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J Thromb Haemost. Author manuscript; available in PMC 2022 January 03.

Published in final edited form as:

J Thromb Haemost. 2020 October ; 18(10): 2732–2743. doi:10.1111/jth.15003.

Quantification of citrullinated histones: Development of an improved assay to reliably quantify nucleosomal H3Cit in human plasma

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Abstract

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AUTHOR CONTRIBUTIONS

CT and ALJ designed and supervised the study. CT, KA, NWH, MRM, AR, MD, MFW, and HW performed experiments and analyzed data. SL, JMB, MJM, MAC, SAH, SSB, DFN, and SZS prepared essential materials to conduct the experiments. ZWS, NSK, MCK, MWC, YH, NM, and HW guided project conception and experimental direction. All authors were involved in interpretation of the data. CT and ALJ wrote the manuscript with input from all authors. All authors approved the final manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information section.

CONFLICTS OF INTEREST

EpiCypher is a commercial developer and supplier of platforms similar to those used in this study: recombinant semi-synthetic nucleosomes (versaNucs, dNucs), antibody validation platforms, and nucleosome-based H3Cit assays (eg EpiCypher Catalog No. R&D143001). NWH, MRM, MCK, MWC, and ALJ are inventors on pending patents covering use of recombinant nucleosomes carrying histone or DNA modifications for antibody validation and assay quantification (PCT/US2019/020283). CT, KA, AR, MD, YH, SSB, DFN, NSK, SZS, SL, NM, and HW declare no conflicts of interest.

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Background: Recent data propose a diagnostic and prognostic capacity for citrullinated histone H3 (H3Cit), a marker of neutrophil extracellular traps (NETs), in pathologic conditions such as cancer and thrombosis. However, current research is hampered by lack of standardized assays.

Objectives: We aimed to develop an assay to reliably quantify nucleosomal H3Cit in human plasma.

Methods: We assessed the common practice of in vitro enzymatically modified histone H3 as calibration standards and the specificity of available intrapeptidyl citrulline antibodies. Based on our findings, we developed and validated a novel assay to quantify nucleosomal H3Cit in human plasma.

Results: We show that enzymatically citrullinated H3 proteins are compromised by high enzyme-dependent lot variability as well as instability in plasma. We furthermore demonstrate that the majority of commercially available antibodies against intrapeptidyl citrulline display poor specificity for their reported target when tested against a panel of semi-synthetic nucleosomes containing distinct histone H3 citrullinations. Finally, we present a novel assay utilizing highly specific monoclonal antibodies and semi-synthetic nucleosomes containing citrulline in place of arginine at histone H3, arginine residues 2, 8, and 17 (H3R2,8,17Cit) as calibration standards. Rigorous validation of this assay shows its capacity to accurately and reliably quantify nucleosomal H3Cit levels in human plasma with clear elevations in cancer patients compared to healthy individuals.

Conclusions: Our novel approach using defined nucleosome controls enables reliable quantification of H3Cit in human plasma. This assay will be broadly applicable to study the role of histone citrullination in disease and its utility as a biomarker.

Keywords

cancer; citrullination; enzyme-linked immunosorbent assay; extracellular traps; histones; nucleosomes

1 | INTRODUCTION

Histones are positively charged proteins that package negatively charged DNA into nucleosomes, the basic subunits of chromatin. Multiple histone residues are decorated by post-translational modifications (PTMs),[1] including one or a combination of methylation, citrullination, acylation, phosphorylation, sumoylation, ubiquitination, adenosine diphosphate (ADP) ribosylation, and biotinylation, among others. These PTMs are key elements in the regulation of chromatin structure and gene expression.[2] In addition to their nuclear function, histones are emerging as critical damage-associated molecular pattern molecules (DAMPs). When translocated from the nucleus and expelled into the extracellular space, they contribute to inflammatory,[3,4] toxic,[5] and pro-thrombotic[4,6] pathways. Levels of histones and histone PTMs in blood are increased in several diseases,[7] and may even serve as novel therapeutic targets.[8,9]

Citrullination of histone H3 (H3Cit) in particular is gaining increasing interest due to its central role in the release of neutrophil extracellular traps (NETs).[10] By releasing web-like

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NETs consisting of decondensed chromatin coated with granule proteins, neutrophils trap and disarm pathogens as part of the innate immune system.[11] However, NETs can also propagate inflammation and have been implicated in a variety of non-infectious diseases such as cancer,[12–18] autoimmunity,[19,20] and thrombosis.[21,22] A prerequisite of one form of NET formation is activation of the calcium-dependent peptidyl-arginine deiminase 4 (PAD4) enzyme, which mediates the conversion of positively charged arginine residues to citrulline on histone tails.[10] While PAD4 is known to hypercitrullinate multiple sites on histone tails including arginine residues on histones H3, H4, and H2A, PAD4-mediated citrullination of histone H3 at arginine residues 2, 8, and 17 (H3R2,8,17Cit) is predominantly associated with NET formation and associated pathologies (Table 1; Figure 1A). Hypercitrullination weakens the histone interaction with DNA, thus unfolding the tightly packed chromatin[10] (Figure 1B) and contributing to NET formation (Figure 1C). H3Cit is therefore considered a useful marker of NET formation.[23–34]

Various enzyme-linked immunosorbent assays (ELISAs) quantifying H3Cit in plasma samples have been described, revealing clear elevations and a prognostic capacity of circulating H3Cit in several pathologic conditions (Table 1). In particular, numerous vascular disorders, including ischemic stroke, trauma, sepsis, myocardial infarction, venous thromboembolism, thrombocytopenia, and aortic stenosis demonstrate elevations in H3Cit. Further, H3Cit-induced NET formation has been shown to play a causative role in rodent models of thrombosis, as PAD4 genetic depletion or inhibition can reduce thrombus formation.[33,35] Thus, there is an urgent need to accurately and reliably quantify H3Cit in order to study its biological function and utility as a biomarker for vascular and other disorders.

As with all rapidly expanding fields, a lack of standardized assays can hamper data interpretation, with the reported levels of circulating H3Cit varying between studies (Table 1). This questions the specificity and reproducibility of the currently used ELISAs, which employ histone proteins for assay development and standardization. Such an approach is problematic because histones are highly unstable in blood.[36] Furthermore, in vitro enzymatically modified histones can demonstrate a high degree of lot variability, undermining their use as calibration standards. In contrast to histones, nucleosomes are relatively stable in circulation, where they are released during cell death and in pathological conditions such as cancer, autoimmune disease, sepsis, trauma, stroke, and deep vein thrombosis.[37–39] We thus examined semi-synthetic nucleosomes containing the PTM of interest for use as calibration standards, because they are homogenous, fully characterized, and display high consistency across lots.

