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Point-of-care quantification of serum cellular fibronectin levels for stratification of ischemic stroke patients☆

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Abstract

The abundance of cellular fibronectin (c-Fn) for ischemic stroke patients and the narrow time-window (<4.5 h) for the decision to administer the thrombolytic treatment with recombinant tissue plasminogen activator (rtPA) are challenging for the development of a point-of-care (PoC) diagnostic platform. We report a case of stratification of ischemic stroke patients based on a magnetoresistive biosensor platform that quantifies the c-Fn levels in a small volume of serum, within the clinically relevant time-window. Our PoC platform uses different ratios of biofunctionalized magnetic nanoparticles (MNPs) as immunoassay labels to adjust the sensitivity within the clinically relevant ranges for c-Fn (1-4 μg/mL). After optimizing the detection range, resolution, and sensitivity, our device was able to stratify ischemic stroke patients who developed hemorrhagic transformation, the main side-effect of rtPA, from those (both non-treated and treated with rtPA) who did not.

Key words: Acute ischemic stroke; Cellular fibronectin; Hemorrhagic transformation; Magnetic nanoparticles; Magnetoresistive biosensor; Stratification

Cellular fibronectin (c-Fn) protein combines the importance of being both a medically relevant biomarker and an excellent model of challenges encountered in developing clinical assays. Specifically, as a biomarker, c-Fn is associated with various conditions, including its role as an important predictive indicator for severe hemorrhagic transformation (HT) after thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) in acute ischemic stroke.[1–4] Developing clinical assays for this condition presents two major practical challenges: the abundance of c-Fn in the serum of ischemic stroke patients and the narrow time-window (<4.5 h) for the decision to administer rtPA. The side effects of administering a thrombolytic drug based on

Abbreviations: c-Fn, cellular-fibronectin; MNPs, magnetic nanoparticles; MR, magnetoresistive (sensors); SV, spin-valve; MF, magnetic focusing; AC, alternating current; DC, direct current; LoD, limit of detection; LoB, limit of blank; LoQ, limit of quantification; HT, hemorrhagic transformation; Sulfo-LC-SPDP, sulfosuccinimidyl 6-[3′(2-pyridyldithio)-propionamido] hexanoate; PDGFC, platelet-derived growth factor C; MMP9, matrix metallopeptidase 9

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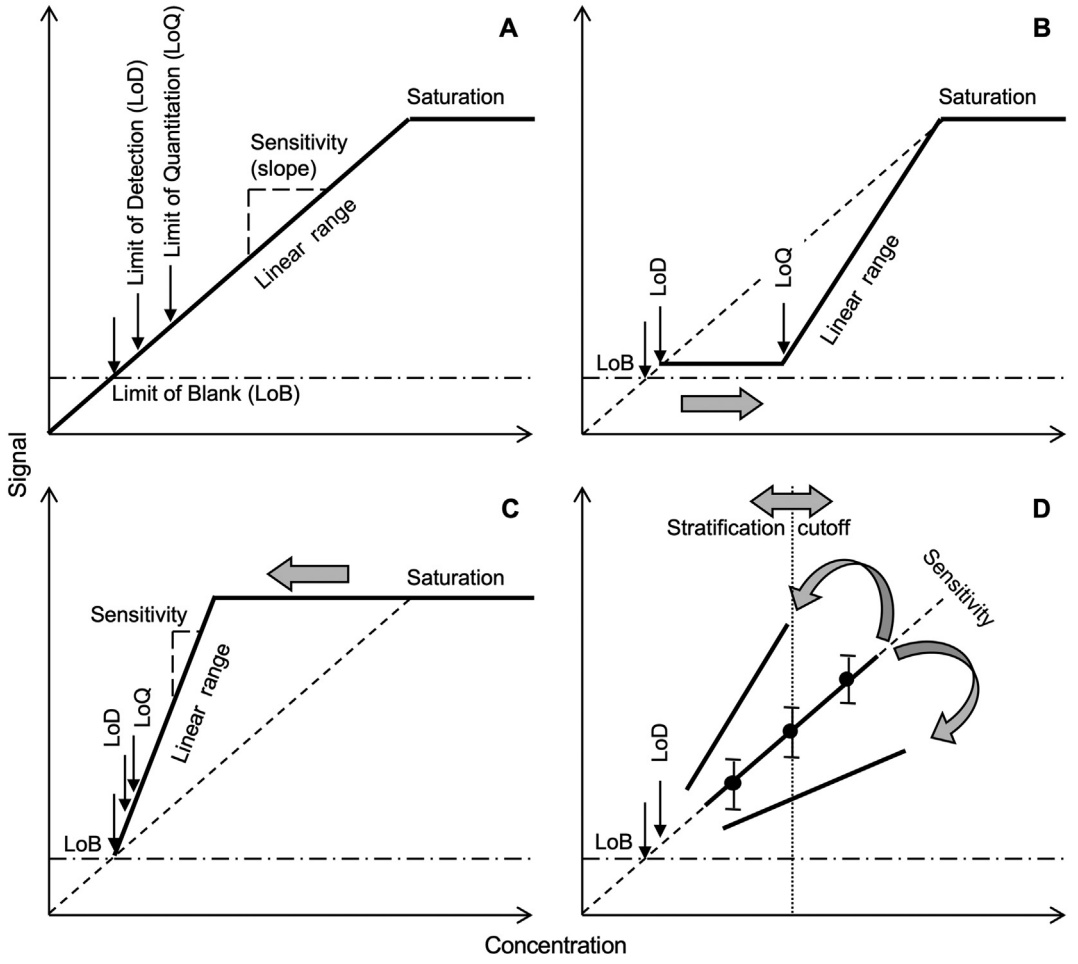

