Extracellular Vesicle Surface Markers as a Diagnostic Tool in Transient Ischemic Attacks

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ABSTRACT

BACKGROUND AND PURPOSE - Extracellular vesicles (EVs) are promising biomarkers for cerebral ischemic diseases, but not systematically tested in patients with transient ischemic attacks (TIAs). We aimed at: (1) Investigating the profile of EV-surface antigens in patients with symptoms suspicious for TIA; (2) Developing and validating a predictive model for TIA diagnosis based on a specific EV-surface antigen profile.

METHODS - We analyzed 40 subjects with symptoms suspicious for TIA, and 20 healthy controls from a training cohort. An independent cohort of 28 subjects served as external validation. Patients were stratified according to likelihood of having a real ischemic event using the Precise Diagnostic Score (PREDISC), defined as: "unlikely" (score 0-1), "possible-probable" (score 2-3), or "very likely" (score 4-8). Serum vesicles were quantified by nanoparticle tracking analysis and EV-surface antigen profile characterized by multiplex flow cytometry.

RESULTS - EV concentration increased in patients with "very likely" or "possible-probable" TIA ($P<0.05$) compared to controls. Nanoparticle concentration was directly correlated with the PREDISC score (R=0.712-$P<0.001$). After EV immuno-capturing, CD8, CD2, CD62P, MCSP, CD42a, CD44, CD326, CD142, CD31, and CD14 were identified as discriminants between groups. ROC curve analysis confirmed a reliable diagnostic performance for each of these markers taken individually and for a compound marker derived from their linear combinations (AUC=0.851). Finally, a random forest model combining the expression levels of selected markers achieved an accuracy of 96% and 78.9% for discriminating patients with a "very likely" TIA, in the training and external validation cohort, respectively.

CONCLUSIONS - The EV surface-antigen profile appears to be different in patients with transient symptoms adjudicated to be very likely caused by brain ischemia compared with patients whose symptoms were less likely to due to brain ischemia. We propose an algorithm based on an EV surface-antigen specific signature that might aid in the recognition of TIA.

Nonstandard Abbreviations and Acronyms

EVs:	Extracellular Vesicles
TIA:	Stroke Ischemic Attack
PREDISC:	Precise Diagnostic Score
MRI:	Magnetic Resonance Imaging
NPs:	Nanoparticles
NTA:	Nanoparticle Tracking Analysis
FC:	Flow Cytometry
MFI:	Median Fluorescence Intensity
Ctrl:	Controls
DWI:	Diffusion-Weighted Imaging

INTRODUCTION

The traditional definition of Transient Ischemic Attack (TIA) is a sudden, focal neurological deficit of presumed vascular origin lasting less than 24 hours. [1] Patients with transient symptoms and evidence of infarction may be classified as brain infarction with transient neurological symptoms.[2, 3] Regardless the definition, the diagnosis of TIA is challenging, because a gold standard is lacking. The diagnostic process is mainly based on clinical history [4] and the inter-rater reliability between vascular neurologists is poor.[5, 6] To overcome these difficulties, the Precise Diagnostic Score (PREDISC) represents a well-structured scoring tool, composed of standardized clinical and neuroimaging elements,[7] that allows a more objective discrimination of patients with symptoms suspicious for TIA, with excellent inter-rater agreement. To improve the diagnostic process, a reliable biomarker is a prerequisite. Diagnostic biomarkers of TIA have been investigated, but none of the potential candidates reached enough accuracy for TIA diagnosis.[8, 9]

Extracellular vesicles (EVs) are new promising biomarker candidates.[10] EVs are secreted nano-sized particles that play an important role in intercellular communication and are involved in numerous biological mechanisms related to cerebrovascular diseases, like immune responses, inflammation and coagulation processes.[11-14] Higher levels of endothelial and platelet-derived EVs have been associated with the diagnosis and prognosis of stroke,[15-17] however, data on patients with TIAs are scarce.[18] Indeed, none of the studies explored the diagnostic performance of EVs as potential biomarkers for TIA.

We aimed to investigate the surface-antigen profile of EVs in patients with symptoms suspicious for TIA. Considering the lack of a reliable gold standard for the diagnosis of TIA, patients were accurately selected using the PREDISC score, which allowed the stratification of patients according to the

likelihood of an ischemic mechanism underlying a transient neurological deficit. In particular, we adopted a combined approach (based on clinical evaluation, MRI, and EV surface markers) for the recognition of TIA, in order to develop and validate a predictive model for the diagnosis of a transient ischemic event based on the specific EV-surface antigen profile.

METHODS

This is a prospective, observational cohort study. A detailed description of patient selection, EV characterization, and statistics is provided as Supplemental Material. All data relevant to the study are included in the article or uploaded as supplementary information.

Inclusion and exclusion criteria

Patients with symptoms suspicious for TIA included in the training cohort were recruited at the Stroke Center of Neurocenter of Southern Switzerland in Lugano, from September 2017 to July 2020. An independent cohort was recruited at the Stroke Center of Lausanne (Switzerland), from Mars 2019 to April 2020, and served as external validation. Patients were included in the study if: 1) presenting in the Emergency Room (ER) affected by symptoms suspicious for TIA (defined as a sudden, focal neurological deficit of presumed vascular origin lasting less than 24 hours); [1] 2) Age ≥18 years; 3) cerebral magnetic resonance imaging (MRI) performed within 48 hours of admission. We excluded patients in case of ocular TIA (amaurosis fugax), symptoms at inclusion, contraindications for MRI, pregnancy, or concomitant acute or chronic inflammatory disease (e.g., infections, cancer, autoimmune disease). Healthy controls (Ctrl) were recruited among ambulatory patients in the same period of enrollment of subjects with suspected TIA. Sixty participants were enrolled, 40 with symptoms suspicious for TIA, and 20 Ctrl, which corresponded to a minimum power of 90% considering the

estimated variability for median fluorescence value of EV-surface markers with a mean effect size (Cohen's d coefficient) of 0.91, and a significance level (alpha error) of 0.05.

Study protocol

For all patients, the 8-points PREDISC score [7] was calculated as previously described (Table I, please see https://www.ahajournals.org/journal/str). Two independent neurologists evaluated medical history, the general and neurological physical exams, to determine ABCD2, ABCD3-I scores[19, 20] and the PREDISC clinical sub-score (0-4 points). Each patient underwent a cerebral 3T MRI, within 48 hours of admission, to define the PREDISC imaging sub-score (0-4 points). Patients were subsequently stratified into three groups, according to the likelihood of having a TIA, according to the traditional definition[1], and based on the final PREDISC score: "unlikely" (0-1), "possible/probable" (2-3) or "very likely" (4-8; Table II, please see https://www.ahajournals.org/journal/str). In addition to routine blood exams, each patient underwent peripheral blood sampling. Blood was collected in heparin-free tubes and serially centrifuged to remove cellular components and larger vesicles.