As an additional complexity, widespread problems with antibody specificity, lot-to-lot consistency, and lack of application-specific validation contribute to the reproducibility crisis.[40] Histone PTM antibodies in particular are notoriously variable in capability[41,42] rendering it critical to evaluate their specificity in the context of the physiological target epitope (eg, linearized histone peptide compared to full-length histone protein or three-dimensional nucleosome). Such issues must be rigorously addressed to ensure reliable quantification of H3Cit.

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The development of nucleosome-based ELISAs using specific antibodies to quantify histone PTMs in human blood samples is urgently needed, and here we have developed such an assay to quantify nucleosomal H3Cit. The incorporation of characterized renewable reagents and extensive optimization of each assay step ensures the accurate and reliable quantification of nucleosomal H3Cit levels in human plasma samples and will support comparisons between different laboratories and disease settings. Accurate and specific methodology is crucial in moving the field forward, and this work demonstrates the need for rigorous, platform-appropriate validations.

2 | METHODS

2.1 | Determination of inter-lot variability of in vitro enzymatically modified histones versus semi-synthetic designer nucleosomes (dNucs) and the stability of histones versus nucleosomes in plasma

The inter-lot variability of in vitro PAD4 citrullinated histone H3 was determined as previously described.[43] Briefly, human recombinant PAD4 (Cayman #10500) and human recombinant histone H3 (Cayman #10263) were mixed at 2.5 U of PAD4 per microgram of histone and incubated at 37°C for 1 hour in reaction buffer (50 mmol/L Trizma base [pH 7.6], 4 mmol/L $CaCl_2$, 4 mmol/L DTT, 1 mmol/L PMSF). A final concentration of 10 000 ng/mL in vitro enzymatically citrullinated H3 was obtained by adding PBS supplemented with 1% bovine serum albumin (BSA) and 1% glycerol. Two lots of in vitro citrullinated H3 were created using two different lots of PAD4 (lot numbers 0470448–1 and 0492528–1) and aliquoted at −80°C. For comparison, three independent lots of human semi-synthetic H3R2,8,17Cit designer nucleosomes (dNucs; EpiCypher #16–1362; "lot 1" refers to commercial lot number 18126001; "lot 2," 18288002; "lot 3," 20080003) were created and aliquoted at −80°C. Inter-lot variability of in vitro enzymatically citrullinated histone H3 and H3R2,8,17Cit dNucs were assessed by ELISA and presented as F(DFn, DFd). Stability in plasma was assessed by spiking semi-synthetic H3R2,8,17Cit histones and nucleosomes into 100% human plasma. The plasma was then diluted down to 5% for ELISA detection of H3Cit and samples normalized to standards prepared in 5% human plasma.

2.2 | Antibody testing

For brevity, the target antigen, vendor, catalog no., lot no., and clonality for each antibody in this study are denoted by a unique ID (Table 2). To test the specificity of these antibodies in a nucleosome context, we assembled a panel by individually ligating histone H3 tail peptides (aa1–31 [A29L] with a modification of interest) to a H3 tailless nucleosome precursor (H3.1N 32 assembled on 147bp 5′ biotinylated 601 DNA). The resulting nucleosomes (EpiCypher versaNuc®) were confirmed to contain <5% free DNA, undetectable levels of peptide precursor, and 90% full-length H3.1 with the modification(s) of interest (Figure S1 in supporting information). The final versaNuc panel included an unmodified control and citrulline at one or a combination of H3R2, H3R8, and/or H3R17. These were individually coupled to saturation on different MagPlex avidin bead regions (Luminex), multiplexed at equivalence, and used to query the binding specificity of twelve antibodies to intrapeptidyl citrulline (see Figure 3A), each at three different concentrations (1:250, 1:1000, 1:4000). Antibody binding to each nucleosome-bead conjugate was detected by anti IgG*PE, read

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on a Luminex FlexMap-3D, and data presented as % on-target signal for the best dilution of each antibody. Pan-peptidyl citrulline antibodies were normalized to the H3R2,8,17Cit nucleosomes as the on-target signal. Inter-lot variabilities of abTriCit-1b, abTriCit-1c, abR8Cit-1a, and abR8Cit-1b were further assessed by ELISA and presented as F(DFn, DFd).

2.3 | Samples

Blood samples were obtained from 40 cancer patients and 30 age-matched healthy individuals enrolled in an ongoing study investigating the role of NETs in cancer (Ethical Review Authority approvals 2015/1533–31/1, 2016/359–32, 2016/1102–32, 2016/2051–32/1, 2017/1837–32 2017/2160–31-1, and 2018/2742–31). Patient characteristics are presented in Table S1 in supporting information. Plasma samples were prepared from citrated whole blood following immediate centrifugation for 20 minutes at 2000 × *g* at room temperature (RT), stored at −80°C, and thawed on ice at time of analysis. All procedures were in accordance with the declaration of Helsinki. All patients and healthy controls signed written informed consent, and the study was approved by the regional ethical review board.

2.4 | H3Cit-DNA ELISA protocol and step-by-step standardized validation

All reagents were equilibrated to RT. Calibration standards were prepared from H3R2,8,17Cit dNucs (EpiCypher #16–1362) in a two-fold dilution series at 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, and 0 ng/mL in standard diluent (50 mmol/L Tris-HCl pH 7.5, 300 mmol/L NaCl, 0.01% [w/v] BSA, 0.01% [v/v] Tween-20). High Bind Clear 96-well microplates (Thermo Fisher Scientific #3855) were coated with abR8Cit-1c at a concentration of 5 μg/mL overnight at 4°C. After three washes (phosphate buffered saline [PBS] with 0.05% [v/v] Tween-20), plates were blocked with 300 μL PBS supplemented with 1% (w/v) BSA for 1.5 hours at RT. Following three washes, 20 μL plasma or calibration standard was added with 80 μL of detection antibody anti-DNA POD (Cell Death Detection ELISA PLUS kit, Roche #11 774425001) and incubated for 2 hours at RT on a 300 rpm shaker. After three washes, 100 μL horseradish peroxidase (HRP) substrate (Thermo Fisher Scientific #34028) was added to each well and incubated in the dark for 10–15 minutes (or until the highest standard had developed a dark blue color). Optical density (OD) was measured at 650 nm using an automatic plate reader (Tecan Infinite Pro). Concentrations were extrapolated versus nominal log concentration applying a sigmoidal 4PL regression to the calibration curve. Concentrations of the standard curve, incubation times, and dilutions of samples and antibodies were optimized in preliminary experiments.

