Platelet-derived extracellular vesicles in Huntington's disease

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Abstract

The production and release of extracellular vesicles (EV) is a property shared by all eukaryotic cells and a phenomenon frequently exacerbated in pathological conditions. The protein cargo of EV, their cell type signature and availability in bodily fluids make them particularly appealing as biomarkers. We recently demonstrated that platelets, among all types of blood cells, contain the highest concentrations of the mutant huntingtin protein (mHtt) – the genetic product of Huntington's disease (HD), a neurodegenerative disorder which manifests in adulthood with a complex combination of motor, cognitive and psychiatric deficits. Herein, we used a cohort of 59 HD patients at all stages of the disease, including individuals in pre-manifest stages, and 54 healthy age and sex-matched controls, to evaluate the potential of EV derived from platelets as a biomarker. We found that platelets of pre-manifest and manifest HD patients do not release more EV even if they are activated. Importantly, mHtt was not found within EV derived from platelets, despite them containing high levels of this protein. Correlation analyses also failed to reveal an association between the number of platelet-derived EV and the age of the patients, the number of CAG repeats, the Unified Huntington Disease Rating Scale total motor score, the Total Functional Capacity score or the Burden of disease score. Our data would therefore suggest that EV derived from platelets with HD is not a valuable biomarker in HD.

Key words – Biomarkers, plasma, immune system, mutant huntingtin protein, Huntington's disease, platelets-derived extracellular vesicles

Introduction

The need to find useful and reliable biomarkers of disease onset and progression in chronic neurodegenerative disorders of the central nervous system are becoming ever more critical as we enter a new therapeutic era with these diseases. In one such disorder, Huntington's disease (HD), this search has included cerebrospinal fluid (CSF) and blood sampling targeting neurotransmitters such as GABA [1, 2], as well as inflammatory markers such as clusterin [3], interleukin 6 and 8 [4] and microglial activation [5, 6]. Magnetic resonance methods assessing structural changes and atrophy have also been explored [7, 8] along with a range of other imaging approaches (see summary **Fig.S1** [3, 4, 9–51]). Of late however, there has been much excitement relating to the concentrations of neurofilament light chain [52, 53] and mutant huntingtin (mHtt) – the genetic product of HD – and their correlations with disease course [18, 54, 55]. These proteins in turn may be released from circulating blood cells in the form of extracellular vesicles (EV).

EV encompass entities such exosomes stored in multivesicular bodies and microvesicles derived from the plasma membrane. Based on the process dictating their release, EV are subdivided into three major groups: 1) exosomes, produced by exocytosis of multivesicular bodies (diameter ranges ≈50–150 nm), 2) microvesicles, also termed microparticles or ectosomes, generated by cytoplasmic membrane budding and fission (diameter ranges ≈100–1000 nm) and 3) apoptotic bodies, released by apoptotic cells (diameter ranges >1000 nm) [56]. However, these definitions are still subject to change given the facts that 250 nm in size exosomes have been described, apoptotic cells can release exosome-like vesicles and there is no specific markers of EV subtypes [57–59]. The International Society of Extracellular Vesicles endorses EV as the term to use, as it more liberally encompasses all vesicle types released by cells [60].

What is particularly appealing about EV is that they are shed from all eukaryotic cell types [61]; a phenomenon that is exacerbated in pathological conditions [62, 63]. All EV are composed of membrane proteins and lipids, as well as cytoplasmic components of the cell from which they originate, such as mRNA and miRNA, organelles or infectious particles (e.g. prions, virus) [57]. Their protein cargo, cell signature and availability in bodily fluids, such as blood plasma, make EV very attractive biomarkers and have been studied in blood, cerebrospinal fluid (CSF), urine and brain in several different neurodegenerative diseases. As platelets are particularly rich in mHtt

[64], we sought to investigate whether EV derived from the circulating blood cells in HD would correlate with various clinical aspects of the disease.

Materials and methods

Ethic statement and participant recruitment

Ethical approval from the institutional review boards of the various centers involved in this study were obtained (CHU de Québec, #A13-2-1096; CHUM, #14.228; Cambridge Central Regional Motor Ethics Committee, REC #03/303 & #08/H0306/26; and Cambridge University Hospitals Foundation Trust Research and Development department, R&D #A085170 & #A091246) in accordance with the Declaration of Helsinki. Written informed consents were further obtained from all participants included in this work.