Figure 1. Parameterization **(A)** and optimization of the detection range, resolution, and sensitivity **(B, C, D)** for a PoC platform.

incorrect stratification of ischemic stroke patients are potentially life-threatening, highlighting the critical importance of ensuring validity and robustness of the c-Fn assay implemented in a point-of-care (PoC) diagnostic platform.

The optimization of a PoC platform requires appropriately matching of the parameters of the sensing device to the clinically relevant detection range and sensitivity for the analyte. Several parameters (Figure 1, *A*) can be used to quantify the sensitivity of an analytical procedure, such as a biorecognition assay implemented in a biosensor. Limit of detection (LoD) corresponds to the lowest analyte concentration that can be reliably distinguished from the limit of blank (LoB), which is determined from replicate measurements of blank samples.[5] While LoD is most commonly assumed to parameterize sensitivity, beyond its use in testing and specification of devices, LoD is not typically as important for clinical measurements as the limit of quantification (LoQ) and the linear dynamic range (Figure 1, *A*).[5] To quantify a diagnostically relevant analyte concentration, the analytical procedure needs to be optimized (Figure 1, *B-D*) to ensure that this concentration is above the LoQ and within the linear dynamic range.

In general, such procedural optimization can be used to shift the LoQ above the LoD (Figure 1, *B*) or the saturation threshold towards the LoD (Figure 1, *C*). These adjustments decrease the range of analyte concentrations mapped onto the linear dynamic range and concomitantly increase its slope, i.e., the sensitivity (cf. Figure 1, *B-C* and *A*). The intrinsic reason for having to make these adjustments is the uncertainty associated with each measurement: the number of concentration values that can be distinguished from one another (i.e., the resolution of an analytical procedure) is determined by the number of the confidence intervals (at the desired uncertainty level) that can fit between the LoD and the saturation threshold (Figure 1, *D*). Accordingly, no single parameter of an analytical procedure can be optimized arbitrarily or independently of the others, but rather, adjusting any one of them implies a change in the rest, as illustrated in Figure 1. In the model case of c-Fn, the optimization must place the diagnostically relevant concentration range in the samples of stroke patients within the quantifiable (linear) range, i.e., between the LoQ and the saturation threshold. In other words, the cutoff value of the c-Fn concentration used to stratify the likelihood of the HT in acute ischemic stroke patients treated with rtPA must be approximately in the middle of the linear range of the PoC platform.

We demonstrate how a PoC platform based on magnetoresistive (MR) biosensors[6,7] is optimized for quantification of the

c-Fn concentration in a range clinically relevant for stratification of ischemic stroke patients. The features of this platform, such as the use of biofunctionalized magnetic nanoparticles (MNPs) as immunoassay labels, enable us to adjust the range and sensitivity of c-Fn quantification, while the automated microfluidic format ensures the full assay time of 100 min, i.e., well within the clinically relevant time-window.

Clinical case

Ischemic stroke is a medical emergency with a short therapeutic time-window (<4.5 h) for thrombolytic therapy with rtPA, the only pharmacological effective treatment available[8,9] for acute cases. The low percentage of the patients receiving the rtPA treatment is due to the inability to timely predict who will benefit from it without suffering severe side effects, such as HT, which causes significant morbidity and mortality. Currently, patient selection for rtPA treatment relies on neuroimaging techniques combined with individual information[10,11]; the resulting stroke prediction models, however, may not accurately predict lesion growth.[12–14] Previous investigations indicate that predictive biomarkers can help to better evaluate the risk–benefit of rtPA,[2] suggesting the use of PoC platforms as an aid in the stratification of stroke patients.