The study complied with the Declaration of Helsinki. We obtained ethics approval from the local ethics committee and all patients gave informed consent (study protocol n° Project-ID 2017-00559 CE TI 3201). Non-blinded investigators recruited participants but were not involved in experimental analysis.

Characterization of extracellular vesicles

We evaluated diameter and concentration of serum nanoparticles by nanoparticle tracking analysis (NTA) using Nano-Sight LM10 (Malvern Instruments, United Kingdom). Since NTA is not specific for EV, hereafter we refer to nanoparticles (NP) for NTA measurement, expressing concentration as number of particles per mL of serum (n/mL) and particle diameter reported in nm. Serum samples underwent

multiplex bead-based EV capture and analysis by flow cytometry (FC) using a MACS-Plex Human Exosome Kit (Miltenyi Biotec; Bergisch Gladbach, Germany), as previously described.[21] Median fluorescence intensity (MFI) was measured by the MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec; Bergisch Gladbach, Germany). The detection of 37 different EV-surface antigens was simultaneously performed. Background was subtracted from the MFI value of each marker, and then normalized using by the mean MFIs of CD9, CD63, and CD81. Levels of expression were reported as normalized MFI (%) for each EV-surface antigen (median value and interquartile range).

Statistical analysis and diagnostic modeling

Variable distribution was assessed with the Kolmogorov–Smirnov test. Student's T-test or ANOVA with post-hoc Bonferroni's test and Mann-Whitney or Kruskal-Wallis tests were used to compare variables with a normal or non-normal distribution, respectively. Categorical variables were compared by a chi square test. Pearson's R test was used to assess correlations between variables. Multivariate logistic regression analysis was used to evaluate the odds ratios (ORs) and 95% confidence intervals (95% CI). An OR greater than 1 indicates an increased likelihood of TIA diagnosis, and an OR less than 1 a decreased likelihood, independently from potential confounders considered for each model. Diagnostic performance and area under the curve (AUC) were evaluated by the analysis of receiver operating characteristic (ROC) curves. *P*-values<0.05 were considered significant. The inter-rater agreement between neurologists in assigning the PREDISC score was calculated with the Cohen's kappa score.

Linear discriminant analysis (LDA) was used as strategy for features reduction to build the 2D/3D-canonical plot and evaluate the performance of EV-surface antigen signatures in discriminating patients with TIA. The diagnostic model was built on patients with symptoms suspicious for TIA (controls were not included in the training process), using a random forest (RF) classification algorithm; each RF was

composed of 20 classification trees. The predicted diagnosis was based on the outcome of each RF classification tree; if at least 11 of 20 trees of the forest predict the diagnosis of TIA, the patient was classified as "very likely" of experiencing a TIA (according to the stratification by PREDISC score). Performance and generalizability of the RF model was assessed by leave-one-out validation algorithm (Supplemental Material) and real external validation on an independent cohort. IBM SPSS Statistics 22 (IBM Corp., Armonk, New York, USA), Python 3.5 (library, scikit-learn), and GraphPad PRISM 7.0a (La Jolla, California, USA) were used for analyses.

RESULTS

Patient Characteristics

Clinical and biochemical parameters of patients included in the training cohort are summarized in Table 1. The 40 patients admitted to the neurology department with symptoms suspicious for TIA were compared to 20 Ctrl; there were no significant differences between Ctrl and patients with symptoms suspicious for TIA for all considered variables. The mean age was 67 years, 48.3% were males, with a relatively high prevalence of cardiovascular risk factors, such as hyperlipidemia (78.3%), hypertension (58.3%), diabetes (5.0%), and renal disease (6.7%). Patients with symptoms suspicious for TIA were stratified with the PREDISC score for the likelihood of having a true ischemic event: [7] "unlikely" (n=10; PREDISC=0-1), "possible-probable" (n=15; PREDISC=2-3), and "very likely" (n=15; PREDISC=4-8) diagnosis of TIA.

ABCD2 and ABCD3-I scores partially discriminate patients with a "very likely" diagnosis from those with an "unlikely" diagnosis ($P<0.05$ and $P<0.001$, respectively). No differences were found regarding other clinical and biochemical variables (Table II, please see https://www.ahajournals.org/journal/str). After MRI evaluation (diffusion-weighted imaging sequences, DWI), 13 patients displayed a

hyperintense ischemic lesion (DWI positive), while 27 patients had a normal imaging result (DWI negative). The inter-rater agreement between the two blinded raters was high for the final PREDISC Score (92.5%; Cohen's Kappa=0.911; p<0.001).

Nanoparticle (NP) tracking analysis (NTA)

After serial centrifugation steps to eliminate cellular debris and larger particles, all serum samples were evaluated by NTA to assess particle concentration and diameter (Figure 1 and Tables III and IV, please see https://www.ahajournals.org/journal/str). The number of NPs per mL of serum was significantly increased in patients with a "possible-probable" or a "very likely diagnosis" of TIA compared to Ctrl (*P*<0.001). NP concentration was similar in Ctrl and patients with an "unlikely" diagnosis of TIA (PREDISC 0-1). The same trend was found after stratification for EV diameter, 30-150 nm vs. 151-500 nm (Figure 1A). The number of NPs/mL was also directly correlated with the ABCD3-I score (R=0.432; *P*=0.005), and PREDISC score (R=0.712; *P*<0.001; Table V, please see https://www.ahajournals.org/journal/str). NP diameter was significantly decreased in patients with a "possible-probable" / "very likely" diagnosis of TIA compared to Ctrl (*P*<0.05; Figure 1B). Patients with a hyperintense ischemic lesion (DWI positive) displayed an increased NP concentration and a decreased diameter, compared to patients with a normal imaging result (DWI negative; *P*<0.01; Figure 1A-1B).

After bead-based EV immuno-capturing, samples were analyzed by FC. The surface expression of EV-specific tetraspanins (CD9, CD63 and CD81) was evaluated to confirm the reliability of quantitative NTA in discriminating serum EVs. Particle concentration in NTA was directly correlated with mean MFIs of CD9, CD63, and CD81 (R=0.780; P<0.001; Figure 1C). Based on the expression of tetraspanins, the amount of circulating EV was significantly higher in patients with a "very likely" diagnosis of TIA (PREDISC score 4-8), compared to controls or patients with TIA considered "unlikely"

(PREDISC score 0-1; Figure 1D). Consistently, DWI positive patients displayed increased levels of fluorescence for the EV specific markers, compared to patients that resulted negative at imaging.