Performance metrics of the assay were methodologically assessed as per standard operating procedures.[44] Trueness and uncertainty could not be assessed due to the lack of a certified reference method. The working range was defined by the lower and upper limits of quantification (LLOQ and ULOQ, respectively) and calculated by the concentration based on the signal of 10 standard deviations (SD) above the mean of 10 blank samples (LLOQ) and the signal of 10 SD below the mean at clear saturation of six different standard curves (ULOQ). Precision was assessed by intra- and inter-assay coefficient of variation (CV) calculated by running one plasma sample in six replicates on the same plate (intra-assay), and four plasma samples in duplicate on four different days (inter- assay), with acceptable

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values of <10% and <15%, respectively. Dilution linearity was determined by spiking two undiluted plasma samples with H3R2,8,17Cit dNucs to expected concentrations of 2000 ng/mL (five-fold the ULOQ). Serial dilutions of the spiked plasma samples (in standard diluent) were performed until the expected concentration was below LLOQ, and analyzed in duplicate on the same plate. Results are presented as the % recovery for the calculated concentration at each dilution within the working range of LLOQ and ULOQ. A % recovery of 80%–120% was accepted.[45] Parallelism was assessed by serial dilutions of two plasma samples containing high endogenous concentrations of H3Cit-DNA complexes (in standard diluent). Neat samples and serial dilutions were analyzed in duplicate in the same run, and compensated for the dilution factor. For each sample, the CV was calculated from the results from the neat sample and the dilutions, with an accepted CV < 20%.[45] Recovery was determined by collecting four aliquots of a plasma sample with a concentration of H3Cit-DNA complexes within the working range. One aliquot was left undiluted and the other three were diluted 1:2, 1:4, and 1:8 (in standard diluent). Ten microliters of H3R2,8,17Cit dNuc was added to the samples to an expected concentration of 400 ng/mL, and analyzed in duplicate in the same run. Results are presented as the % recovery, with an accepted % recovery of 80% to 120%.[45] Selectivity of the assay was assessed by analyzing H3R2,8,17Cit dNucs and unmodified recombinant nucleosomes in the same run. Clinical samples from 40 cancer patients and 30 healthy controls were analyzed to ensure that the levels of H3Cit-DNA complexes found in plasma are within working range of the assay. Sample stability was assessed by one to four freeze-thaw cycles of aliquots from two plasma samples with concentrations within the range of LLOQ and ULOQ. Frozen samples were thawed to RT slowly on an ice bed and mixed gently before being refrozen at −80°C for 24 hours before repeating the freeze-thaw cycle. All samples were analyzed in duplicate in the same run, and results presented as concentrations after one to four freeze-thaw cycles.

To further investigate sample stability, blood was drawn from two patients with H3Cit concentrations within the working range of the assay. Plasma samples stored at −20°C for 1 week before transfer to −80°C were compared to plasma samples stored immediately at −80°C. Samples with a 6-hour delay to centrifugation were compared to samples centrifuged within 45 minutes of blood draw. Samples were analyzed in quadruplicate on the same run. Interference was assessed for bilirubinemia, lipemia, and hemolysis. As no total allowable error was available for H3Cit-DNA, a difference of <15% in paired samples was considered acceptable. The interference of bilirubin and lipemia were tested by spiking five samples within the working range of the assay. A stock solution of bilirubin (17 500 μmol/L) was made by dissolving bilirubin powder (Sigma-Aldrich #B4126) in 0.1 mol/L NaOH. Spiking into plasma was performed in the spiking ratio 1:50 by volume corresponding to concentrations of bilirubin ranging from 117 to 350 μmol/L. Spiking with only 0.1 mol/L NaOH was used as control. To simulate lipemia, intralipid 20% (Sigma-Aldrich #I141) was spiked into samples (1:20 by volume) corresponding to concentrations of triglyceride ranging from 3.87 to 11.3 mmol/L. Spiking with only isotonic NaCl was used as control. The interference of hemolysis was tested on two samples within the working range of the assay in four replicates. Citrated blood was divided into two pools. One pool was stored for 2 hours at −80°C before it was thawed for 1 hour at RT and centrifuged. To achieve hemoglobin concentrations of 1.3–6 g/L in plasma, the top layer was mixed with plasma

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from the second pool. Plasma from the second pool, with a hemoglobin concentration of 0.1 g/L, was used as control.

2.5 | Statistics

The extra sum-of-squares *F* test was used to compare curves. D'Agostino and Pearson normality test was used to test for normality of distribution, and statistical methods were chosen to fit non-normal distributions when appropriate. Continuous variables are presented as medians with interquartile ranges (IQR) and compared with the Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc, La Jolla, CA, USA). A *P*-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Synthetically modified nucleosomes provide optimal calibration in plasma samples compared to modified histone proteins

In vitro PAD4-mediated citrullination of histone H3 is a commonly used practice in ELISA standardization (Table 1). However, this approach can contribute to significant inter-lot variability of ELISA signal, as demonstrated by side-by-side serial dilutions of citrullinated histones prepared using two different lots of PAD4 (Figure 2A). It would be expected that fully defined standards could be a superior approach to assay standardization. However, when semi-synthetic H3R2,8,17Cit histone protein was spiked-in to 100% human plasma, we observed close to no detectable recovery (Figure 2B). In contrast, nucleosomes containing H3R2,8,17Cit were recovered at expected levels (Figure 2B). Two separate series of experiments comparing a total of three production lots of H3R2,8,17Cit dNucs showed that the ELISA calibration curves generated by each lot were highly similar (Figure 2C and D), suggesting that defined recombinant nucleosomes could provide a standardized approach to plasma-based assay calibration.

3.2 | Commercial intrapeptidyl citrulline antibodies show poor specificity and lot variability

The specificity of 12 commercially available intrapeptidyl citrulline antibodies (Table 2) was tested on a Luminex multiplex platform to a panel of semi-synthetic nucleosomes (unmodified or citrullinated at one or a combination of defined residues). The resulting data reveal that the majority of antibodies showed a complete inability to discern the H3Cit target compared to unmodified nucleosomes (Figure 3A). Notably, abTriCit-1 (polyclonal), which is included in many of the currently used H3Cit ELISAs (Table 1), displayed a high cross-reactivity to H3R2Cit and low signal-to-baseline (S/B) relative to unmodified nucleosomes (Figure 3B). In contrast, two lots of monoclonal abR8Cit-1 (1a and 1b) antibody displayed negligible cross-reactivity to off-target PTMs with high S/B (Figure 3C). Further investigation showed significant lot-to-lot variability for ELISA standard curves generated with the widely used polyclonal abTriCit-1 antibody (1b and 1c; Figure 3D), while the monoclonal abR8Cit-1 antibody produced stable calibration curves across lots (1a and 1b; Figure 3E).