Blood samples were collected from HD patients and their respective healthy age- and sex-matched donor controls (CTRL) (**Table 1**). The patients' scores on the Unified Huntington Disease Rating Scale (UHDRS) motor score, Total Functional capacity (TFC) and burden of disease (BDS) were collected. All clinical evaluations were conducted within 6 months of the blood being taken. Participants were further asked to fill out a questionnaire related to health issues and medication, and a full blood count was performed on all individuals on the day of blood sampling. Comorbidities were determined based on medical information provided by the participant and/or caregiver (see **Table 1**). None of the comorbidities reported in these CTRL subjects had an impact on the number of EV derived from platelets (**Fig. S2A**).

Comparative blood samples were also obtained from positive control patients with another neurodegenerative disorder, i.e. mild stage Parkinson's disease (PD) patients (2 men, 1 woman, mean 70 years of age). Their clinical evaluation included measures on the Unified Parkinson Disease Rating Scale (UPDRS, mean score = 39), Hoehn and Yahr stage (H&Y, mean score = 1) stage, the Mini Mental State Examination (MMSE, mean score = 29), the Addenbrooke's Cognitive Examination (ACE, mean score = 95) and the Beck Depression Inventory (BDI, mean score = 2).

Preparation of platelets

Platelets – Citrated blood tubes were centrifuged for 10 minutes at 282g at room temperature. Platelet-rich plasma was collected and to this was added 1/5 of the volume of acid citrate dextrose (ACD) and 1/50 of the volume of ethylenediamine tetraacetic acid (EDTA) 0.5M, before the complete solution was centrifuged twice for 2 minutes at 400g and 5 minutes at 1300g. The subsequent platelet pellet was dissolved in 100µl of Tyrode pH 6.5, 900µl of Tyrode pH 7.4, 200µl of ACD and 20µl of EDTA 0.5M. A fraction of the platelets' pellet was centrifuged for 5 minutes at 1300g and dissolved in 100µl of lysis buffer with protease and phosphatase inhibitor for Western blot analysis. All samples were stored -80°C.

Preparation of EV samples

EV were quantified by flow cytometry within two distinct types of bio-samples: platelet-free plasma (PFP) or from activated platelets. In order to obtain these distinct bio-fluids, series of centrifugations were used.

PFP – Citrated blood tubes (BD Vacutainer #363083) was centrifuged twice for 15 minutes at 2500g at room temperature and the supernatant was harvested and stored at -80°C within 2 hours of sampling. All samples were stored -80°C.

Activated platelets – For experiments related to activated platelets, platelet pellets were prepared as indicated in the section "*Preparation of platelets*" above. A fraction of the pellet was centrifuged for 5 minutes at 1300g and diluted at a concentration of 100 million/ml in Tyrode pH7.4. Following this step, 5mM of calcium chloride was added and the platelets were separated in three fractions for activation: incubation for 30 minutes at 37°C (Resting); incubation for 30 minutes at 37°C with 5 µg/ml of collagen (Collagen activation); incubation for 30 minutes at 37°C with 0.5U/ml of thrombin (Thrombin activation). In all three fractions after activation, 20mM of EDTA was added and centrifuged for 5 minutes at 3200g to separate the activated-platelet supernatants from the platelet proteins. All samples were stored -80°C.

Flow cytometry quantification

For platelet EV quantification, we used a FACS Canto II Special Order Research Product equipped with a forward scatter (FSC) coupled to a photomultiplier tube (FSC-PMT) and a small particle

option, as described previously [56, 62, 65]. The forward scatter (FSC) on this dedicated equipment is coupled to a photomultiplier tube (PMT) with a 488 nm solid state, 100 mW output blue laser (rather than the conventional 20 mW), and includes a 633 nm HeNe, 20 mW output red laser and a 405 nm solid state diode, 50 mW output violet laser. The flow cytometer includes an FSC-PMT and a Fourier optical transformation unit, which reduces the background/noise and increases the angle of diffusion, thereby enhancing the detection of small-diameter particles. The assigned voltage for FSC-PMT was 300 volts (V). For side scatter (SSC), the assigned voltage was 460 V and the threshold 200. Voltage was set to 360 V for FITC, 450 V for PE-Cy7, 500 V for Deep Red (or APC), 400 V for PE, 500 V for Alexa Fluor 700 and 450 V for V450. Acquisition was performed at low speed (~10 µ l/min) and, to remain quantitative, a known quantity of polystyrene microsphere beads (Polybead® polystyrene 15.0 µm microspheres Alexa Fluor700, Polysciences: 18328) was added to each tube. Silica particles (Kisker Biotech GmbH & Co. Steinfurt, Germany) of known dimensions (100 nm, 500 nm and 1 µ m in diameter) were used for instrument set-up standardization [56, 62, 65].