Analysis of Extracellular Vesicle (EV)-surface markers

To investigate a potential role for specific EV-derived surface biomarkers in the stratification of patients according to their likelihood of TIA, we performed a multiplex FC analysis that simultaneously evaluated the MFI of 37 different antigens expressed on the surface of EVs (see Supplemental Material). In order to reference fluorescence of individual antigens to EV-specific marker expression, the MFI of single antigen was normalized to the mean MFI of CD9-CD63-CD81. Adopting LDA as feature reduction strategy, we drew the 3D-canonical plot to visualize patient classification according to levels of expression of the 37 analyzed EV-surface antigens and the final likelihood of TIA diagnosis according to PREDISC score (Figure 2A). Panels in Figure 2A show two different plot perspectives highlighting discrimination of Ctrl (in green) and patients admitted for symptoms suspicious for TIA (left-panel) or patients with a "very likely" diagnosis of TIA (in red) and all others (right-panel). The expression level of 10 antigens increased progressively with the PREDISC score (Figure 2B) and was significantly higher in patients with a "very likely" diagnosis compared to Ctrl, or patients with an "unlikely" diagnosis of TIA (Figure 3A and Table VI, please see https://www.ahajournals.org/journal/str): CD8 (T-cell transmembrane glycoprotein), CD2 (T-cell surface antigen), CD62P (P-selectin, alpha-granule membrane protein), MCSP (Melanoma-associated Chondroitin Sulfate Proteoglycan), CD42a (platelet membrane glycoprotein IX), CD44 (T-cell surface receptor), CD326 (epithelial cell adhesion molecule), CD142 (tissue factor), CD31 (PECAM-1, Platelet-Endothelial Cell Adhesion Molecule-1), and CD14 (monocyte differentiation antigen). No differences were found between controls and patients with an unlikely/possible-probable diagnosis of TIA.

The expression levels for 6 out of 10 of the markers directly correlated with the PREDISC score (R range 0.343-0.461; $P<0.05$; Figure 3B). Other significant correlations were also found with PREDISC clinical and imaging sub-scores, and with ABCD2 and ABCD3-I scores (Table V, please see https://www.ahajournals.org/journal/str).

To exclude potential confounding effects related to age and/or cardiovascular risk factors on EV analysis, we performed a multivariate regression analysis. After correction for age, systolic blood pressure, hypertension and hyperlipidemia (Table VII, please see https://www.ahajournals.org/journal/str), we confirmed the significant association of the 10 EV markers with a "very likely" diagnosis of TIA (PREDISC score 4-8; OR ranging between 1.003-1.016; $P<0.05$). NP concentration was no longer associated with suspected TIA, after correction for confounders.

Among EV-markers differentially expressed in patients stratified for PREDISC score, CD42a, CD31, and CD14 discriminate patients with an ischemic lesion in DWI compared to patients with negative imaging (Table VIII, please see https://www.ahajournals.org/journal/str). No significant correlations were found between NP concentration or EV surface antigens expression and the volume of ischemic lesion at DWI (Table V, please see https://www.ahajournals.org/journal/str).

Models for the diagnosis of TIA

Diagnostic performance of each EV-surface antigen as biomarker of "very likely" TIA (according to the PREDISC score) was evaluated by analysis of ROC curves (Figure I A-C, please see https://www.ahajournals.org/journal/str) in the training cohort. Figure I B (please see https://www.ahajournals.org/journal/str) reports the AUC and sensitivity/specificity for the 10 differentially expressed EV antigens and for a compound EV marker derived from their weighted linear combination. All the evaluated EV markers displayed acceptable performance (AUC 0.641-0.851;

sensitivity 60.0-93.3%; specificity 51.1-86.7%; $P<0.05$), with the compound marker showing a sensitivity of 93.3% and a specificity of 64.4%.

As for patients with confirmed ischemic lesions in DWI, the diagnostic performance of CD42a, CD31, and CD14 reached a maximum sensitivity and specificity of 92.3% and 88.9%, respectively (Figure I D, please see https://www.ahajournals.org/journal/str; AUC 0.695-0.729). The compound EV marker (weighted linear combination of CD42a-CD31-CD14) displayed the highest performance (AUC=0.813; sensitivity=76.9%; specificity=77.8%).

Next, we developed a diagnostic model combining the expression levels of the 10 differentially expressed EV-surface antigens, using an RF classification algorithm. We first focused on the discrimination of patients stratified for the PREDISC score according to the likelihood of TIA. At training, the RF model discriminating patients with a PREDISC score 0-1 *vs.* 2-3 was able to correctly classify 88% of patients (n=25; model 1; Figure 4A), whereas the RF model discriminating patients with PREDISC score 2-3 *vs.* 4-8 correctly classified 86.7% of patients (n=30; model 2; Figure 4B); both models displayed a sensitivity of 93.3% and a specificity of 80% for detecting patients at higher likelihood of TIA. Finally, the RF model discriminating patients with a PREDISC score 0-1 *vs.* 4-8 (n=25**;** model 3; Figure 4C) displayed a higher performance, correctly classifying all patients except one (accuracy=96.0%); notably, in this model all patients with a "very likely" diagnosis of TIA (PREDISC 4-8) were correctly detected (sensitivity=100%).

We also built a diagnostic model able to detect patients with an ischemic lesion on MRI (n=40; DWI model; Figure 4D). An RF model discriminating DWI positive *vs.* negative patients was able to correctly classify patients with high accuracy and specificity (respectively, 90% and 96.3%).

To exclude overfitting bias, RF models were internally validated by leave-one out algorithm and then tested in an independent external validation cohort (Figure 4 A-D and Figure 5A).

The internal validation confirmed an acceptable performance for model 3 (PREDISC 0-1 vs. 4-8) and DWI model with an accuracy of 76% and 75%, respectively. On the other side, patients with "unlikely" TIA compared to "possible-probable", and those with TIA "possible-probable" compared to "very likely", were hardly distinguishable (accuracy at internal validation 56.0% and 66.7%, respectively).

Characteristics of patients enrolled in the independent validation cohort (n=28) are reported in Table IX and Table X (please see https://www.ahajournals.org/journal/str). Clinical and biochemical parameters did not differ between training and validation cohort, even after stratification for the PREDISC score. Consistently with internal validation, the external validation confirmed a reliable performance for model 3 (PREDISC 0-1 vs. 4-8) and for the model discriminating DWI positive *vs.* negative patients, with an accuracy of 78.9% and 71.4%, respectively (Figure 5).

Considering the combined training and validation cohorts, 27 of 68 patients displayed symptoms suspicious for TIA lasting less than 1 hour (Table XI, see https://www.ahajournals.org/journal/str). NP diameter was lower in patients exhibiting symptoms lasting < 1 h compared to Ctrl (P=0.002); NP concentration was higher in both groups of patients (with symptoms < 1 h or ≥ 1 h; P<0.001), compared to Ctrl. Among the 10 EV surface antigens differentially expressed in patients stratified according to the PREDISC score, the MFI for CD2, CD62P, CD42a, CD44, and CD326 was higher in patients exhibiting symptoms lasting less than 1 h compared to Ctrl, whereas CD8, CD2 and CD62P were higher in patients with symptoms for more than (or equal to) 1 h compared to Ctrl. Interestingly, HLA-DRDPDQ, CD209, CD41b, and CD142 were found as discriminants of patients exhibiting symptoms lasting less than 1 hour (Table XI and Figure IIA, see https://www.ahajournals.org/journal/str). Overall, the EV specific signature was able to discriminate patients exhibiting symptoms lasting less than 1 hour and those with symptoms for more than (or equal to) 1 hour from Ctrl, as shown by LDA and reported in the 2D canonical plot (Figure IIB, see https://www.ahajournals.org/journal/str).

