally A, Wagner DD and Ebert BL. Increased neutrophil extracellular trap formation promotes thrombosis in myeloproliferative neoplasms. *Sci Transl Med*. 2018;**10**.
6. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V and Zychlinsky A. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007;**176**:231-41.

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Appendix: Study survey

You are receiving a questionnaire about neutrophil extracellular traps (NETs).

The purpose of this survey is to evaluate current practice in research involving NETs among researchers in the field of thrombosis and haemostasis.

We invite you to answer the following questions. It will take you 5-7 minutes to answer the questionnaire. Your participation is very important in order to evaluate feasibility for a standardization study proposed by the Vascular Biology SSC.

Thank you in advance.

By completing this field with your email you are agreeing to participate in the study as mentioned above.
All data are confidential and will be used for scientific research purposes and will be published at the end of the research.

Please enter your email address to start:
* must provide value

What is your job title/function?
PhD student
Postdoctoral fellow/researcher
Laboratory technician
Instructor
Assistant Professor (or equivalent)
Associate Professor (or equivalent)
Professor
Other (please explain)

In which type of institute is your research group located?
Academic (university)
University Hospital
Clinical
For-profit company
Other (please explain)

How many years of laboratory experience do you (personally) have?
0-3 years
3-6
6-12
more than 12

How many years of experience do you (personally) have working with NETs?
Less than 1
1-2

3-5
5-10
More than 10

Which disease settings/ research focuses does your lab investigate? (please explain in 1-2 sentences)

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In which species do you investigate NETs? (select all that apply)

Human
Mice
Rats
Rabbits
Sheep
Cows
Pigs
Non-human primates
Zebrafish
Other (please explain)

In which biological materials do you investigate NETs? (select all that apply)

Isolated neutrophils
Whole blood
Blood plasma
Blood serum
Bronchoalveolar lavage
Peritoneal fluid
Tissue samples (fresh frozen)
Tissue samples (fixed)
Other (please explain)
None (I do not study NETs in biological materials)

For cell isolation: what is the source of neutrophils you use? (select all that apply)

Blood
Bone marrow
Spleen
Other (please explain)
None (I do not isolate neutrophils as part of my research)

For Blood: which anticoagulant do you use for blood draws?

EDTA

Sodium citrate
Acid citrate dextrose (ACD)
Heparin
Serum collection tube
No anticoagulant
Other (please explain)
Not applicable

How much time elapses before you begin sample preparation?

Sample preparation is immediate
Less than 30 minutes
Less than 2 hours
More than 2 hours (same day)
Overnight or longer
Not applicable

How do you store your samples for the long-term? (select all that apply)

Room temperature
4 degrees
-20 degrees
-80 degrees
Not applicable

How long are your samples stored before analysis (select all that apply)

Less than one day
1-7 days
7-14 days
14-30 days
More than 30 days
Not applicable

Which methodological approaches/targets do you use to identify NETs? (select all that apply)

Ex vivo neutrophil stimulations paired with microscopy (not including immunostaining)
Ex vivo neutrophil stimulations paired with microscopy (including immunostaining)
Ex vivo neutrophil stimulations paired with flow cytometry
Ex vivo neutrophil stimulations paired with plate-based assays (fluorescence, luminescence, colorimetric, etc)
MPO-DNA complexes
Citrullinated histones

NE-DNA complexes
Nucleosome ELISA (for example, Cell Death Detection Kit)
ELISA (other)
DNA-dyes (cell membrane permeable)
DNA-dyes (cell-membrane impermeant)
Histology (not including immunostaining)
Histology (including immunostaining)
Western Blot
RT-PCR
Commercial NETosis assay kit
Other (please explain)
Not applicable (I do not identify NETs as part of my research)

If using immunostaining as part of your experimental approaches, against which epitopes are the antibodies you use to identify NETs?
DNA
DNA-histone
Histone
Neutrophil elastase
Myeloperoxidase
Citrullinated histones
Other (please expand)
Not applicable (I do not perform immunostaining to identify NETs)

Which methodological approaches do you use to quantify NETs? (select all that apply)
Ex vivo neutrophil stimulations paired with microscopy (not including immunostaining)
Ex vivo neutrophil stimulations paired with microscopy (including immunostaining)
Ex vivo neutrophil stimulations paired with flow cytometry
Ex vivo neutrophil stimulations paired with plate-based assays (fluorescence, luminescence, colorimetric, etc)
MPO-DNA complexes
Citrullinated histones
NE-DNA complexes
Nucleosome ELISA
ELISA (other)
DNA-dyes (cell membrane permeable)
DNA-dyes (cell-membrane impermeant)
Histology (not including immunostaining)
Histology (including immunostaining)
Western Blot

RT-PCR
Commercial NETosis assay kit
Other (please explain)
Not applicable (I do not quantify NETs in my research)

Do you perform digestion on NETs samples prior to analysis?
Yes
No
Sometimes

If yes, which of the following do you use?
DNase(s)
MNase(s)
Restriction enzymes
Other (please explain)

Would you be interested in participating in a standardization study for quantification of NET biomarkers in human plasma?
Yes
No

If yes, would you be interested in participating for (select all that apply):

A single measurement using your existing protocols
A single measurement for validation of a proposed standardized methodology
The entire study (repeated measurements in your laboratory, up to 3 years)

Would you be interested in providing reagents for a standardization study for quantification of NET biomarkers in human plasma?

Yes
No

If yes, please indicate which types of reagents you would be able/willing to provide to contribute to the study. (Please explain)

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Do you have any further questions/comments/suggestions? If so, please expand in the below text box.

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