For all experiments, diluted annexin-V buffer (BD Pharmingen, Mississauga, ON, Canada) and phosphate buffered saline (PBS) were filtered on 0.2 µm pore size membranes (PALL, PN4612). To quantify platelet-derived EV, we used the surface markers CD41+ (platelets) (5µl) coupled to annexin-V staining (3µl). PFP or activated-platelet supernatants (5µl) were incubated with Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK) 10mM (Calbiochem, Etobicoke, ON, Canada) for 5 minutes followed by a 30-minute incubation with V450-conjugated mouse anti-human CD41a (1/20, BD Pharmingen: clone HIP8) and PerCP-Cy™5.5-conjugated annexin-V (1/20, BD Pharmingen: 561431) in a final PBS volume of 100µl, all at room temperature. Finally, samples were diluted to a volume of 2ml prior to FACS analysis. To determine background noise levels, antibody mixes were incubated in the absence of PFP sample and unlabeled PFP was used as a negative control. Every sample was re-identified to ensure that the experimenter was blind to clinical status.

Quantification of Htt and αSynuclein by Western blot

Quantification of Htt and αSynuclein, the latter being the main protein involved in pathology of PD, were determined by western blot analysis on pellets of resting or activated (thrombin or collagen) platelets from HD and from positive control patients with PD, respectively. Fifteen µg

of platelet proteins were mixed with 1x DTT and 5x Sample buffer and then water was added to give a final volume of 30µl. The samples migrated for 105 minutes at 100V on a Tris-acetate gel (3-8%) and were transferred to a polyvinylidene difluoride (PVDF) membrane overnight at 20V followed by 20 minutes at 100V. Membranes were incubated with the primary mouse anti-huntingtin antibody (1:1000, Milipore: Mab2166) or the mouse anti-αSyn (1:16,5, AbCam: Ab75305) followed by an HRP-goat anti-mouse (1:250 000, Jackson ImmunoResearch laboratories: 115.035.166) to detect the protein of interest. Chemiluminescence was quantified using a ThermoScientific MyELC Imager. Immunoblot band intensity was quantified with ImageJ Analysis Software (National Institutes of Health, http://imagej.nih.gov/ij).

Quantification of soluble mHtt in platelet-derived EV

EV from thrombin-activated supernatants (see section *Preparation of EV samples - Activated platelets*) were concentrated/enriched by centrifugation performed at 20000g for 90 minutes and dissolved in 150µl of lysis buffer with protease and phosphatase inhibitor. Total protein in cell lysates was quantified using a BCA Protein Assay Kit (catalogue #23225; Thermofisher) and around 180 ng/ml of proteins *per* samples were obtained. mHtt protein levels in each lysate was quantified by performing a dilution series (from 30µg/ml to 18,75µg/ml) of the EV lysates using the 2B7-MW1 Singulex detection assay, as previously described [55]. This technology can detect solid signals of mHtt as low as 4 pg/ml. In brief, samples were diluted in 6% BSA, 0.8% Triton X-100, 750 mM NaCl, and complete protease inhibitor. Samples were placed onto a 96-conical assay plate (catalogue P-96-450V-C; Axygen) and the 2B7 antibody coupled to magnetic particles was added. The plate was sealed, incubated and stirred at room temperature for 1 hour. After washing, MW1 antibody was added to the plate. The plate was sealed, incubated and stirred at room temperature for 1 hour. Plates were washed and the antibody-antigen complex was transferred to a new 96-conical assay plate to eliminate non-specific binding events to the plastic. After washing, elution buffer and the eluted detection antibody were transferred to a Nunc 384-well analysis plate (catalogue 264573; Sigma-Aldrich) and neutralized with Tris buffer (Tris, 1 M, pH 9). The analysis plate was spun down to eliminate foaming and bubble formation, sealed, and subsequently analyzed with the Erenna Immunoassay System (Singulex).

