Additional file 3. Supplementary method information

Additional file 3A. Method for sample labeling

The samples were directly labeled using biotin as described previously [23, 24]. In brief, 3 µL of plasma samples were diluted in 22 µL 0.1 M sterile filtered phosphate buffered saline (PBS) (09-9400, Medicago) in 96-well microtiter plates (732-4828, Thermo Scientific). Samples were allocated in a stratified distribution plate layout in regards to sample subgroup, patient sample age at sampling, sex and collection year. We used two types of plate controls; replicate plasma sample pools, to control for plate variability, and wells without sample. Subsequently, 5 µL NHS-PEG_4 biotin (21329, Thermo Scientific), dissolved in DMSO (276855, Sigma-Aldrich) and diluted in PBS, was added to the diluted plasma samples using a liquid handler (SELMA, CyBio). This corresponds to 20 ng NHS-biotin per well and a 10-fold theoretical excess of biotin over plasma protein. The samples were incubated for 2 hours at 4°C upon which the reaction was quenched by addition of a 250-fold molar excess of 0.5 M Tris-HCl pH=8 (T6066, Sigma-Aldrich) over biotin followed by a 20 minutes incubation at room temperature at 650 rpm. The biotin-labeled samples were stored at -20°C until further use.

Additional file 3B. Method for copuling of antibodies to beads

Antibodies were diluted to 16 µg/mL in 100 µL 0.1 M 2[N-Morpholino]ethanesulfonic acid (MES)-buffer (Sigma-Aldrich) pH=5 using a pipetting robot (TECAN EVO150) and coupled to carboxylated color-coded magnetic beads (MagPlex-C, Luminex Corp) as described previously [23, 24]. Beads (n = 500 000/ID), located in 96-well microtiter plates (781 101, Greiner BioOne), were washed with 80 µL 0.1 M NaH_2PO_4 (phosphate buffer) pH 6.2 (S3139, Sigma Life Science) with a plate washer/dispenser (EL406, Biotek) on a magnet before being suspended in 50 µL phosphate-buffer. Activation buffer, consisting of 10 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (ProteoChem) and 10 mg/mL Sulfo-N-hydroxysulfosuccinimide (Sulfo-NHS) (24510, Thermo-Fisher Scientific) in phosphate buffer, was subsequently added to the beads, resulting in 0.5 mg EDC and 0.5 mg Sulfo-NHS per well. The beads suspended in activation buffer were incubated for 20 minutes at 650 rpm at room temperature and washed two times with 100 µL 0.1 M MES. The pre-diluted antibodies were added to the activated beads and incubated

for 2 hours at 650 rpm at room temperature. After incubation, the antibody-coupled beads were washed three times in 100 µL 1 × PBS, 0.05% Tween20 (BP337, Fisher Bioreagents) (PBS-T) and re-suspended in 50 µL storage buffer (Blocking Reagent for ELISA, Roche Applied Science). The individual bead IDs were pooled together after overnight blocking at 4°C, creating a bead stock containing all coupled bead IDs.

The coupling efficiency was tested using R-Phycoerythrin-conjugated (RPE) goat anti-rabbit IgG (111-116-144, Jackson), RPE-conjugated goat anti-mouse IgG (115-116-146, Jackson) and RPE-conjugated donkey anti-goat IgG (795-116-147, Jackson). 100 µL RPE-conjugated antibodies diluted to 0.5 µg/mL in PBS-T were added to 5 µL antibody-coupled bead stock in different wells, followed by incubation for 20 minutes at 650 rpm at room temperature. After incubation, the wells were washed three times with 100 µL PBS-T before analyzed on a Flexmap 3D instrument (Luminex corp.).