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3.3 | H3Cit-DNA ELISA performance metrics

In light of the above we developed an ELISA quantifying nucleosomal H3Cit, implementing the highly specific and consistent abR8Cit-1 monoclonal capture antibody, a dsDNA detection antibody, and an H3R2,8,17Cit dNuc calibration standard (referred to as "H3Cit-DNA ELISA"). A systematic validation was performed assessing the working range, precision, linearity, parallelism, recovery, and selectivity (Figure 4A–F). The working range was defined by a LLOQ of 20.5 ng/mL and ULOQ of 383.4 ng/mL. The results show a high precision with low intra- and inter-assay CVs of 3.3 and 8.9%, respectively (Figure 4A and B). Samples with spiked concentrations above the ULOQ could be diluted to concentrations within the working range and thus accurately recovered (Figure 4C). Samples with high levels of endogenous H3Cit-DNA complexes displayed a low CV upon serial dilutions (Figure 4D), ensuring that the antibody binding characteristics to endogenous H3Cit-DNA complexes is the same as for the H3R2,8,17Cit dNuc standard. The concentration–response relationship was furthermore similar in the calibration standard and the plasma samples as shown by recovery experiments spiking known concentrations of H3R2,8,17Cit dNucs into plasma diluted in assay buffer (1:1–1:8; Figure 4E). The assay showed a high selectivity to citrullinated nucleosomes as shown by the detection of H3R2,8,17Cit dNucs but not unmodified recombinant nucleosomes (Figure 4F).

Analysis of plasma samples from 40 cancer patients and 30 healthy individuals demonstrated that the levels of H3Cit-DNA complexes in human plasma were within working range of the assay after dilutions, and revealed significantly higher levels of H3Cit-DNA in cancer patients than in healthy individuals; median (IQR) 295.0 (151.5–489.1) versus 27.9 (9.9–78.9), $P < .001$ (Figure 4G). To better understand best practices for plasma sample preparation, we assessed pre-analytical variables including freeze/thaw cycles, variations on standard collection protocols, and common sources of potential interfering factors in clinical samples. Clinical plasma samples displayed a high stability after one to three freeze-thaw cycles (Figure S2A in supporting information). A 6-hour delay to centrifugation compared to <45 minutes yielded similar concentrations of H3Cit-DNA (Figure S2B), as did plasma storage at −20°C compared to −80°C for 1 week (Figure S2C). The addition of bilirubin and triglycerides yielded changes in H3Cit-DNA levels below <15% for all levels tested (Figure S2D and E). Finally, hemolysis yielded a positive bias exceeding 15% (Figure S2F), indicating that hemolyzed samples should not be used for the assay.

4 | DISCUSSION

NETs are generating considerable interest due to their suggested role in a wide variety of disease settings.[20,22,46] Since their discovery, the field is rapidly expanding with >3000 PubMed search results on "neutrophil extracellular trap" (May 2020). However, a lack of reliable methodologies has hampered the interpretation of often contradictory results, demonstrating the need for standardized assays. Here we discuss challenges with currently used approaches to quantify the NET marker H3Cit in human plasma samples. We draw attention to the weakness of using in vitro enzymatically citrullinated histone H3 (or indeed any form of free histone) as calibration standards in human plasma, and demonstrate the

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clear advantage of fully defined semi-synthetic nucleosomes. We furthermore show that the majority of available antibodies against intrapeptidyl citrulline are hampered by low capacity to distinguish their reported target, particularly in the physiologically relevant context of an intact nucleosome. Finally, we present a robust assay implementing semi-synthetic nucleosomes citrullinated at H3R2, H3R8, and H3R17 as a calibration standard, and highly specific monoclonal antibodies. Rigorous methodological validation of the assay performance metrics shows the capacity to accurately and reliably quantify nucleosomal H3Cit levels in human plasma samples.

Reliable and reproducible calibration standards are key for accurate quantification. The currently used H3Cit ELISA approaches employ in vitro enzymatically citrullinated histone H3 for calibration standard generation (Table 1). However, histones are unstable in plasma, as shown by the rapid degradation of both calf thymus histones[36] and in vitro enzymatically citrullinated histone H3[47] spiked into plasma. A rapid clearance of circulating histones is crucial because of their potentially harmful effects on host cells.[3–5] While the mechanisms of in vivo free histone degradation are unclear, endogenous proteases, such as active protein C,[36] or the NET associated enzymes neutrophil elastase, myeloperoxidase,[48] and cathepsins,[49] likely contribute. Indeed, H3Cit bound to DNA in nucleosomes could be protected against degradation, as shown here by the stability of semi-synthetic nucleosomes containing H3Cit. This further supports the relative stability of circulating extracellular nucleosomes[37] and ensures a linear recovery of the nucleosome based calibration standard.

In vitro enzymatic modification of histones (or nucleosomes) could be expected to show some variability between preparations possibly due to variable modification of the target sites, generation of off-site modifications that impact antibody recognition, or lack of standardized production protocols between labs. Indeed, we show that two lots of PAD4 enzyme generate distinct H3Cit calibration curves even when histones were citrullinated and assayed in parallel (Figure 2A). This could be a major source of assay variability that may impair comparison of results across labs and overall utility of H3Cit quantification as a disease biomarker. In contrast, we show negligible inter-lot variability of H3R2,8,17Cit dNucs, supporting the superiority of semi-synthetic PTM-containing nucleosomes over in vitro enzymatically modified histones or nucleosomes as calibration standards.

Antibody specificity is crucial to robust assay performance, where poor-quality reagents would be expected to undermine biological interpretations. Histone peptides containing the PTM of interest are often used to examine antibody specificity (Table 1), even though antibody binding in this context does not resemble the in vivo interaction with full-length histones or nucleosomes (with their extensive three-dimensional structures and electrostatic forces). This is of direct relevance because histones are unstable in blood.[36,47] Rather, they circulate bound to DNA in the context of a nucleosome.[50,51] Notably, many currently used assays fail to distinguish between free histones and nucleosomes, and the terms histones and nucleosomes are often used interchangeably.[52] Our results extend prior data on the poor specificity of many histone PTM antibodies,[41,42] showing that the majority of commercial intrapeptidyl citrulline antibodies display no discernible ability to detect citrullinated histones in a nucleosomal context. Of note, the widely used polyclonal abTriCit-1 (cited in > 200 publications; CiteAb), displayed not only high off-target cross-reactivity to non-

citrullinated histones, but also substantial inter-lot variability. Our results instead support the use of the highly specific monoclonal abR8Cit-1, showing an off-target cross-reactivity of 0.2% as well as negligible inter-lot variability.

Finally, we present a robust assay quantifying nucleosomal H3Cit levels in human plasma. The use of stable semi-synthetic nucleosome calibrants and specific monoclonal antibodies enables high assay precision with low intra- and inter-lot variability. The step-by-step control of the assay performance metrics, including dilution linearity, parallelism, and recovery, and application to clinical samples ensures accurate and reliable results, allowing for comparison of nucleosomal H3Cit levels in plasma between laboratories and patient cohorts. Importantly, although the present study focused on validating the assay for use with plasma samples, H3Cit has also been shown to be elevated in serum samples, including a study showing it may be a reliable biomarker for sepsis.[53] Stability in plasma compared to serum samples has been found to differ for some analytes, particularly when there is a delay to centrifugation.[54] Future studies should rigorously evaluate assay compatibility with serum samples as well as pre-analytic performance metrics as we have done here for plasma.