Methods

Baseline characteristics of the study population

We studied two different cohorts of patients with acute ischemic stroke: the first group included patients treated with intravenous rtPA (n = 20) and the second, those not treated (n = 10). Likewise, patients treated with rtPA were analyzed separately due to the potential effect of the interaction between rtPA treatment and c-Fn levels on clinical outcome, especially HT [rtPA patients who developed HT (n = 10) and those who did not (n = 10)]. Sample size was calculated using the statistical EPIDAT software, based on a 10% prevalence of symptomatic HT according to previous studies.[15] The minimum sample size calculated to detect this effect was made accepting an alpha level of 5% and 80% power.

The first cohort consisted of (n = 20) patients with a first-ever acute ischemic stroke treated with intravenous rtPA (0.9 mg/kg for 1 h) within 4.5 h from symptom onset at the University Hospital of Santiago de Compostela. All patients were prospectively evaluated by using cerebral CT and National Institute of Health Stroke Scale (NIHSS) and modified Rankin Scale (mRS) and were evaluated during a follow-up period of 90 days. Patients with prior disability (modified Rankin score [mRS] > 1) and known infectious, inflammatory, or cancer diseases at the time of treatment were excluded.

The second cohort was selected from consecutive patients with a first-ever ischemic stroke non-treated with rtPA (n = 10) admitted to the same hospital within 12 h of symptom onset who were previously independent in their daily living activities. Patients with prior disability (modified Rankin score [mRS] > 1) and known infectious, inflammatory, or cancer diseases at the time of treatment were excluded.

Paired healthy subjects from the same hospital, free from overt vascular disease, were also included as controls. Briefly, we identified a subgroup of (n = 10) subjects with a similar age and sex distribution without evidence of symptomatic cardiovascular disease according to the following criteria: (i) no history of heart disease, stroke, or peripheral arterial disease; and (ii) normal electrocardiogram and chest X-ray.

Other exclusion criteria for all study groups were: substantial alteration of renal function (glomerular filtration <60 mL/min), presence of chronic inflammatory disease, and administration of anti-inflammatory medication or antithrombotic or hormone therapy in the two previous weeks. Subjects with acute infection based on clinical criteria were also excluded from all of study groups. Patients were considered to be hypertensive if they had systolic blood pressure $\geq$ 140 mmHg and/or diastolic pressure $\geq$ 90 mmHg and/or were using antihypertensive drugs.

This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association (2000) and approved by the Ethics Committee of the Servizo Galego de Saúde. Informed consent was obtained from each patient or their relatives after full explanation of the procedures.

Clinical variables

All patients were admitted to an acute stroke unit and treated following the European Stroke Organization guidelines.[16] Medical history recording potential vascular risk factors, blood and coagulation tests, 12-lead electrocardiogram, chest X-ray and carotid ultrasonography were performed at admission. Stroke subtype was classified according to the TOAST criteria,[17] and stroke severity was assessed by a certified neurologist using NIHSS at admission (or before rtPA treatment, when applicable).

Neuroimaging variables

CT scans were carried out at admission and between days 4 and 7. Patients who received rtPA were studied before infusion and 24-36 h after treatment. Early CT signs of infarction were evaluated at admission, and infarct volume and HT were assessed on the follow-up CT. HT was classified according to the European–Australasian Cooperative Acute Stroke Study (ECASS II)[18] and defined as symptomatic when it was associated with Early Neurological Deterioration END (Increment $\geq$4 points in the NIHSS within 24 h). Infarct volume was calculated on the follow-up CT by using the formula $0.5 \times a \times b \times c$, where a and b are the largest perpendicular diameters and c is the number of 1-cm thick sections that contain the lesion.

Biofunctionalization of the MR biochip platform

An MR biochip with an array of 30 U-shaped spin-valve (SV) sensors (Figure S1) was microfabricated as described previously.[19] Gold pads (12.9 × 35.4 μm^2) above SV sensors were cleaned (isopropanol, deionized water, UV-ozone for 5 min) and treated for 20 min with 1 mg/mL in phosphate buffer (PB) of sulfosuccinimidyl 6-[3′(2-pyridyldithio)-propionamido] hexanoate (Sulfo-LC-SPDP, Thermo Scientific) as a

heterobifunctional surface linker. After linker deposition, a monoclonal anti-human c-Fn (or anti-human PDGFC, as a non-specific control, both from Immunostep) antibody (Ab) in buffered glycerol (250 μg/mL of Ab in PB, 5% Glycerol) was contacted for 1 h with the chip surface, followed by blocking for 1 h with 1% of bovine serum albumin (BSA, Sigma) in PB. Negative-control sensors were blocked for 1 h in 5% BSA in PB.