EV immunoproteomic characterization by Western blot

Supernatant of thrombin-activated platelets (see section *Preparation of EV samples - Activated platelets*) from 3 healthy donors (women, average age of 26 years with no-comorbidities nor medicament intake) were concentrated/enriched by centrifugation performed at 20000g for 90 minutes. The pellet – containing EV derived from platelets – was dissolved in 40µl of lysis buffer with protease and phosphatase inhibitor. Total protein in EV lysates was quantified using a BCA Protein Assay Kit (catalogue #23225; Thermofisher) at a concentration of approximately 500 ng/ul. Ten ug of EV protein derived from platelets and platelets lysate (as control for the experiment) were mixed with 5x Laemmli buffer and sufficient water to make up a final volume of 40 µl. Samples were migrated on a 10% SDS-PAGE for 1 hour and 45 minutes at 100 V and transferred to a PVDF membrane at 20 V. Membranes were subsequently incubated overnight at 4˚C with primary antibodies. The candidate proteins were detected using antibodies against platelet microvesicle marker CD41a (1:1000, Abcam: ab63983); cytoskeleton marker β-actin (1:5000, Abm: G043); the EV markers, tumor susceptibility 101 (TSG101) (1:100, Abcam: ab83 clone 4A10) and ALG-interacting protein X (ALIX) (1:200, Santa Cruz: sc-53538 clone 3A9); and the mitochondrial marker voltage-dependent anion channel (VDAC) (1:1000, Cell Signaling: 4661). Membranes were washed with PBS-Tween 0.1% and incubated with the corresponding secondary antibody conjugated with horseradish peroxidase: goat anti-rabbit (1:25000, Jackson ImmunoResearch, West Grove, PA: 111-035-144) or goat anti-mouse (1:25000, Jackson ImmunoResearch: 115-035-166) for 1 hour at room temperature. Membranes were visualized by myECL imager (Thermo Fisher Scientific, Waltham, MA) after a 2-minute incubation in chemiluminescence reagents (Luminata Forte; EMD Millipore). Immunoblot band intensity was evaluated with ImageJ Analysis Software and the most representative result of the 3 donors compared is shown in **Fig.S2B** (National Institutes of Health, http://imagej.nih.gov/ij).

Statistical analyses

For **Table 1, Fig.1A-B and Fig.S2A**, comparisons between groups were obtained by Mann-Whitney U test or Kruskal-Wallis ANOVA. Correlations reported in **Fig.1C-G** were determined using Pearson's correlation test. For analyses pertaining to **Fig.1H**, data were expressed as the mean on a theoretical ratio of 1 on the western blot intensity and compared using the Wilcoxon signed rank test. Theoretical intensity came from non-activated platelets. Statistical analyses were performed using a one-sample t-test. In **Fig.1I**, comparisons between groups were

obtained using a one-way Anova followed by Bartelett's test. All statistical analyses were performed using Prism®6.0 (GraphPad Software, LaJolla, CA) or JMP®13 (JMP® Software, SAS Institute Inc., Cary, NC).

Results

This study involved cohorts of HD patients (n=59) at all stages of disease, along with healthy CTRL (n=54) (**Table 1**) for a total of 113 participants. In PD, αSynuclein is also expressed in platelets. Thus, for comparative measures on protein content and release by EV, a much smaller pool of PD patients was also included in the study (see **Materials and Methods section**).

We first evaluated the release of platelet-derived EV in PFP by FACS (**Fig.S2C**). We did not find differences between HD patients at any stage of disease (CD41+EV/ml p=0.485, CD41+EV/platelets p=0.145, **Fig.1A**) and their respective CTRL. Similar results were obtained when patients of all disease stages were pooled (data not shown). We confirmed the presence and origin of EV derived from activated platelets by immunoblot and that EV preparations contained proteins reportedly present in platelet-derived EV [60, 66, 67] such as the platelet-surface protein CD41a, the cytoskeleton protein actin, the EV proteins TSG101 and ALIX, and the mitochondrial proteins VDAC (**Fig.S2B**).

We further quantified platelet-derived EV in PFP from non-activated and activated platelets exposed to either collagen or thrombin, two agents known to trigger platelet activation and EV release. The total number of platelet-derived EV in either the resting or activated platelet supernatant was not significantly different between pre-manifest, manifest HD and their respective CTRL (Resting platelets p=0.998, Collagen activation p=0.221, Thrombin activation p=0.725, Resting/Collagen p=0.221, Resting/Thrombin p=0.783, **Fig.1B**). This data combined demonstrates that the presence of the mutant form of Htt in platelets does not impact on EV release *in vivo* and *in vitro*.