Accordingly, the RF models displayed a similar accuracy when applied to patients with symptoms lasting < 1 hour (accuracy ranging between 72.7% and 93.8%), or to patients with symptoms for ≥ 1 hour (accuracy 80.0-85.7%; Figure III, see https://www.ahajournals.org/journal/str). The highest accuracy was reached by the model discriminating patients with symptoms suspicious for TIA and PREDISC score 0-1 from patients with a PREDISC score 4-8, in case of symptoms lasting less than 1 hour (15 of 16 patients correctly classified; RF model 3, Figure IIIB-D, see https://www.ahajournals.org/journal/str).

DISCUSSION

We have found that the profile of EV-surface antigens appears to be different in patients with symptoms suspicious for TIA, showing a significant increase in the number and reduced diameter of serum-derived nanoparticles, compared to healthy controls. We also documented a relevant correlation between the nanoparticle concentration and the likelihood of having a TIA, as determined by the PREDISC score. Among EV-surface markers, some of them, of different cellular origin (leucocytes, platelets, endothelium) were identified as potential discriminants and were associated to the likelihood of having a TIA at multivariate analysis, after correction for potential confounders. On these premises, we proposed a diagnostic model based on a combined clinical, imaging and biological approach.

We observed an increase in the total number of circulating EVs in patients at highest likelihood of TIA. Our results are in line with previous studies, describing increased levels of PEVs in patients with TIA or lacunar infarcts.[22] More recently, Agouni et al. demonstrated an increased number of EVs positive for CD146, CD62E, CD62P, CD235a, CD66b, and CD45[18] in patients with acute ischemic stroke or with a TIA. However, these studies did not focus on a selected population of TIA patients, and they did not explore the diagnostic potential of EVs. A systematic meta-analysis evaluating 988 patients with stroke,

demonstrated a significant increase in the total number of EVs, and of specific EV subpopulations (endothelial- EEV(s), platelets- PEV(s), erythrocyte-, and leucocyte- derived EVs).[15, 17] Although less recent and small sample-size studies did not find a significant difference in the number of EVs in patients with stroke compared to subjects with TIA and controls,[18, 23] we think that these discrepancies may be due to the heterogeneity of the evaluated cohorts and to protocols for EV isolation and characterization. Nevertheless, the association between NP concentration and TIA was no longer significant, after correction for potential confounding factors, and it was not included in the diagnostic models. The release of EVs may be triggered by hypoxia, shear stress, vascular inflammation, thrombosis, endothelial dysfunction, and other ancillary processes shared by many cardiovascular disorders.[24, 25] In addition, levels of all EVs, have been correlated to severity, volume of ischemic lesions, and outcome in acute stroke.[26-29] In patients with TIAs, in analogy to ischemic stroke, EV increase appears to be a marker of brain ischemia, probably reflecting a cellular response to the complex mechanisms that underlie ischemic brain injuries.

We observed a reduced particle diameter in patients classified as “possible/probable” and “very likely” to have a TIA, according to the PREDISC stratification. In TIAs, where an early interruption of the ischemic injury occurs, smaller EVs could play a role of mediator, similar to what observed in ischemic strokes.[15] On the other hand, we could speculate that smaller EVs may be markers of inflammation, atherosclerosis, and/or vascular repair.[15, 17, 30, 31] Further studies are necessary to clarify these findings with potential diagnostic/therapeutic implications.

We found that 10 different EV-surface antigens were able to stratify patients for probability of having a TIA according to the PREDISC score. Indeed, the expression levels of the majority of them derived from leucocytes, endothelium and platelets (see supplemental methods for details), were found to be elevated

in patients with the highest probability of TIA (PREDISC 4-8), compared to controls and patients with a PREDISC score of 0-1. Moreover, three of them (CD42a, CD31, and CD14) were also increased in patients with an ischemic lesion in DWI compared to negative imaging. These findings are consistent with previous data showing higher numbers of PEVs and EEVs in patients with an ischemic stroke.[16, 18, 23, 27-29] PEVs play a central role in hemostasis and thrombosis; it has been suggested that the release of PEVs increased after platelet activation or apoptosis, and that these vesicles can trigger the coagulation cascade, promote leukocyte and endothelial cell adhesion, stimulate the secretion of inflammatory molecules, and contribute to thrombin generation and clot formation,[32-34] thus favoring atherosclerosis and ischemic stroke. Indeed, as a marker of activated platelets, CD62P was increased in 44 patients with stroke and 21 with TIA compared to matched healthy controls,[18, 27] whereas the number of CD142 (coagulation factor III, or tissue factor) -positive micro-particles was higher in stroke subjects, displaying an enhanced pro-coagulant activity.[35, 36] Similarly, EEVs have been associated with vascular inflammation in stroke patients, with disease progression and worsening of patient outcome.[37] Even if it is still unclear whether EVs may represent the cause or the consequence of endothelial dysfunction, endothelial injury can induce the formation of thrombosis, leading to the occurrence of ischemic stroke. In accordance with these hypotheses, CD31-positive EEVs increased in patients with acute stroke and were associated with disease severity.[16, 23] Hence, the qualitative analysis of surface antigens, which reflects inflammatory or pro-thrombotic processes that underlie cerebral ischemia, seems to be a promising measurable biomarker in patients with TIA.

Moreover, we provided a proof-of-concept that EV profiling could be integrated into a structured clinical and imaging approach and could facilitate the discrimination of patients based on the likelihood that their transient symptoms were most likely caused by brain ischemia as stratified by the PREDISC score. Even

in the absence of a comparable gold standard test, ROC curve analyses revealed a reliable diagnostic performance for each evaluated surface antigen and for a compound EV marker, calculated from their linear weighted combination to identify patients with a PREDISC score of 4-8. A similar performance was observed for discriminating patients with an ischemic lesion at DWI compared to negative imaging. Supervised machine learning algorithms were used, finally, to develop and validate diagnostic models discriminating patients at higher probability of a true ischemic event. The combination of EV antigen expression into a specific signature, accurately classified each subject according to their likelihood of TIA, and the RF algorithms were able to correctly discriminate the majority of subjects at training and validation of the models. In particular, an RF model detected those patients with an ischemic lesion in DWI with high accuracy and specificity. The relevance of symptom's duration in TIA is currently strongly debated.[2] We therefore wondered if the duration of symptoms could affect the performance of the diagnostic model. The proposed RF algorithms displayed a comparable accuracy when applied to patients exhibiting symptoms lasting less than 1 hour, or to those with symptoms for more (or equal to) 1 hour. These results strongly suggest the valuable potential of EVs as biomarkers for the diagnosis of a true ischemic TIA. Noteworthy, this method could be fully automated and easily applied to clinical routine.

