SUPPLEMENTARY APPENDIX

# A novel combinatorial technique for simultaneous quantification of oxygen radicals and aggregation reveals unexpected redox patterns in the activation of platelets by different physiopathological stimuli

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**Online supplement**

**Supplemental Methods Section**

## Chemicals and reagents

Cyclic hydroxylamines 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine (PPH) were purchased from Noxygen (Elzach, Germany). Prostaglandin E1 (PGE1), indomethacin, superoxide dismutase (SOD), polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), catalase (CAT), polyethylene glycol-conjugated catalase (PEG-CAT), N-acetylcysteine, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (or TEMPOL), heparin and thrombin were obtained from Sigma-Aldrich (Poole, UK). Stock solutions of cyclic hydroxylamines (10 mM) were prepared in EPR grade Tyrode's-HEPES buffer (THB) containing 25 µM deferoxamine methanesulfonate salt (DF) and 5 µM sodium diethyldithiocarbamate trihydrate (DETC) purchased from Noxygen, (Elzach, Germany). Stock solutions were kept under argon on ice to keep an oxygen free atmosphere and were prepared daily. Native LDL (nLDL) and oxidised LDL (oxLDL) were purchased from BioRad (Oxford, UK). Horm collagen was from Nycomed (Linz, Austria). The NOX inhibitory peptides were obtained from Prof J Pagano: Nox2ds-tat (YGRKKRRQRRRCSTRIRRQL), scNox2ds-tat (RKKRRQRRRCLRITRQSR), NoxA1ds (EPVDALGKAKV) and scNoxA1ds (LVKGPDAEKVA).

## Platelet preparation

Human blood was drawn from healthy volunteers by median cubital vein venepuncture under local ethics committee approval. Sodium citrate was used as anticoagulant (0.5% w/v). PRP was separated from whole blood by centrifugation (250 × g, 17 min), and platelets were separated from PRP by a second centrifugation step (500 × g, 10 min), in the presence of prostaglandin E1 (PGE1, 40 ng/ml) and indomethacin (10 µM). Washed platelets were then resuspended in a modified THB with a pH 7.4 (10 mM HEPES, 145 mM NaCl, 2.9 mM KCl, 1 mM MgCl2, 5 mM glucose, in ESR grade water (NOX-07, Noxygen, Germany) at a density of $2 \times 10^8$/ml.

## EPR/turbidimetry assay

$2\times10^8$ platelets/ml were prepared as described in the supplemental methods section. Prior to adding stimuli, 200 µM CMH or PPH was added to platelets. Platelet suspensions were loaded onto a Chronolog 700-2 aggregometer with continuous stirring (1,200rpm at 37°C) and the turbidimetry readings were immediately started. After 1 minute, stimuli were delivered and aggregation was measured for 10 minutes. Aggregation was quantified as % absorbance decrease = (initial absorbance – final absorbance) / initial absorbance. After 10 min of aggregation (as determined to optimise the signal-to-noise and reproducibility of the assay), 50 µl of platelet-free supernatant were transferred into the Hirschmann precision micropipettes and read using an e-scan (Noxygen, Germany). EPR spectra were recorded using the following EPR settings: centre field 3,492.5 G, field sweep 60 G, modulation amplitude 2 G, sweep time 10 s, number of scans 10, microwave frequency 9.39 GHz, microwave power 20 mW, conversion time, 327.68 ms, time constant, 5242.88 ms. Samples for calibration curves were obtained from a 10 mM stock solution of a standard $CM^{\bullet}$ diluted to 0.3, 1, 3, 10, and 30 µM. EPR signal from samples was utilised to calculate

the concentration of $CM^{\bullet}$ as described in Supplemental Figure 2 and the CMH oxidation rate was obtained using the formula below:

$$\textbf{CMH oxidation rate} = \frac{[CM^{\bullet}] \times \text{Volume}}{\text{Platelet density} \times \text{Volume} \times \text{Time}}$$

**Thrombus formation under physiological flow assay**

The Bioflux200 system (Fluxion, South San Francisco, CA) was used to analyse thrombus formation in human and mouse whole blood under flow. Microchannels were coated with 0.1/0.05 mg·mL−1 collagen I (monomeric collagen from calf skin, Sigma, UK) for 1–2 hours at 37°C before blocking with 1% BSA in Tyrode's-HEPES buffer and washing with Tyrode's-HEPES buffer. Heparin-anticoagulated whole blood was incubated with scrambled or the NOX inhibitory peptides, NoxA1ds and Nox2ds-tat before the addition of 1 µM DiOC6 for 10 minutes before the blood was added to the wells. Thrombus formation was visualized by fluorescence microscopy at a shear rate of 200 or 1,000 $sec^{-1}$. Representative pictures were taken at 10 min and surface area coverage was determined using Image J.