We next evaluated the potential of platelet-derived EV to serve as a biomarker with respect to various clinical features/measures of the disease. However, correlation analyses failed to reveal an association between the number of platelet-derived EV and the age of the patients (p=0.804, **Fig.1C**), the number of CAG repeats (p=0.899, **Fig.1D**), the UHDRS score (p=0.553, **Fig.1E**), the

TFC score (p=0.481, **Fig.1F**) or the Burden of disease score (p=0.121, **Fig.1G**). We obtained similar results when we pooled patients at all disease stages (data not shown).

Finally, western blotting analysis revealed that platelets derived from HD patients contained the same amount of normal Htt (Htt) protein even in activated states (*p<0.05; **p<0.01, **Fig.1H**), suggesting that Htt is not released by activated platelets. This was confirmed by TR-FRET analysis (One-way Anova p=0.994; Bartlett's test p=0.532, **Fig.1I**) where the soluble form of the mutant protein, mHtt, was found to be equivalent across all HD stages and not different from the control subjects, suggesting that mHtt is also not present in EV released from platelets in HD. This result contrasts with αSynuclein which we evaluated in an independent cohort of positive control of PD patients and which we showed to be released by activated platelets (**Fig.1H**).

Discussion

We evaluated EV derived from platelets as a biomarker of disease in a cohort of 59 HD patients at all stages of the disease, including pre-manifest individuals, and 54 age and sex-matched controls. This undertaking built on our most recent reports demonstrating that platelets, which are the second most abundant cell type in the blood, show the highest concentrations of mHtt [64]. Based on this, we sought to investigate whether EV derived from platelets could serve as a biomarker of HD. We first observed that platelets derived from pre-manifest and manifest HD patients do not release a greater number of EV when they are either in a resting or activated state. Consequently, correlation analyses failed to reveal an association between the number of platelet-derived EV and the age of the patients, the number of CAG repeats, the UHDRS score, the TFC score or the Burden of disease score whether patients were analyzed according to individual disease stages or when they were all pooled together. This is in contrast to a recent study we have undertaken which shows correlations between the number of vesicles derived from erythrocytes of PD patients and their total UPDRS score [68]. This therefore highlights the difference in EV behaviour in PD compared to HD [68].

To our knowledge, EV have never been explored as a potential biomarker in HD and our study would suggest that EV derived from platelets are not likely to be useful in this disease. In particular, activated platelets in HD do not release more EV indicating that this pro-

inflammatory context is not perturbed by mHtt load - given this protein is known to be highly expressed in these cells. The role of Htt in platelets is unknown, but the fact that HD patients do not overly suffer from bleeding-related disorders [20], suggest that despite its abundance in platelets, its mutated form does not interfere with platelet EV release.

In summary, we would conclude that platelet derived EV are not useful biomarker in HD despite these cells containing high levels of mHtt. Thus the search for better circulating blood measures continues, although the recent work looking at NFL levels in the plasma of HD patients, holds promise.

Figure legends

Table 1. Participant information. Disease severity was evaluated within 6 months of blood sampling. Comorbidities were determined from medical information reported by the participant or caregiver. Statistical analyses were performed using Mann-Whitney U test or Kruskal-Wallis test (**p*<0.05). **Abbreviations**: BDS, Burden of Disease Score; CTRL, Control; F, Female; HD, Huntington's disease; M, Male; NA, not applicable/available; Pre-HD, premanifest Huntington's disease patient; TFC, Total Functional Capacity; UHDRS, Unified Huntington's Disease Rating Scale.