Our study has different strengths. We provide evidence of a potentially reliable biomarker for TIA recognition. We systematically characterized a standard panel of 37 different EV-surface antigens and evaluated their accuracy in the diagnosis of TIA, after stratification for the PREDISC score. The diagnostic model was developed in a highly selected and well-characterized cohort of patients, accurately screened using a previously validated clinical and radiological scale (PREDISC score)[7] and tested by both a theoretical internal validation algorithm and in a real independent external validation cohort. We

propose the first machine-learning diagnostic algorithm based on an EV-specific signature, which may be integrated into management of these patients for identifying true ischemic events.

The present study has also several limitations. First, the PREDISC score was applied to stratify patients according the likelihood of an ischemic mechanism underlying a transient neurological deficit. This approach includes a structured neuroimaging analysis to assess the likelihood of an ischemic etiology for the symptoms. Formally, the term "TIA", as used in this study and in the PREDISC score is defined by the traditional terminology ("a sudden, focal neurological deficit of presumed vascular origin lasting less than 24 hours"). [1] As a result, our observations may not be generalizable to patients selected with a different TIA definition. Nevertheless, we propose that in the future, the term "TIA" should initially be used in a broader "acute cerebrovascular syndrome" concept to reflect that fact that until the evaluation is complete the etiology of the symptoms, and whether they are caused by brain ischemia, is typically uncertain.[38] Second, the relatively small sample size currently limits the generalizability of our diagnostic model. Third, all patients included in the analysis were enrolled in 2 Swiss referral centers; a selection bias cannot be excluded and further validation on a larger independent cohort should be provided in future studies to definitely confirm our results. Fourth, we did not perform a quantitative non-volumetric signal analysis of DWI and of its correlation with EV markers; this interesting research question was beyond the aim and the power of our study and might be addressed in a dedicated study. Finally, it is well known that EVs provide autocrine and paracrine signals to target cells[13] and surface antigens play important roles in mediating such effects. In the present study, we did not assess whether circulating EVs from patients with TIA may also act as signaling molecules, contributing to the ischemic process in these patients. This approach requires ex-vivo experiments performed on target cells (i.e., neurons and/or endothelial cells), which were not performed here but might help define whether EVs not only represent potential biomarkers, but also active players in the pathogenesis of ischemic stroke.

CONCLUSIONS

An EV-specific signature may help discriminate patients according to their likelihood of TIA. The profiling of EV surface antigen can be performed using a standardized and low-cost assay. This approach is minimally invasive for patients and could represent a potential “point-of-care testing” tool. The proposed diagnostic model could be applied to discriminate true ischemic TIA from TIA mimics, bringing promising diagnostic potential for EVs in acute cerebrovascular diseases. Larger, multicentric interventional studies are necessary to confirm our findings and evaluate the applicability of this novel approach in clinical practice.

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Supplemental Materials

Supplemental methods.

Supplemental Tables I-XI.

Online Figures I-III.

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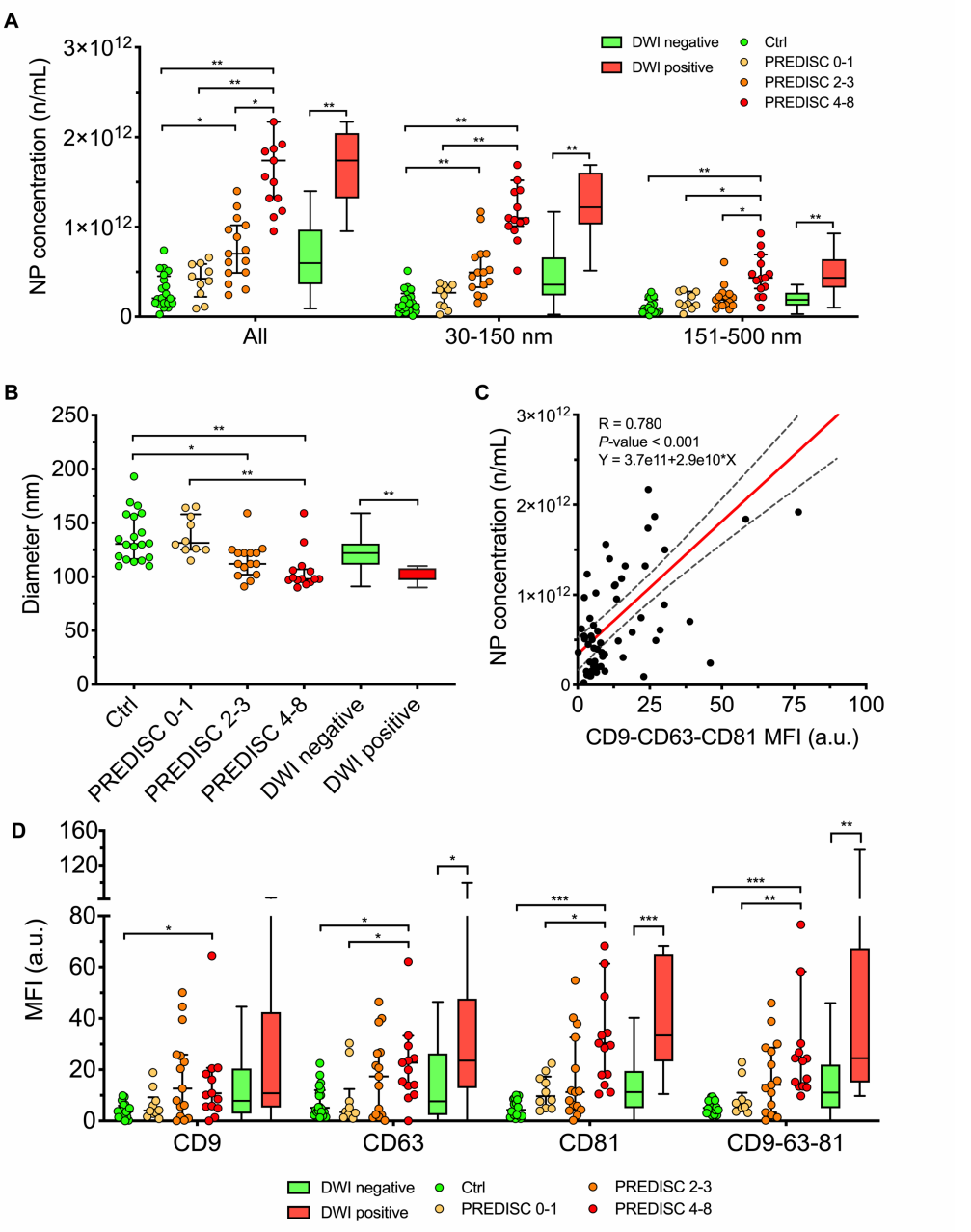


Figure 1. Nanoparticle tracking analysis.