Homogeneous magnetic labeling of stroke biomarkers

Streptavidin-conjugated 250-nm MNPs (2 μL of 4.9×10^{11} mL^{-1}, Nanomag, Micromod) were incubated for 1 h in solution (50 μg/mL in PB with 0.05% Tween®20, PB-T) of biotinylated polyclonal anti-human c-Fn (or anti-human MMP9 as a specificity control, both from Immunostep); after functionalization with secondary Abs, the MNPs surface was blocked for 1 h with 5% BSA in PB. The stock solution of MNPs was diluted 1:4 (2 μL of stock MNPs solution added to 6 μL of PB-T) before incubation for 1 h at room temperature with 60 μL of patient serum samples; undiluted and 1:8 diluted MNP solutions were also tested in optimization experiments. To prepare spiked standards for the calibration curve, serum samples from healthy patients were depleted to exclude proteins >3 kDa (Amicon® Ultra-0.5 centrifugal filter). After incubation with patient samples, MNPs were washed once in PB-T to remove unbound proteins.

Heterogeneous capture and detection of MNP-labeled biomarkers

The MNP-labeled biomarkers obtained from the previous step are loaded through a microfluidic system, which is connected to an automated syringe pump (New Era Pump Systems), and contacted with the Ab-functionalized MR biochip. To enhance (beyond the diffusion limit) the probability of MNP-labeled biomarkers being captured by the monoclonal probe primary Abs on the sensor, magnetic focusing (MF) was applied for 2 min, whereby the MNPs are attracted towards and oscillate around the sensors, to provide multiple capture opportunities. After MF, MNP-labeled biomarkers were left in contact with Ab-functionalized sensors for 30 min, followed by a wash to remove unbound biomarkers. The difference (ΔVbinding) between the signal from each Ab-functionalized sensor at this end-point vs the initial baseline is normalized over the baseline signal of the sensor (Vsensor). This normalized sensor signal (ΔVbinding/Vsensor) is proportional to the number of MNP-labeled targets captured at that sensor surface. The details of the MR measurement are described in the supplementary data file (Figure S2).

Statistical analysis

All molecular data are presented as mean ± 95% confidence interval (CI), based on at least four replicates (different sensors). Standard statistics were calculated using built-in functions of Microsoft Excel. For the clinical data, the results were expressed as percentages for categorical variables and as mean (SD) or median [quartiles] for the continuous variables depending on whether their distribution was normal or not. Proportions were compared using the chi-square or Fisher test, while the continuous variables between groups were compared with the Student's *t* or the Mann–Whitney tests. ANOVA was used to analyze the relationship between ischemic stroke subtypes and controls. The statistical analysis was conducted using IBM SPSS Statistics (version 20.0).