The capacity of neutrophils to release NETs has fascinated researchers since its discovery, but much remains to be learned about the disease relevance and therapeutic potential of this cellular mechanism. H3Cit is widely used as a marker of NET formation, but the lack of standardized methods to detect and quantify this PTM is a critical issue that needs to be addressed. Notably, citrullinated histones may derive from cells other than neutrophils, such as cancer cells expressing PAD4.[55,56] Our work strongly emphasizes the importance of establishing more stringent validations of currently used methods, and provides a novel path to ensure more robust and reproducible data that can be compared between disease settings and laboratories. Our validation of available antibodies, introduction of semi-synthetic nucleosomes containing the PTMs of interest, and the herein presented nucleosomal H3Cit ELISA may be an important step toward understanding the in vivo source of circulating H3Cit as well as its clinical significance in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by a postdoctoral fellowship from the Stockholm County Council (CT) and grants from the Jochnick Foundation (CT), the Regional Agreement on Medical Training and Clinical Research (ALF) between Stockholm County Council and Karolinska Institutet (HW) and the National Institutes of Health (YH and MW T32 HL007149). EpiCypher is supported by the National Institutes of Health under award numbers R44CA214076 (MCK), R44GM136172 (ZWS), R43AI134162 (ALJ), and R44AI134162 (ALJ).

Funding information

Jochnick Foundation; Regional Agreement on Medical Training and Clinical Research (ALF) between Stockholm County Council and Karolinska Institutet; Foundation for the National Institutes of Health, Grant/Award Number: R44CA214076, R44GM136172, R43AI134162, and R44AI134162; Stockholms Läns Landsting

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Essentials

- Neutrophil extracellular traps (NETs) may be diagnostic and prognostic in several diseases.
- Quantifying histone citrullination, a marker of NETs, is hampered by lack of standardized assays.
- We present a robust assay quantifying nucleosomal histone citrullination (H3Cit) in human plasma.

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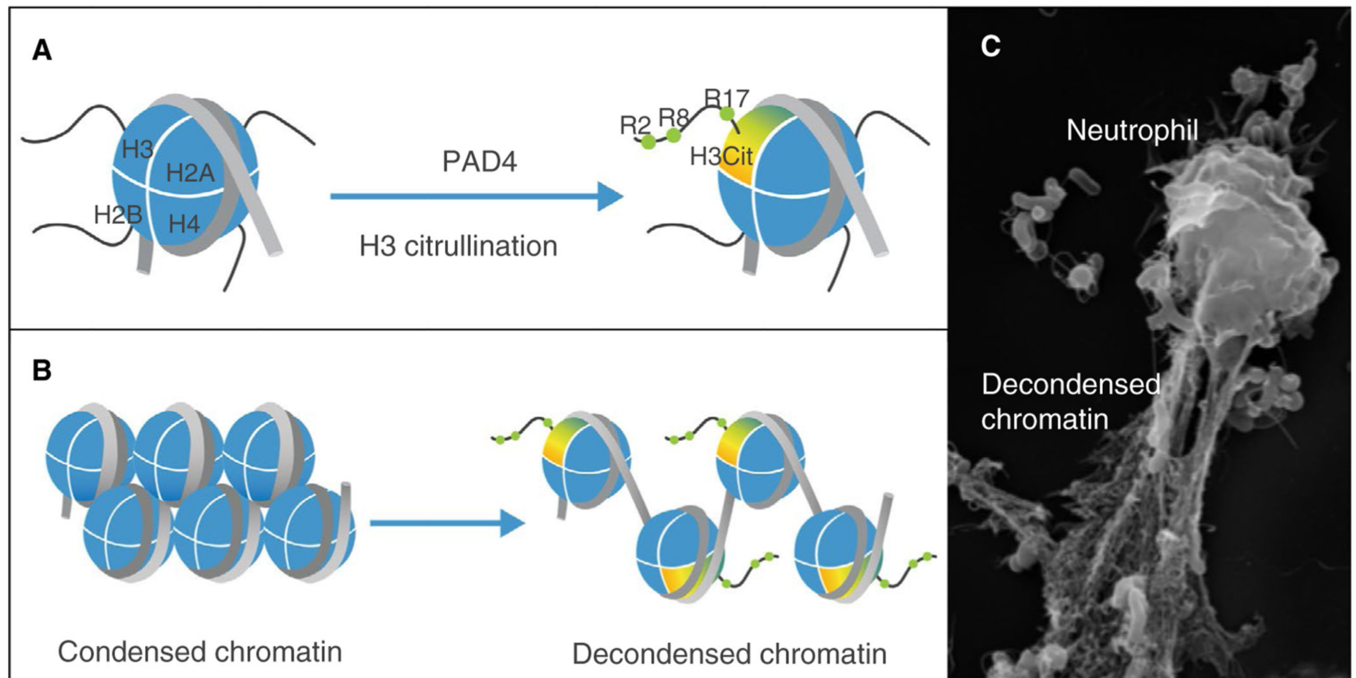

FIGURE 1.

Peptidyl-arginine deiminase 4 (PAD4) facilitates chromatin decondensation and subsequent neutrophil extracellular trap (NET) formation through citrullination of histone tails. A, Histone proteins are packaged into nucleosomes, which contain dimers of histones H2A, H2B, H3, and H4 wrapped by 147 bp DNA within higher order chromatin. The PAD4 enzyme citrullinates positively charged arginines on histone tails, including H3 at arginine residues 2, 8, and 17 (H3R2,8,17Cit). B, Hypercitrullination weakens the interaction between histones and DNA, thus unfolding the tightly packed chromatin. C, Chromatin decondensation is a prerequisite for the neutrophil release of web-like strands of citrullinated nucleosomes and associated granule proteins into the extracellular space. In contrast to free histones, nucleosomes are stable in blood,[37] making them ideal markers of NETs. Panel © adapted from Thalin et al[22]