**Immunoprecipitation and pull-down assay**

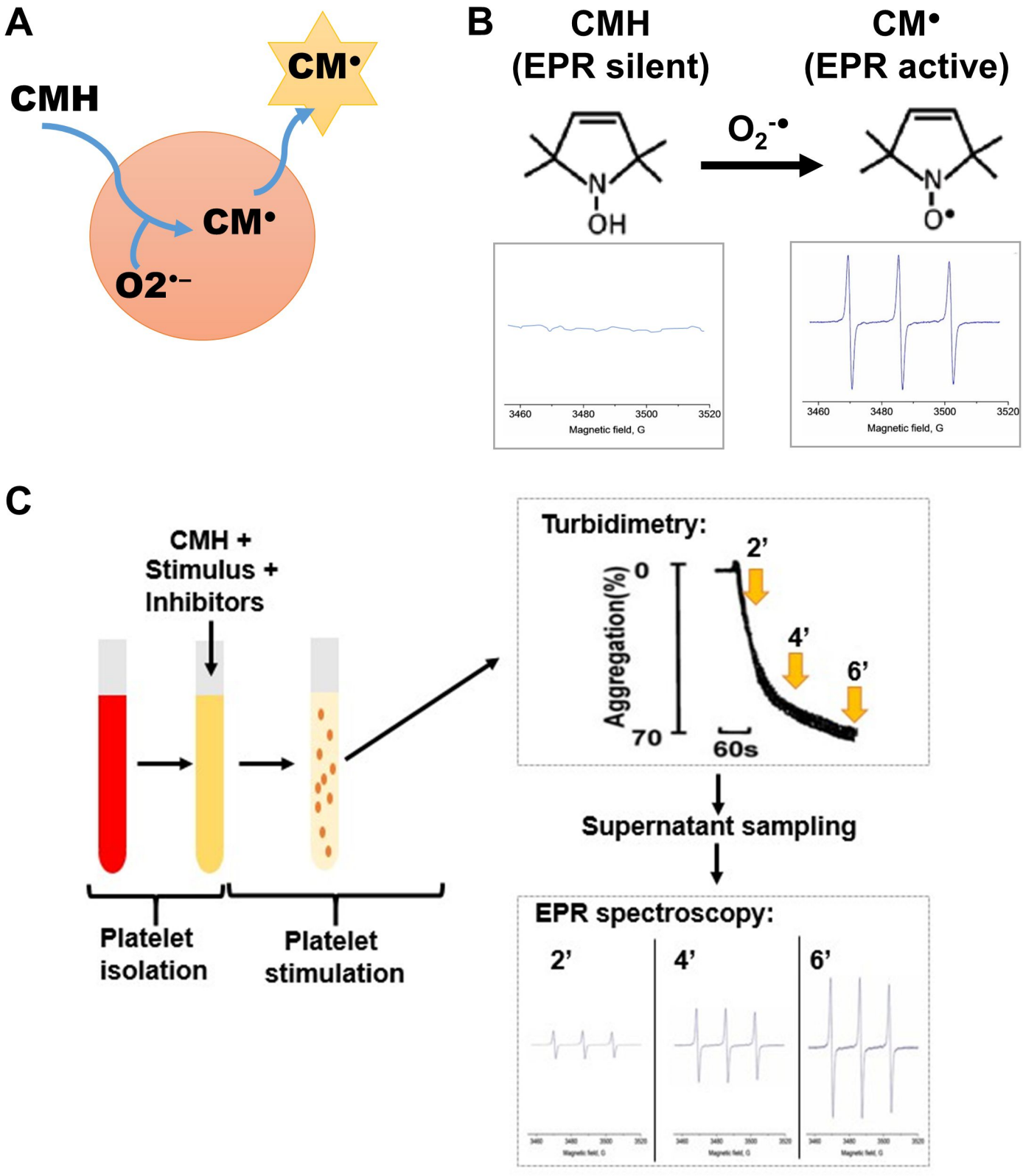
After stimulation, platelet were lysed under milder condition using a gentle lysis buffer (Tris 50 mM, NaCl 200 mM, Nonidet P-40 1%, Na deoxycholate 0.5%, and pH 7.4) with protease and phosphatase inhibitors. NOX1 or NOX2 was immunoprecipitated with 1 µg of rabbit mAb (Novus and Abcam respectively) overnight at 4 °C and the immunocomplexes were precipitated by addition of protein A/G Plus Agarose beads for 2 hours at 4 °C. Following brief centrifugation, immunocomplexes were washed five times with 1 ml of lysis buffer, and finally resuspended in 100 µl of SDS sample buffer 2X. Protein complexes were then analysed by Western blotting.