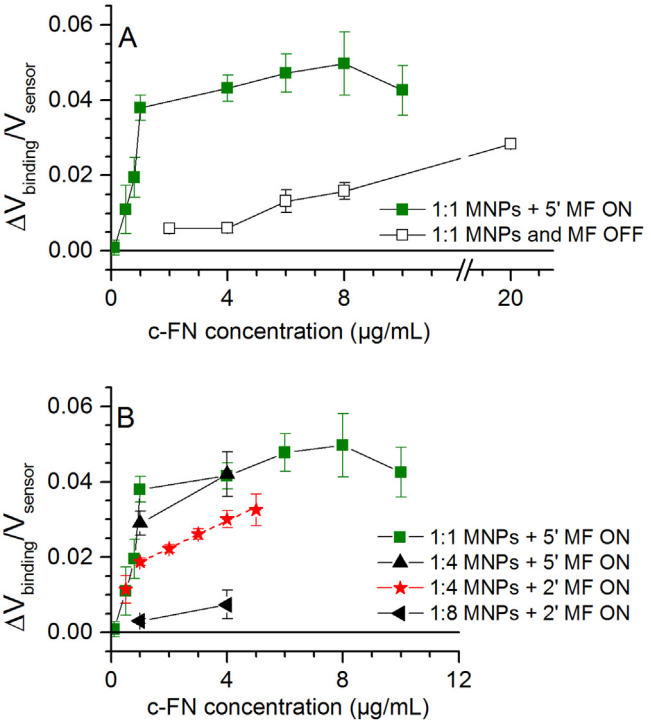

Figure 2. Optimization of the detection range, resolution, and sensitivity for MR biochip. **(A)** Influence of the magnetic focusing (MF) on the sensor signal: filled squares for MF ON, open squares for MF OFF. **(B)** Systematic shift of the sensor signal as the function of increasing MNP concentration and reducing the MF duration, for 4 pairs of values specified in the legend. The "1:1 MNPs + 5' MF" dataset is shown in both panels as green squares to help compare the data; the data for optimized conditions are indicated by red stars and a linear fit in **(B)**.

Results

Assay optimization

The methodology summarized in Figure 1 was followed to optimize the analytical procedure. Without MF enhancement, the cutoff value for c-Fn concentration (3.6 μg/mL) is at the LoD of the assay (open symbols in Figure 2, *A*, cf. Figure 1, *B*). Enabling the MF, dramatically increases the assay sensitivity (linear slopes for filled [c-Fn 0-1 μg/mL] vs open [c-Fn 4-20 μg/mL] symbols in Figure 2, *B*) but pushes the saturation threshold to lower concentration (cf. Figure 1, *C*), i.e., below the cutoff value. To place the cutoff value within the linear range (cf. Figure 1, *D*), the sensitivity was optimized (Figure 2, *B*) by systematically reducing the concentration of the MNP labels (1:4 and 1:8 dilutions) and the duration of MF (from 5 to 2 min). The optimized calibration curve (red star symbols in Figure 2, *B*) exhibits low uncertainty and is linear around the cutoff value.

Table 1
Characteristics of the study groups.

	Control $n = 10$	IS non-rtPA $n = 10$	IS rtPA $n = 10$	IS rtPA+HT $n = 10$	P
Age, years	68.4 ± 9.5	72.4 ± 12.3	67.0 ± 7.9	74.3 ± 11.5	0.213
Male, %	60	60	50	60	0.873
Stroke subtype					0.312
Atherothrombotic, %	-	0	20	20	
Cardioembolic, %	-	40	60	50	
Lacunar, %	-	0	0	0	
Undetermined, %	-	60	20	30	
History of hypertension, %	60	80	70	80	0.523
History of diabetes, %	20	20	30	30	0.621
History of dyslipemia, %	30	40	50	50	0.531
Smoking habit, %	20	10	40	10	0.153
Glucose levels, mg/dL	125.7 ± 39.7	143.5 ± 50.2	161.1 ± 59.0	149.6 ± 58.3	0.189
NIHSS score	-	16 [13, 19]	17 [14, 20]	16 [13, 18]	0.886
Lesion volume, cc	-	124.6 ± 176.1	115.2 ± 153.0	112.7 ± 112.9	0.909

Stroke patient stratification

The characteristics of the study population are included in Table 1. The study groups are comparable in terms of age, sex and vascular risk factors.

The calibration curve data (Figure. 3, red star symbols, top axis), obtained using the optimized assay parameters (cf. Figure 2, *B*, red star symbols), demonstrate that the clinically relevant cutoff[3] at 3.6 μg/mL (vertical dotted line) is approximately in the middle of the linear signal range. The signals for healthy controls as well as ischemic stroke patients with and without rtPA treatment are clearly below the cutoff (horizontal dotted line), while for the HT ischemic stroke patient (undiluted samples) the signal within 95% CI is above the cutoff. For three additional HT stroke patients, the c-Fn concentration was so high that the samples had to be five-fold diluted to avoid saturating the sensor.

Discussion

Patient stratification summary

Our results indicate that the stratification of ischemic stroke patients based on sensor signals (Figure 3) was in agreement with the development of HT. Signals above that corresponding to the 3.6 μg/mL cutoff (horizontal dotted line in Figure 3) were only associated with patients who developed HT. Conversely, signals below the cutoff only came from non-HT subjects: either healthy controls or ischemic stroke patients who did not develop HT, with or without the rtPA treatment. In this last group, the detected c-Fn concentration was <1 μg/mL, in agreement with values reported previously.[20] The ability to identify the last group is particularly interesting in terms of clinical practice, as they represent the ischemic stroke patients for whom the rtPA treatment should be safe to administer. Notably, this information was available within 90 min from receiving the serum samples, i.e., within the 4.5 h treatment-decision time-window. A routine test for c-Fn does not exist. Currently, c-Fn levels are determined by commercial ELISA kits, which are not useful due to short therapeutic window for rtPA treatment. Furthermore, the sample volume (60 μL) required for the measurement using the MR biochip is approximately one order of magnitude lower than that typically used in 96-well ELISA format for a comparable number of replicates. Our PoC method is simple and inexpensive and could be used in the future within emergency departments to facilitate the selection of candidates for recanalization therapies.