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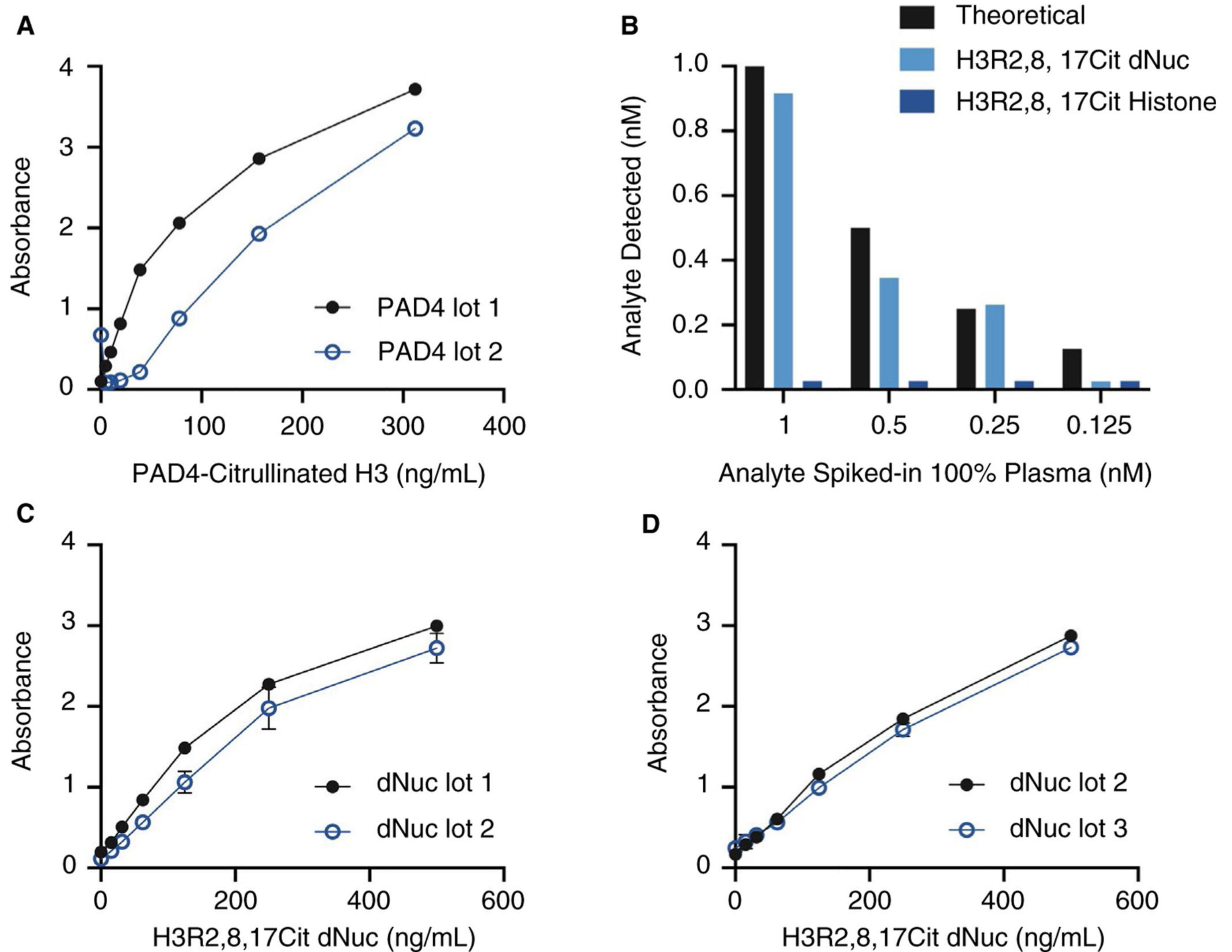

FIGURE 2.

Recombinant nucleosomes are superior calibration standards for plasma-based enzyme-linked immunosorbent assays (ELISAs) compared to either recombinant or enzymatically modified histone proteins. A, In vitro citrullinated histone H3 calibration standard curves were prepared using two different lots of peptidyl-arginine deiminase 4 (PAD4) enzyme (2.5 U PAD4 per µg histone), with serial dilutions of each preparation tested by ELISA in parallel. The lots produce statistically distinct calibration curves; F(DFn, DFd) 133.3 (4,6), $P < .0001$. B, Recombinant H3R2,8,17Cit designer nucleosomes (dNucs), but not recombinant H3R2,8,17Cit histones, are recovered based on expected levels after direct dilution into 100% human plasma. C, ELISA standard curves for lots 1 and 2 of H3R2,8,17Cit dNucs display high inter-lot consistency. There is no statistical difference between the curves; F(DFn, DFd) 2.186 (4,34), $P = .0915$. D, ELISA standard curves for lots 2 and 3 of H3R2,8,17Cit dNucs (lot 1 was exhausted at the time of these experiments) also show no statistical difference between the curves; F(DFn, DFd) 2.004 (4,10), $P = .1698$

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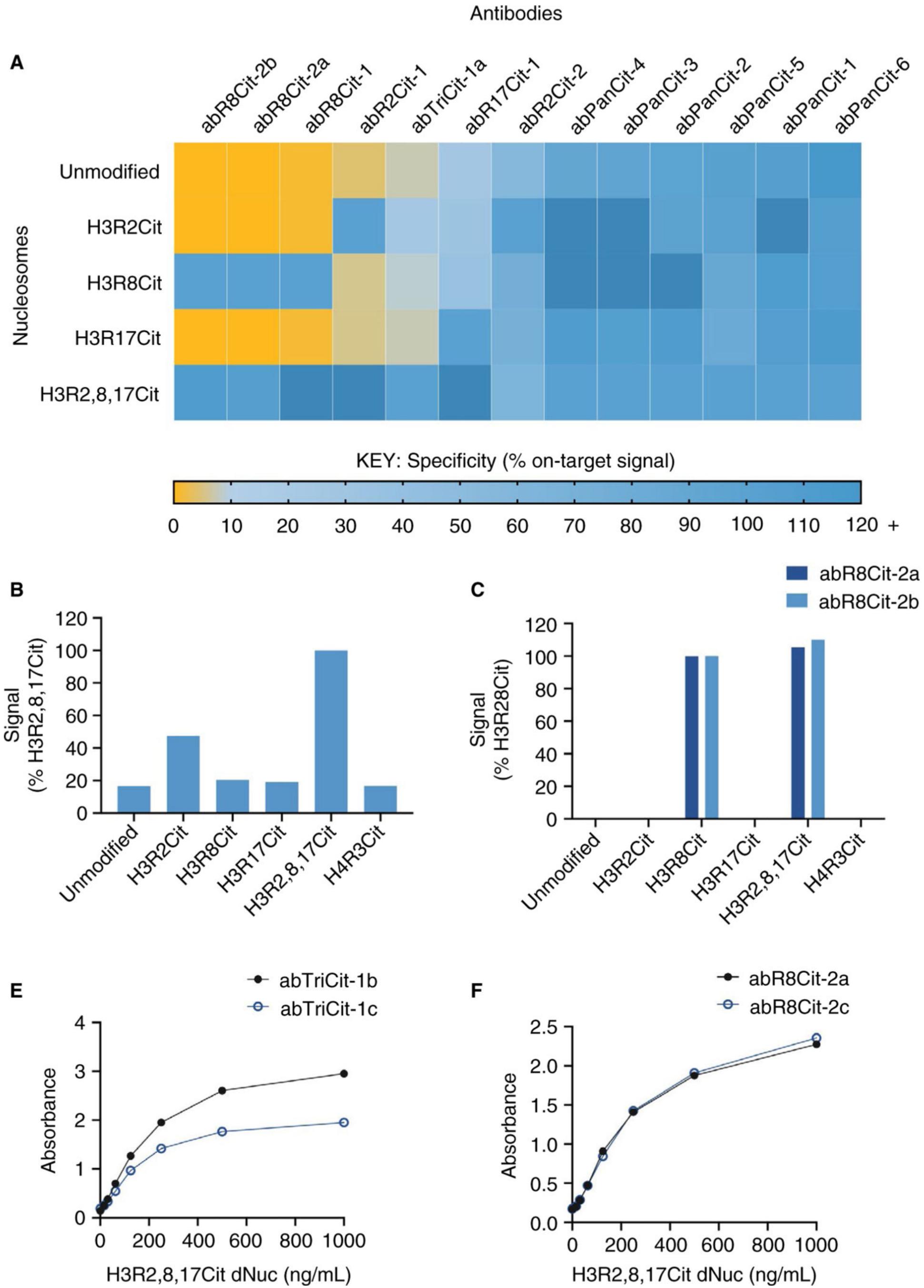