Nanoparticle tracking analysis (NTA) characterization of particles (NPs) from patients with different likelihood of final diagnosis of transient ischemic attack (TIA) stratified by the PREDISC score (TIA "unlikely", n=10, yellow; TIA "possible-probable", n=15, orange; TIA "very likely", n=15; red), compared to controls (Ctrl, n=20, green). Whisker plots report data on patients with or without evidence of an ischemic lesion on multimodal magnetic resonance imaging (DWI, Diffusion-Weighted Imaging; green, DWI negative, n=27; red, DWI positive, n=13). (**A**) NP concentration (n/mL serum); data are shown for all NPs compared to smaller (30-150 nm) and larger (151-500 nm) fractions. (**B**) NP diameter (nm); (**C**) Correlation between NP concentration in NTA (y-axis) and CD9-CD63-CD81 median fluorescence intensity (MFI; x-axis) in flow cytometry (n=60): the regression line is reported in red with its 95% confidence interval. (**D**) Median fluorescence intensity (MFI, arbitrary unit; a.u.) of CD9, CD63, CD81, and mean fluorescence for CD9-CD63-CD81. Data are expressed as median and interquartile range. *P*-values of less than 0.05 were considered significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

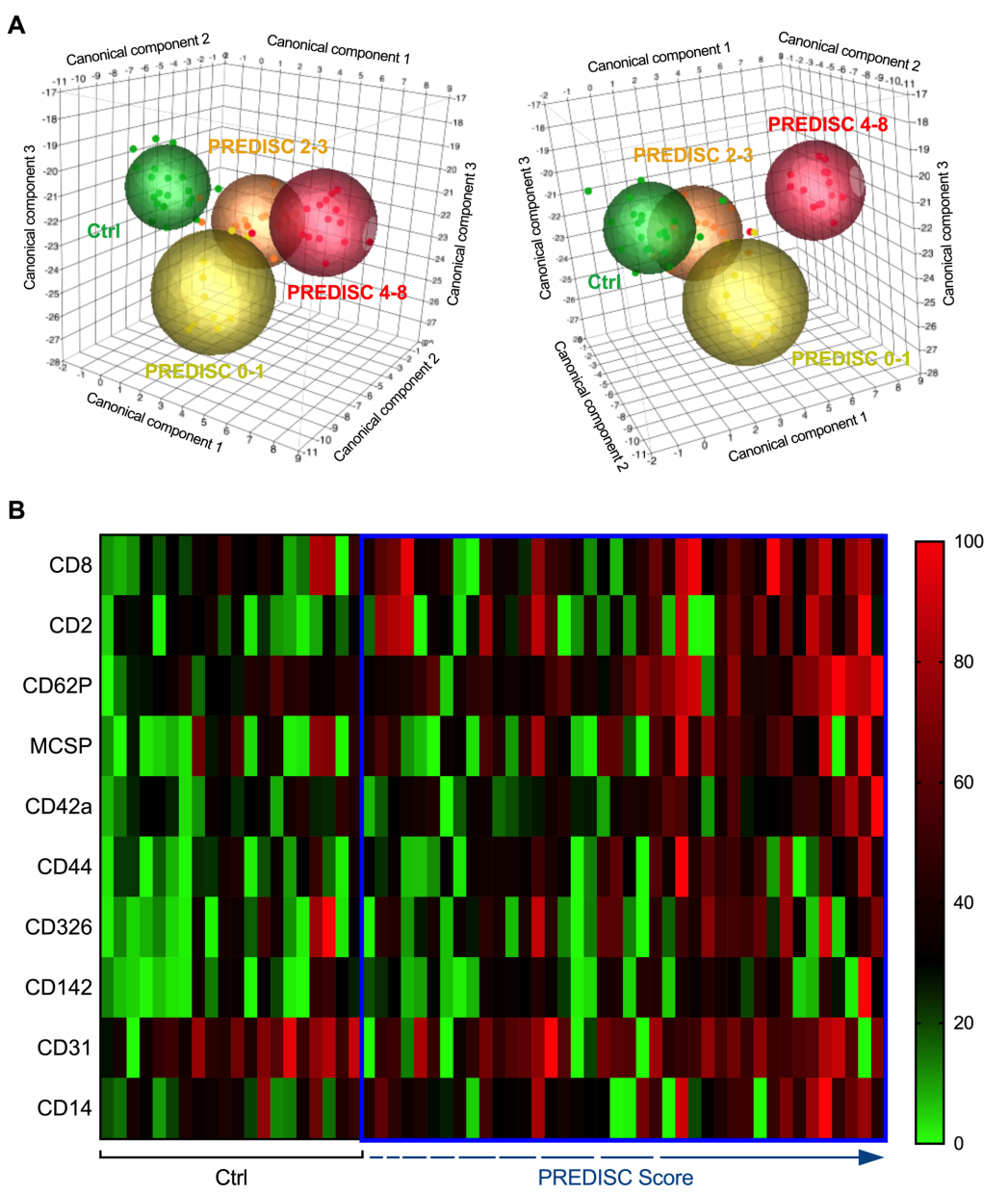


Figure 2. Flow cytometry analysis of EV-surface markers.
Flow cytometry (FC) characterization of EV-surface antigens. (**A**) 3D canonical plot (2 different perspectives) showing patients according their diagnosis (each patient is indicated by a point and diagnoses are represented by colored spheres: controls, Ctrl, green; TIA "unlikely", yellow, TIA "possible-probable", orange; TIA "very likely", red). The canonical axes of the plot (canonical 1, 2, and 3) are calculated by the LDA algorithm from weighted linear combinations of the 37 markers analyzed by FC. The spheres include patients with a linear combination coefficient that falls within the mean +/- SD (canonical 1, 2, and 3 +/- SD). (**B**) Heat map representing median fluorescence intensity (MFI) for 10 EV-surface antigens differentially expressed in patients with symptoms suspicious for TIA compared to Ctrl. Patients with symptoms suspicious for TIA were stratified according to the likelihood of a final diagnosis of TIA according to the increasing PREDISC score (from left to right, blue arrow; green = low fluorescence; red = high fluorescence).