The statistical parameters of our measurements are appropriate for performing the stratification of stroke patients. For all the patient groups, the 95% CIs of the measurements (error bars in Figure 3) were comparable to or lower than the standard deviations (SDs) among the patients within each group, which were within the ranges reported in literature.[4] Similarly, the 95% CIs of the measurements on a patient sample repeated within three days were lower than the inter-day SD, which was lower than the SD within the group of HT patients. Out of 33 patient samples, only two (6%) resulted in false negative HT stratification, both patients treated with rtPA with parenchymal hematoma type I (the least severe of the parenchymal hematomas within the ECASS II Criteria).

Challenges of c-Fn assays

The general analytical challenge for performing assays on c-Fn as a stroke biomarker is related to the range of clinically significant concentrations between 0.1 and 60 μg/mL.[4] This range is not wide enough for using analytical methods with logarithmic resolution but is difficult to match to the dynamic range of linear-resolution methods. For example, a representative calibration curve for a customized ELISA kit (after difficulties encountered using commercial kits, Figures S4 and S5) in the range of c-Fn concentrations similar to that in Figure 3 is presented in Figure 4 (red symbols). This calibration curve directly indicates that distinguishing concentration values around the cutoff at 3.6 μg/mL is not possible using this assay. Unexpectedly, even after diluting the samples to bring their concentration into the better resolved 0 to 1 μg/mL range, the c-Fn concentration could not be reliably quantified nor could the patients be stratified using this assay (bars in Figure 4).

In contrast, an analogous dilution approach for handling samples with c-Fn concentrations at the high end of the clinical range worked successfully for our PoC platform (diluted samples in Figure 3 had initial concentrations of 32 ± 14 μg/mL). The discrepancy between the PoC and ELISA results is particularly intriguing because both assays used the same antibodies. We speculate that the use of MNPs and the reversed biorecognition sequence (starting with the polyclonal homogeneous recognition followed by the MF and monoclonal recognition on the surface) was beneficial for effectively purifying complex samples before capture on the MR biochip. Furthermore, the complexity of

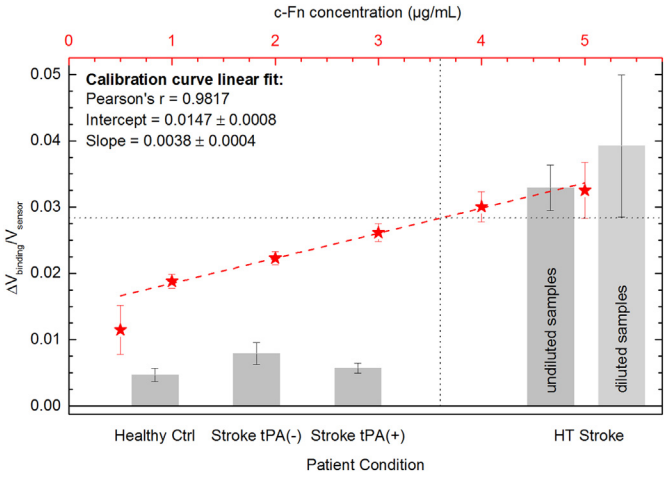

Figure 3. Stratification of stroke patients based on sensor signal. Signals (gray bars) for four patient conditions (bottom axis): healthy controls, stroke patients without(−)/with(+) rtPA treatment, and HT stroke patients, can be compared to the calibration data obtained by spiking c-Fn in depleted serum (red star symbols, dashed line, top axis). Dotted lines indicate the 3.6 µg/mL stratification cutoff (vertical) and the corresponding sensor signal threshold (horizontal).