FIGURE 3.

Identification of an optimal anti-citrulline antibody. A, Multiplexed screening of intrapeptidyl citrulline antibodies against a panel of unmodified and citrullinated semi-synthetic nucleosomes showed that the majority of intrapeptidyl citrulline antibodies fail to differentiate their reported target. Antibodies are sorted by signal-to-baseline (S/B, on-target/unmodified control, with highest values on the left descending to lowest values on the right). B and C, The widely used polyclonal abTriCit-1 displayed an average of 13.6% cross-reactivity to off-target post-translational modifications (PTMs; B) with a 13.6-fold

S/B, whereas two lots of monoclonal abR8Cit-1a and abR8Cit-1b displayed an average of 0.20% and 0.19% cross-reactivity to off-target PTMs with a 476.1-fold and 486.3-fold S/B, respectively (C). D and E, H3R2,8,17Cit designer nucleosome (dNuc) calibration curves assayed with two different lots of polyclonal abTriCit-1 displayed significant variability; F(DFn, DFd) 982.5 (4,6), $P < .0001$ (D), whereas dNuc calibration curves generated using two lots of monoclonal abR8Cit-1 (1a and 1b) displayed no significant variability; F(DFn, DFd) 1.2 (4,6) $P = .397$ (E).

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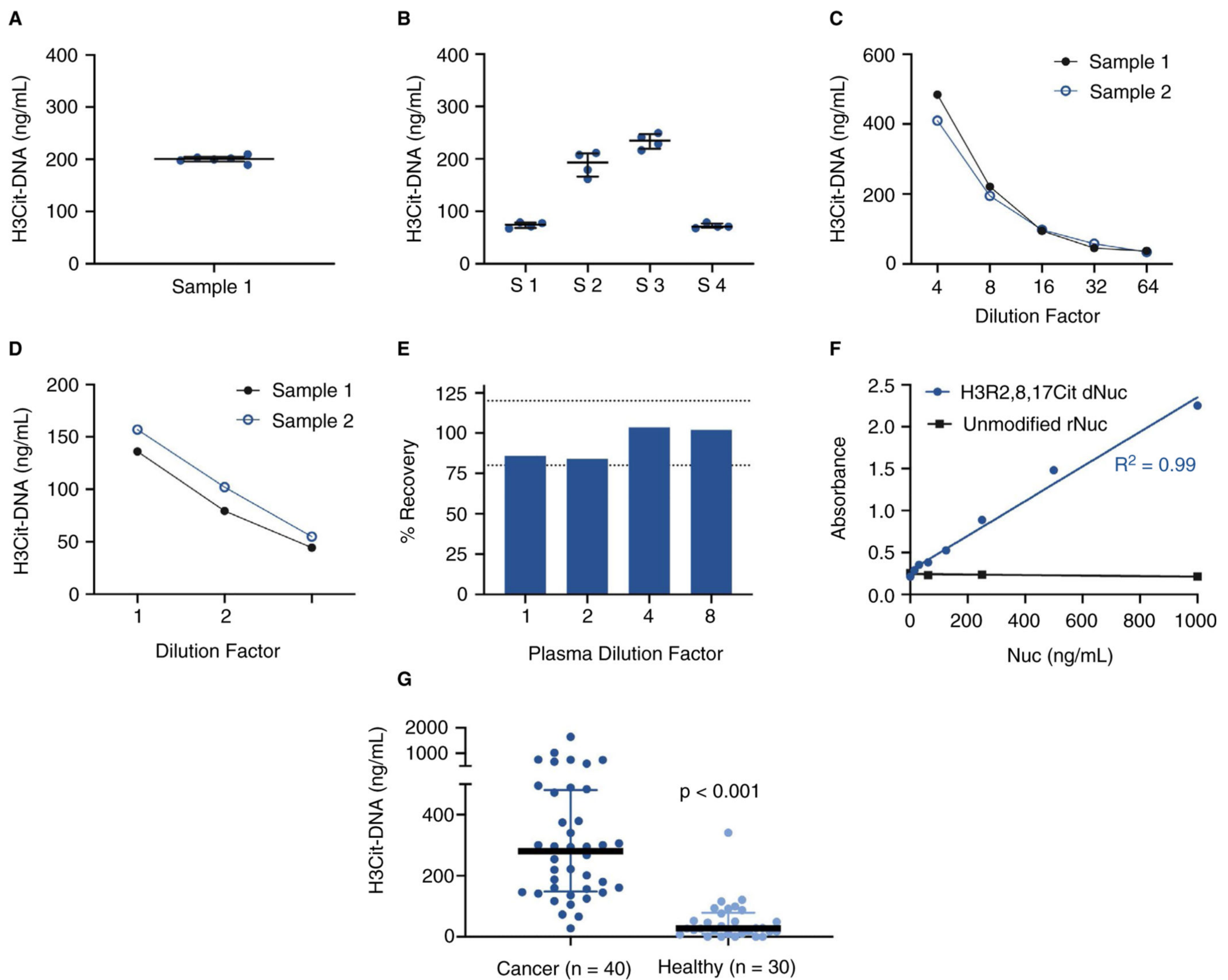

FIGURE 4.