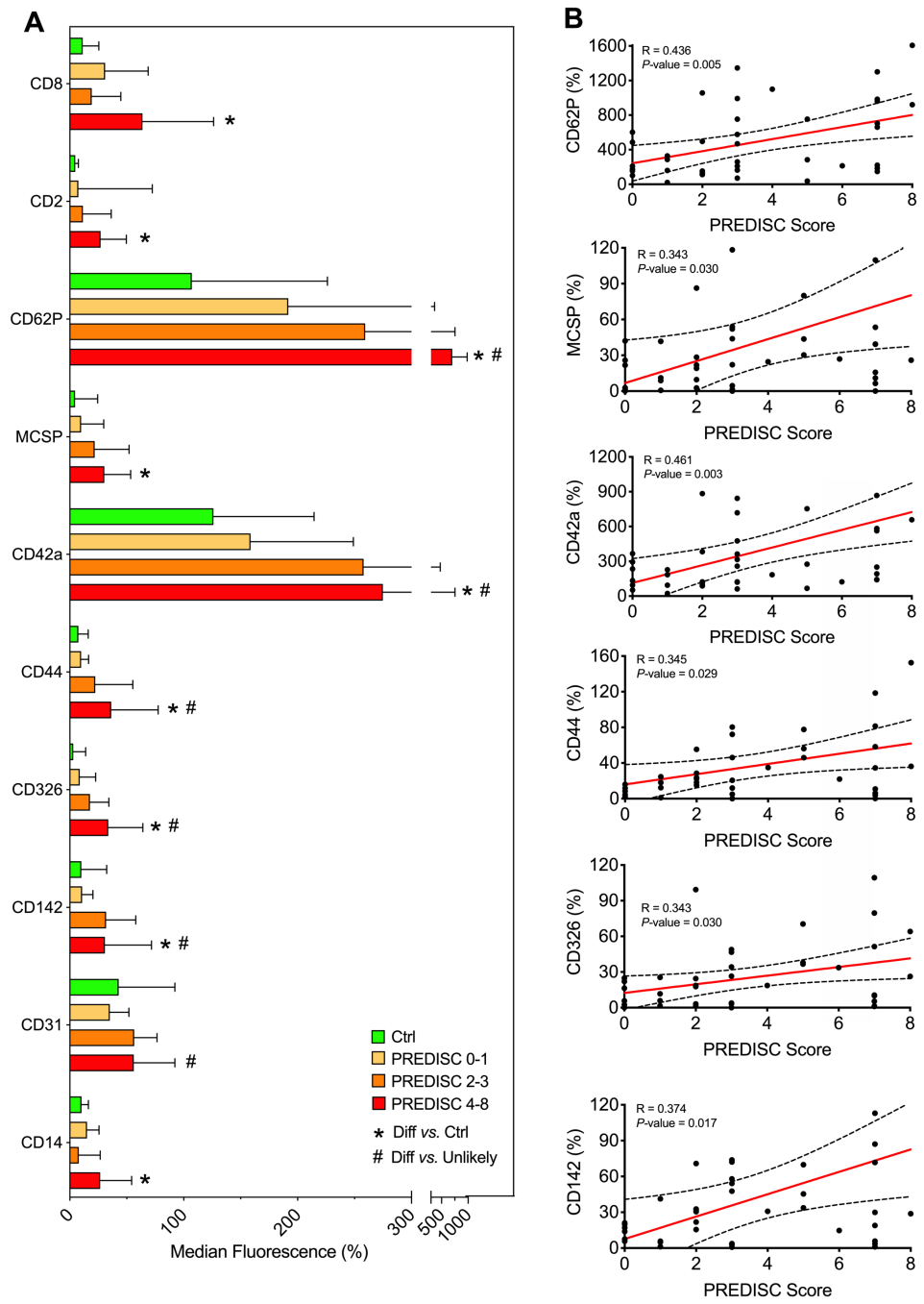


Figure 3. EV-surface marker correlations with PREDISC score.

(**A**) Median fluorescence intensity (MFI, expressed as percentage [%], after normalization to mean MFIs of CD9, CD63, and CD81) for 10 extracellular vesicle (EV) -surface antigens differentially expressed in patients with symptoms suspicious for TIA (stratified according to the likelihood of TIA according to the PREDISC score: TIA "unlikely", n=10, yellow; TIA "possible-probable", n=15, orange; TIA "very likely", n=15; red) compared to controls (Ctrl, n=20, green). *Significant difference compared to Ctrl; #Significant difference compared to TIA "unlikely" (**B**) Correlation between 6 selected EV-surface antigens and the PREDISC score was assessed by Pearson's R test (n=40); the regression line is reported in red with its 95% confidence interval. Data are expressed as median and interquartile range.

A

RF model 1		PREDICTED	
		PREDISC 0-1	PREDISC 2-3
REAL	PREDISC 0-1	8	2
	PREDISC 2-3	1	14
Model Accuracy			88.0%
Leave-one out validation			56.0%
Sensitivity			93.3%
Specificity			80.0%

B

RF model 2		PREDICTED	
		PREDISC 2-3	PREDISC 4-8
REAL	PREDISC 2-3	12	3
	PREDISC 4-8	1	14
Model Accuracy			86.7%
Leave-one out validation			66.7%
Sensitivity			93.3%
Specificity			80.0%

C

RF model 3		PREDICTED	
		PREDISC 0-1	PREDISC 4-8
REAL	PREDISC 0-1	9	1
	PREDISC 4-8	0	15
Model Accuracy			96.0%
Leave-one out validation			76.0%
Sensitivity			100.0%
Specificity			90.0%

D

RF model DWI		PREDICTED at Training		Leave-one out Validation	
		DWI (-)	DWI (+)	DWI (-)	DWI (+)
REAL	DWI (-)	26	1	24	3
	PREDISC 0-1	10	0	10	0
	PREDISC 2-3	14	1	12	3
	PREDISC 4-8	2	0	2	0
	DWI (+)	3	10	7	6
	PREDISC 0-1	0	0	0	0
	PREDISC 2-3	0	0	0	0
	PREDISC 4-8	3	10	7	6
Accuracy (%)		90.0		75.0%	
Sensitivity (%)		76.9		46.2%	
Specificity (%)		96.3		88.9%	

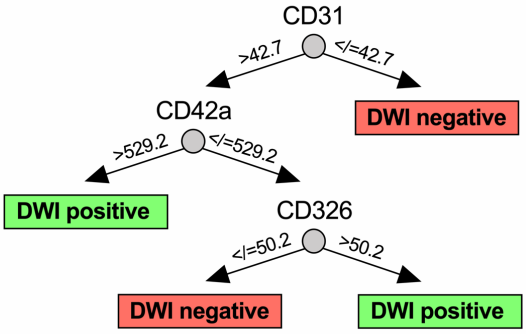
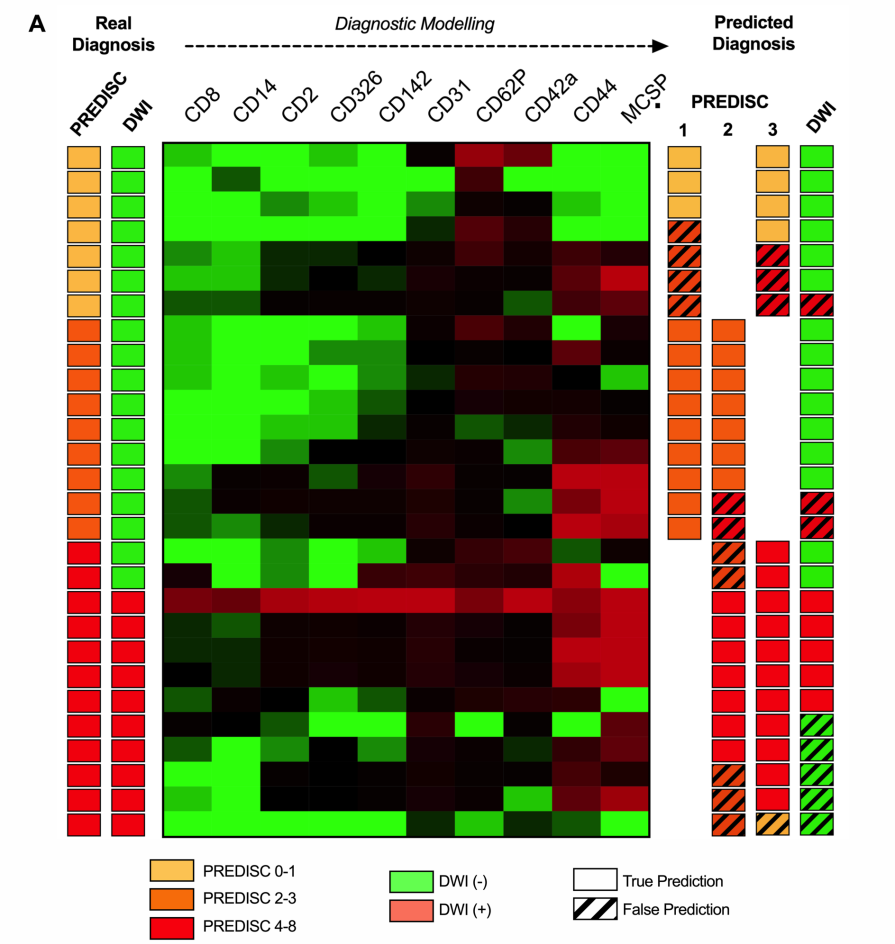