Figure 1. Quantification of platelet-derived EV and their relation to various clinical aspects. (**A**) Quantification of platelet-derived EV in PFP of Pre-HD and HD patients as well as their age and sex-matched healthy CTRL revealed no significant differences for the CD41+EV/ml (CTRL, n=53; Stage 1, n=14; Stage 2, n=13; Stage 3, n=12; Stage 4, n=9; Pre-HD, n=10) and the number of CD41+EV/platelets (CTRL, n=53; Stage 1, n=14; Stage 2, n=12; Stage 3, n=11; Stage 4, n=9; Pre-HD, n=10). (**B**) Quantification of EV in the activated-platelet supernatants of Pre-HD/HD patients and their age and sex-matched healthy CTRL revealed no significant difference (CTRL, n=5. Pre-HD, n=5, HD all stages, n=5). No statistically significant correlations were found between CD41+EV/platelets and (**C**) age, (**D**) CAG repeats, (**E**) UHDRS score, (**F**) TFC score nor (**G**) Burden of disease score. Correlations were determined using Pearson's correlation **p*<0.05. (**H**) Quantification of Htt and αSynuclein in platelets and activated platelets revealed the release of αSynuclein (n=3) but not Htt (n=4). Data are expressed as mean using a theoretical ratio of 1 for the western blot intensity. Statistical analyses were performed using a one-sample t-test, **p*<0.05; ***p*<0.01. (**I**) Quantification by 2B7-MW1 Singulex detection assay revealed the absence of soluble mHtt within isolated extracellular vesicles (each group is represented by a pool of 5 participants). **Abbreviations:** αSyn, α-synuclein; EP, event photons; EV, extracellular vesicles; HD, Huntington's disease; Htt, Huntingtin; Pre-HD, Pre-manifest; S/B, Signal to Background; TFC, Total Functional Capacity; UHDRS, Unified Huntington's Disease Rating Scale; yrs, years.

Figure S1. Identified biomarkers in HD. Summary of the literature for all reported biomarkers of HD in CSF, blood, urine and brain. **Abbreviations:** DOPA: 3,4-dihydroxyphenylalanine; DOPAC: 3,4-dihydroxyphenylacetic acid; IL-6: Interleukin 6;IL-8: Interleukin 8; NMDA: N-methyl-D-aspartate; mHtt: mutant huntingtin; Cu/Zn-SOD: Cu/Zn Superoxide Dismutase; HD: Huntington disease; PCYT1A: Phosphate Cytidylyltransferase 1, Pre-HD : premanifest, Choline, Alpha; YKL-40: chitinase-like protein-40; 5-HIAA: 5-hydroxyindoleacetic acid; 8-OHdG: 8-hydroxy-2' -deoxyguanosine; 8-oxodG6: 8-oxo-7,8-dihydro-2'-deoxyguanosine; ↑ increase; ↓ decrease; √ presence

Figure S2. (**A**) Absence of statistical differences in comorbidities of healthy control patients - which including depression (p=0.2455), diabetes (p=0.0749), hypertension (p=0.9664), hypercholesterolemia (p=0.3615), allergies (p=0.9904) and anxiety (p=0.4675) - and counts of EV derived from platelets. EV from patient without comorbidities were further compared to patients with one or more comorbidities. Again, no statically significant differences were found (p=0.6745). Statistical analyses were performed using the non-parametric Mann Whitney test. (**B**) Immunoblot of CD41a, ALIX, TSG101, actin and VDAC in platelet-derived EV or platelets (Plts). Data are representative of 3 independent experiments. (**C**) **Left panel:** After the

acquisition of fluorescent signals, an initial gating was performed on all data to exclude counting beads from files. In this experiment, 144 beads were counted. **Right panel:** Representation of SSC-H (granularity) and FSC-PMT-H (relative size) dot plots of platelet-derived EV in PFP detecting using PerCP-CyTM5.5-conjugated annexin V and V450-conjugated antibodies directed against CD41. The size of platelet-derived EV ranged between 100 and 1000nm. **Abbreviations:** ALIX, programmed cell death 6 interacting protein; EV, extracellular vesicles; plts, platelets; TSG101, tumor susceptibility gene 101 protein; VDAC, Voltage-dependent anion-selective channel 1.

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Author Contributions

H.L.D. participated in experiments, data analysis and interpretation and preparation of figures. She wrote the first draft of manuscript and helped with subsequent revisions.

J.L.P. participated in the design of the experiments and various aspects of the study including blood collections, experiments and data analysis.

I.S.-A. participated in the design of the experiments and blood collections, took part in some data analysis and interpretation.

S.L.M. helped with patient recruitment in Cambridge and participated in the preparation of blood collection in Cambridge.

A.W. performed T-FRET analyses related to Figure 1I.

S.C. recruited patients in Montreal.

R.A.B. recruited patients in Cambridge, participated to data interpretation and revised the manuscript.

E.B. initiated the study and was involved in the experimental design. He also revised the manuscript.

F.C. initiated the study and was involved in the experimental design. She supervised the project and wrote the manuscript.

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