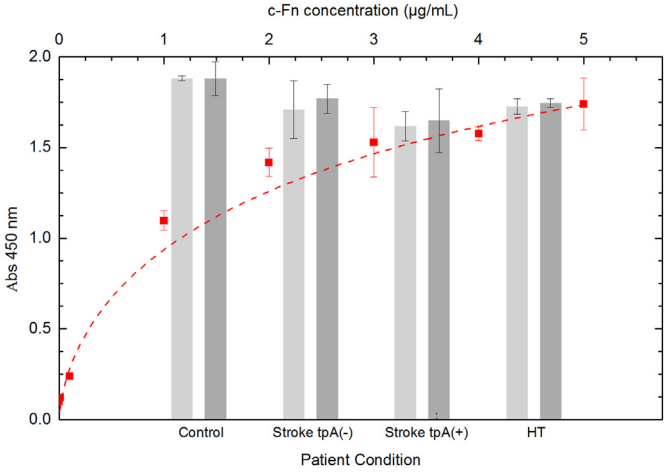

Figure 4. Attempted stratification of stroke patients using an ELISA assay. Absorbance values for samples diluted 1:100 and 1:50 (light and dark gray bars, respectively) for four patient conditions (bottom axis) can be compared to the calibration data (top axis, red symbols ± SD, dashed line provided to guide the eye).

serum samples from stroke patients should not be underestimated: they often present elevated levels of peptides, free-floating hemoglobin, as well as complex cross-reactivity of proteins such as plasma fibronectin, IgG, or hemoglobin, all of which can be challenging to handle in any assay format. Nevertheless, our PoC platform performed robustly, because it offers several adjustable parameters for the optimization of the detection range, resolution, and sensitivity (Figure 2) to address complex samples.

Sequential target discrimination

The literature suggests the use of multiple biomarkers for improving the stratification of stroke patients, for example by detecting both c-Fn and the matrix metalloproteinase-9 (MMP-9).[3] As a proof-of-concept test of the ability of our c-Fn assay to discriminate between c-Fn and MMP-9 targets, we carried out a sequential assay test summarized in Figure 5. Two groups of sensors in the same microfluidic channel were functionalized with anti-human c-Fn and anti-human platelet derived growth factor-CC (PDGF-CC) Abs. Exposing the sensors to MMP-9 functionalized MNPs (non-specific for both mAbs) produced minimal signals, while subsequent exposure to c-Fn functionalized MNPs produced a strong signal for c-Fn (positive control) sensor and a minimal signal for PDGF-CC (non-specific control). The successful sequential discrimination of the two targets opens the possibility of implementing multiplexed assays on the MR biochip platform. To address the additional constrains that arise when measuring multiple targets, additional optimization parameters, such as the concentration of antibodies on the MNP surface, will need to be systematically investigated.

We acknowledge that the limited sample size of this case study will need to be addressed in the future via prospective clinical studies in larger cohorts of patients, to validate the utility of our PoC for the stratification of ischemic stroke patients who are candidates for thrombolytic treatment with rtPA.

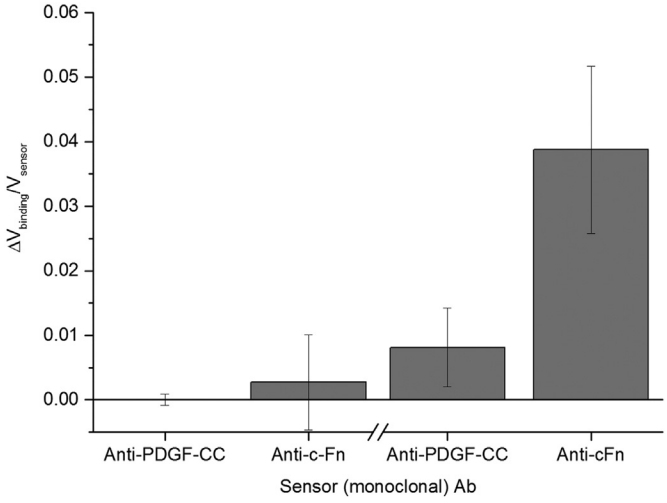

Figure 5. Sequential test of target discrimination. Sensor signals produced by sequentially flowing MMP-9 at 10 μg/mL (non-specific target for both mAbs) and c-Fn at 20 μg/mL (specific target for anti-cFn mAb).

In conclusion, we demonstrated a PoC diagnostic platform for the stratification of ischemic stroke patients based on an MR biochip that quantifies the c-Fn levels in a small volume of serum. In the presented case, our PoC platform uses different ratios of biofunctionalized MNPs as immunoassay labels to adjust the sensitivity within the clinically relevant ranges for c-Fn (1-4 μg/mL). After the optimization of the detection range, resolution, and sensitivity, our PoC platform was able to stratify ischemic stroke patients who developed hemorrhagic transformation, the main side-effect of the thrombolytic treatment, from those (both non-treated and treated with rtPA) who did not.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2020.102287.