Figure 4. Diagnostic modelling.

A random forest (RF) model was developed in the training cohort (n=40) to discriminate patients with symptoms suspicious for transient ischemic attack (TIA) after stratification by the PREDISC score (**A, B, C**). The model was trained on the median fluorescence intensity levels of the 10 EV-surface antigens differentially expressed among the groups. (**A**) RF model 1 to discriminate patients with TIA "unlikely" (PREDISC 0-1; n=10) from "possible-probable" TIA (PREDISC 2-3; n=15); (**B**) RF model 2 to discriminate patients with "possible-probable" TIA (PREDISC 2-3; n=15) from TIA "very likely" (PREDISC 4-8; n=15); (**C**) RF model 3 to discriminate patients with TIA "unlikely" (PREDISC 0-1; n=10) from TIA "very likely" (PREDISC 4-8; n=15). (**D**) Representative classification tree for the DWI RF model and diagnostic performance in the discrimination of patients with an ischemic lesion in diffusion-weighted imaging (DWI positive; n=13) from patients with a negative imaging result (DWI negative; n=27). For each model, the confusion matrix reports real and predicted diagnosis, accuracy, sensitivity, specificity, and validation by the leave-one-out algorithm.



B

RF model 1		PREDICTED	
		PREDISC 0-1	PREDISC 2-3
REAL	PREDISC 0-1	3	4
	PREDISC 2-3	0	9
Model Accuracy			75.0%
Sensitivity			100.0%
Specificity			42.9%

RF model 2		PREDICTED	
		PREDISC 2-3	PREDISC 4-8
REAL	PREDISC 2-3	7	2
	PREDISC 4-8	5	7
Model Accuracy			66.7%
Sensitivity			58.3%
Specificity			77.8%

RF model 3		PREDICTED	
		PREDISC 0-1	PREDISC 4-8
REAL	PREDISC 0-1	4	3
	PREDISC 4-8	1	11
Model Accuracy			78.9%
Sensitivity			91.7%
Specificity			57.1%

RF model DWI		PREDICTED	
		DWI (-)	DWI (+)
REAL	DWI (-)	15	3
	DWI (+)	5	5
Model Accuracy			71.4%
Sensitivity			50.0%
Specificity			83.3%

Figure 5. External validation of machine learning models.
Diagnostic performances of random forest (RF) model 1, 2, 3 and DWI were confirmed by external validation in an independent cohort of patients (n=28). (**A**) Heat map representing median fluorescence intensity (MFI) for 10 EV-surface antigens differentially expressed in patients with symptoms suspicious for TIA compared to Ctrl (green = low fluorescence; red = high fluorescence). Final likelihood of TIA diagnosis according to the PREDISC score and imaging (DWI) results were shown by colors (TIA "unlikely", n=7, yellow; TIA "possible-probable", n=9, orange; TIA "very likely", n=12; red; DWI positive, n=10, light green; DWI negative, n=18, light red; true *vs.* false prediction are reported as blank vs. dashed square). (**B**) Confusion matrix reporting real and predicted diagnosis, accuracy, sensitivity, and specificity for the 4 RF models.

Table 1. Patient Characteristics

Variable	Ctrl [n=20]	TIA [n=40]	*P*-value
Age (years)	64±8.8	69±10.9	0.067
Sex (ref. male)	10(50.0)	19(47.5)	0.855
SBP (mmHg)	140±13.9	152±24.6	0.091
DBP (mmHg)	83±5.2	85±14.1	0.531
BMI (Kg/sqm)	27.4±3.01	25.7±4.40	0.202
Risk factors and past medical history			
Previous stroke (ref. yes)	0(0.0)	2(5.0)	0.309
Previous minor stroke (ref. yes)	2(10.0)	4(10.0)	1.000
Hypertension (ref. yes)	9(45.0)	26(65.0)	0.139
Hyperlipidemia (ref. yes)	13(65.0)	34(85.0)	0.076
Smoking (ref. yes)	5(25.0)	9(22.5)	0.829
Chronic heart disease (ref. yes)	0(0.0)	4(10.0)	0.143
Peripheral vascular disease (ref. yes)	1(5.0)	2(5.0)	1.000
Diabetes mellitus (ref. yes)	1(5.0)	2(5.0)	1.000
Renal disease (ref. yes)	2(10.0)	2(5.0)	0.464
Drugs at the time of TIA			
Antiplatelets (ref. yes)	5(25.0)	12(30.0)	0.685
Anticoagulants (ref. yes)	0(0.0)	1(2.5)	0.476
Antihypertensives (ref. yes)	9(45.0)	21(52.5)	0.584
Hypolipemic agents (ref. yes)	5(25.0)	12(30.0)	0.685
Biochemical parameters			
Glucose (mmol/L)	6.6±2.87	5.8±1.18	0.136
HDL (mmol/L)	1.4±0.49	1.4±0.46	0.899
LDL (mmol/L)	3.1±0.72	3.5±1.17	0.333
Triglycerides (mmol/L)	2.3±1.86	3.1±1.89	0.173
Creatinine (umol/L)	80±24.4	82±18.9	0.792
Platelets (n/L*1000)	244±52.4	252±65.1	0.636
White blood cells (n/L*1000)	7.5±2.48	8.6±8.76	0.608
C reactive protein (mg/L)	2.4±1.54	2.3±2.31	0.823

Clinical and biochemical characteristics of patients with symptoms suspicious for transient ischemic attack (TIA) compared to controls (Ctrl). SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body Mass Index. *P*-values of less than 0.05 were considered significant and are reported in bold.