Citrullinated histone H3 (H3Cit)-DNA enzyme-linked immunosorbent assay (ELISA) performance metrics. A, Intra-assay variability. The same plasma sample in six replicates on the same plate showed an intra-assay coefficient of variation (CV) of 3.3%. B, Inter-assay variability. Four plasma samples (S1-S4) analyzed in duplicate on four different days showed an inter-assay CV of 7.4, 12.5, 6.2, and 6.5%, respectively, with a mean (standard deviation) inter-assay CV of 8.9% (2.9%). C, Dilution linearity. Two samples were spiked with H3R2,8,17Cit designer nucleosomes (dNucs) to 2000 ng/mL and serially diluted in assay buffer. Mean (standard deviation [SD]) recovery for dilutions within the working range of lower and upper limits of quantification was 88% (18%). D, Parallelism. Two samples with high endogenous H3Cit-DNA complex levels were serially diluted in assay buffer. Mean (SD) CV was 16.9% (3%). E, Recovery. Human plasma was prepared in different dilutions. Known concentrations of H3R2,8,17Cit dNucs were spiked into plasma diluted 1:1–1:8 to theoretical concentrations of 400 ng/mL. Recovery were all within 80%–120%, with a mean (SD) % recovery of 93.3% (10.4%). F, Selectivity. The assay detected

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H3R2,8,17Cit dNucs, but not unmodified recombinant nucleosomes. G, Analysis of plasma samples from 40 cancer patients and 30 healthy individuals ensured that the levels of H3Cit-DNA complexes in human plasma were within working range of the assay, and revealed significantly higher levels in cancer patients than in healthy individuals; median (interquartile range) 295.0 (151.5–489.1) versus 27.9 (9.9–78.9), $P < .001$

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TABLE 1

Published data on ELISA-quantified H3Cit levels in human plasma

Assay	Study population	Main findings	Range plasma H3Cit	Ref
In-house ELISA. Ab: abTriCit-1. Ab specificity tested against peptide arrays. No calibration standard.	31 stroke patients with (n = 8) and without (n = 23) cancer. 10 healthy controls.	Plasma H3Cit elevated in stroke patients with cancer.	NA	23
	243 stroke patients. 27 healthy controls.	Plasma H3Cit elevated in stroke patients, and associated with stroke severity, atrial fibrillation, and all-cause mortality.	NA	24
In-house ELISA.[47] Ab: abTriCit-1. Ab specificity tested against peptide arrays. Calibration standard: in vitro enzymatically citrullinated H3.	60 cancer patients. 51 severely ill patients without cancer. 50 healthy controls	Plasma H3Cit elevated in cancer patients, and associated with mortality.	Median cancer patients 18.9 ng/ mL (IQR 7.1–29.8), healthy controls 7.1 ng/mL	25
	936 cancer patients. No healthy controls.	Plasma H3Cit associated with VTE in patients with lung and pancreatic cancer.	Median cancer patients 26.0 ng/mL (IQR 2–88.3)	26
	957 cancer patients. No healthy controls.	Plasma H3Cit associated with mortality.	Median cancer patients 25.8 ng/mL (IQR 1.5–87.8)	27
	77 trauma patients and 49 septic patients. 26 healthy controls.	Plasma H3Cit elevated in septic patients.	Median septic patients appr. 100 ng/mL, trauma patients appr. 50 ng/mL, healthy controls 0 ng/ mL. (Data deduced from figures)	28
	48 patients with acute STEMI undergoing PCI. 21 healthy controls.	Plasma H3Cit elevated in blood from culprit site (CS) compared to femoral site (FS).	Median CS 332 ng/mL (IQR 123–810), FS 235 ng/mL (IQR 113–434), healthy controls 192 ng/mL (IQR 150–399)	29
	Human model of endotoxemia including 22 healthy individuals.	Plasma H3Cit elevated after iv injection of LPS (2 ng per kg body weight).	Median 4 h post LPS 113.5 ng/mL (IQR 62.3–164.3).	30
CitH3 ELISA kit (Cayman #501602). Ab: monoclonal H3R2,8,17Cit (Cayman #17939). Ab specificity tested against peptide arrays. Calibration standard: in vitro enzymatically citrullinated H3.	113 patients with type 2 diabetes mellitus. No healthy controls.	Plasma H3Cit associated with pro-thrombotic clot properties (K_s and CLT).	75th percentile 7.4 ng/mL	31
	369 patients with unprovoked VTE. No healthy controls.	Plasma H3Cit elevated in patients diagnosed with cancer during follow-up, and associated with pro-thrombotic clot properties (K_s and CLT).	Median 7.74 ng/mL (IQR 4.23–12.95).	32
	21 patients with heparin-induced thrombocytopenia (HIT). 18 healthy controls.	Plasma H3Cit elevated in HIT patients.	Median HIT patients 1.8 ng/mL (IQR 0.4–3.5), healthy controls 0.2 ng/mL (IQR 0–0.7).	59
CitH3 ELISA kit (BlueGene Biotech). ELISA characteristics NA.	50 patients with severe aortic stenosis (AS). 20 healthy individuals.	Plasma H3Cit elevated in patients with AS.	Median AS patients 12.24 ng/mL (IQR 8.7–18.0), healthy 6.7 ng/mL (IQR 4.4–8.6).	34

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Abbreviations: Ab, antibody; AS, aortic stenosis; CLT, clot lysis time; CS, culprit site; ELISA, enzyme-linked immunosorbent assay; FS, femoral site; HIT, heparin-induced thrombocytopenia; IQR, interquartile range; K_S, fibrin clot permeability; LPS, lipopolysaccharide; NA, not available; PCI, percutaneous coronary intervention; STEMI, ST elevation myocardial infarction; VTE, venous thromboembolism.

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TABLE 2

Unique ID, target, vendor, catalog number, lot numbern and clonality for antibodies used in this study

ID	Target	Vendor	Catalog no.	Lot no.	Clonality
abPanCit-1	Citrulline	Millipore Sigma	AB5612	3040568	PC
abPanCit-2	Peptidyl-Citrulline	Abcam	ab100932	GR3181417-7	PC
abPanCit-3	Peptidyl-Citrulline	Abcam	ab6464	GR3231447-1	PC
abPanCit-4	Peptidyl-Citrulline	Millipore Sigma	07–377	3088392	PC
abPanCit-5	Peptidyl-Citrulline	Millipore Sigma	MABN328	3108477	MC
abPanCit-6	Peptidyl-Citrulline	Millipore Sigma	MABS487	3068537	MC
abTriCit-1a	H3R2,8,17Cit	Abcam	ab5103	GR3218374-1	PC
abTriCit-1b	H3R2,8,17Cit	Abcam	ab5103	GR314058-3	PC
abTriCit-1c	H3R2,8,17Cit	Abcam	ab5103	GR3218144-1	PC
abR2Cit-1	H3R2Cit	Abcam	ab174992	GR3190441-1	PC
abR2Cit-2	H3R2Cit	Abcam	ab176843	GR208214-2	MC
abR8Cit-1a	H3R8Cit	Abcam	ab232939	GR3285980-1	MC
abR8Cit-1b	H3R8Cit	Abcam	ab232939	GR3268477-2	MC
abR8Cit-1c	H3R8Cit	Abcam	ab232939	GR-3235856-4	MC
abR17Cit-1	H3R17Cit	Abcam	ab219407	GR3216814-1	MC

Abbreviations: MC, monoclonal, PC, polyclonal.