References

1. Serena J, Blanco M, Castellanos M, Silva Y, Vivancos J, Moro MA, et al. The prediction of malignant cerebral infarction by molecular brain barrier disruption markers. *Stroke* 2005;**36**(9):1921-6.
2. Rodríguez-Yáñez M, Sobrino T, Arias S, Vázquez-Herrero F, Brea D, Blanco M, et al. Early biomarkers of clinical–diffusion mismatch in acute ischemic stroke. *Stroke* 2011;**42**(10):2813-8.
3. Castellanos M, Sobrino T, Millán M, García M, Arenillas J, Nombela F, et al. Serum cellular fibronectin and matrix metalloproteinase-9 as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke: a multicenter confirmatory study. *Stroke* 2007;**38**:1855-9.
4. Leira R, Sobrino T, Blanco M, Campos F, Rodríguez-Yáñez M, Castellanos M, et al. A higher body temperature is associated with haemorrhagic transformation in patients with acute stroke untreated with recombinant tissue-type plasminogen activator (RtPA). *Clin Sci* 2012;**122**(3):113-9.
5. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* 2008;**29**(1):S49-52.
6. Freitas PP, Cardoso FA, Martins VC, Martins SAM, Loureiro J, Amaral J, et al. Spintronic platforms for biomedical applications. *Lab Chip* 2012;**12**(3):546-57.
7. Freitas PP, Ferreira HA, Graham DL, Clarke LA, Amaral MD, Martins V, et al. Magnetoresistive DNA chips. *Magnetoelectronics* 2004;**7**:331-86.
8. Barber PA, Zhang J, Demchuk AM, Hill MD, Buchan AM. Why are stroke patients excluded from TPA therapy?: an analysis of patient eligibility. *Neurology* 2001;**56**(8):1015-20.
9. Troke STS, Roup STG. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med* 1995;**333**(24):1581-8.
10. Marks MP, Olivot JM, Kemp S, Lansberg MG, Bammer R, Lawrence RW, et al. Patients with acute stroke treated with intravenous tPA 3-6 hours after stroke onset: correlations between MR angiography findings and perfusion- and diffusion-weighted imaging in the DEFUSE study. *Radiology* 2008;**249**(2):614-23.
11. Albers GW, Thijs VN, Wechsler L, Kemp S, Schlaug G, Skalabrin E, et al. Magnetic resonance imaging profiles predict clinical response to early reperfusion: the diffusion and perfusion imaging evaluation for understanding stroke evolution (DEFUSE) study. *Annals Neurol* 2006;**60**(5):508-17.
12. Mishra NK, Albers GW, Davis SM, Donnan GA, Furlan AJ, Hacke W, et al. Mismatch-based delayed thrombolysis: a meta-analysis. *Stroke* 2010;**41**(1):e25-33.
13. Rivers CS, Wardlaw JM, Armitage PA, Bastin ME, Carpenter TK, Cvoro V, et al. Do acute diffusion- and perfusion-weighted MRI lesions identify final infarct volume in ischemic stroke? *Stroke* 2005;**37**(1):98-105.
14. Cvoro V, Marshall I, Armitage PA, Bastin ME, Carpenter T, Rivers CS, et al. MR diffusion and perfusion parameters: relationship to metabolites in acute ischaemic stroke. *J Neurol Neurosurg Psychiatry* 2009;**81**(2):185-92.
15. Sobrino T, Millán M, Castellanos M, Blanco M, Brea D, Dorado L, et al. Association of growth factors with arterial recanalization and clinical

outcome in patients with ischemic stroke treated with TPA. *J Thromb Haemost* 2010;**8**(7):1567-74.
16. Executive Committee ESO, Writing Committee ESO. Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. *Cerebrovasc Dis* 2008;**25**(5):457-507.
17. Harold P, Adams Jr MD, Birgitte H, Bendixen PhD MD, L. Jaap Kappelle M, Jose Biller MD, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke* 1993;**24**(1):35-41.
18. Larrue V, von Kummer R, Müller A, Bluhmki E. Risk factors for severe hemorrhagic transformation in ischemic stroke patients treated with recombinant tissue plasminogen activator. *Stroke* 2001;**32**(2):438-41.
19. Cherré S, Fernandes E, Germano J, Dias T, Cardoso S, Piedade MS, et al. Rapid and specific detection of cell-derived microvesicles using a magnetoresistive biochip. *Analyst* 2017;**142**(6):979-86.
20. Castellanos M, Leira R, Serena J, Blanco M, Pedraza S, Castillo J, et al. Plasma cellular-fibronectin concentration predicts hemorrhagic transformation after thrombolytic therapy in acute ischemic stroke. *Stroke* 2004;**35**(7):1671-6.