Immunoprecipitates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) for immunoblotting. After blocking with blocking buffer (Odyssey® Blocking Buffer LI-COR), the corresponding PVDF membranes were incubated with anti-NOX1 goat polyclonal antibody (Sigma-Aldrich) with anti-NOXA1 mouse polyclonal antibody (Abcam) or anti-NOX2 goat polyclonal with anti-p47phox mouse monoclonal antibody (Santa Cruz, USA) overnight at 4 °C. The PVDF membranes were washed three times with wash buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.0, and 0.05 % Tween 20) and then incubated with secondary antibodies (1:15,000) for 1 hour, washed with wash buffer, and imaged on the Odyssey Clx. Secondary antibodies were produced in goat and mouse to the species of the primary antibody and were conjugated with IRdye fluorophores visible in the 700 and 800 channels of the CLx imager. Images were acquired on the CLx imager at 169 µm resolution and all processed images were free of pixel saturation.

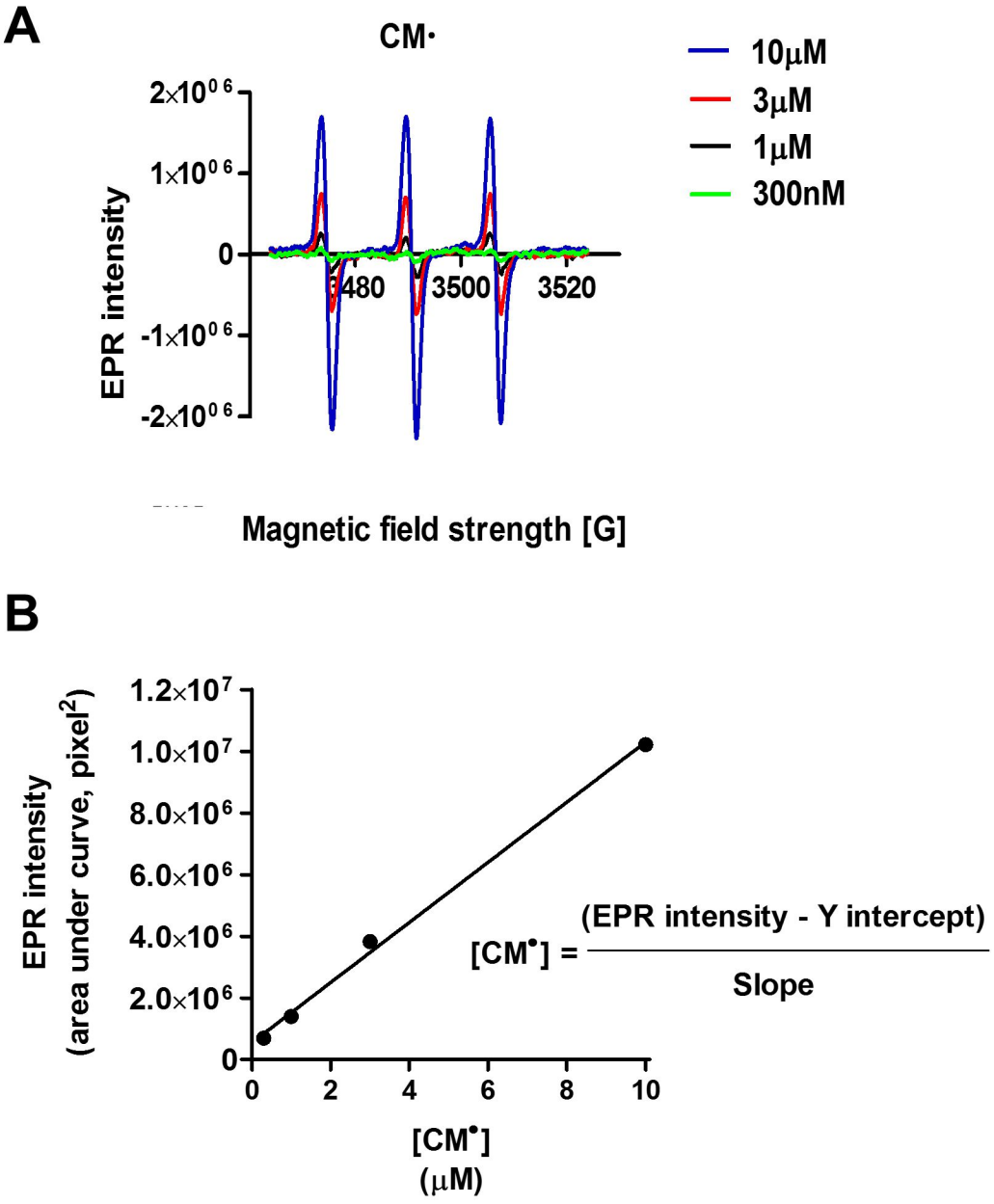
**Statistical analysis**

We utilised the statistical software Graphpad Prism 7 for Windows (version 7.03, February 20th 2017). For dual comparisons we utilised t-test, while for multiple comparisons we used either Analysis Of Variance (ANOVA) plus multiple comparison post-tests (such as Bonferroni's or Tukey's test) or Non Parametric Tests such as Kruskal-Wallis. The decision on whether using ANOVA or non-parametric tests was

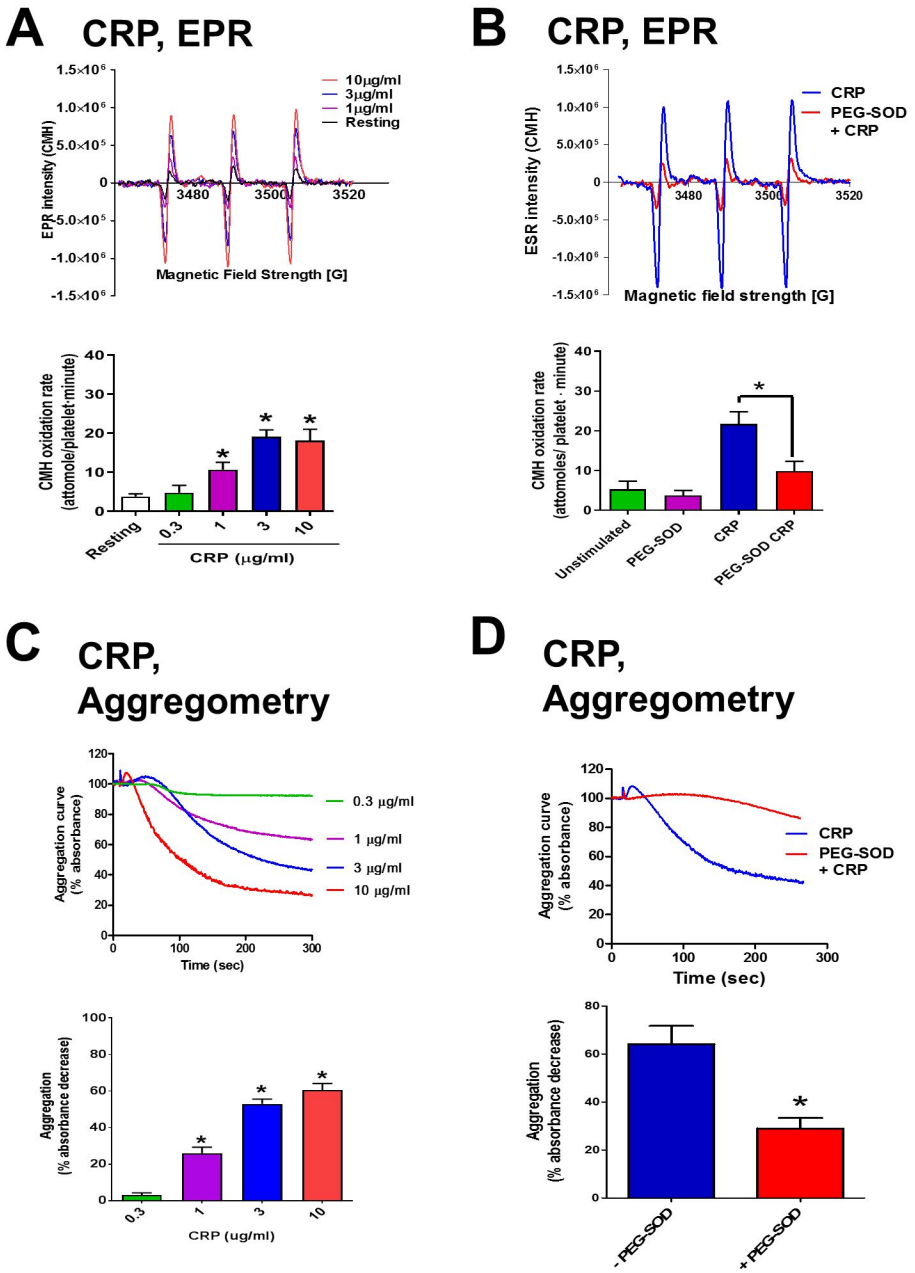
based on the analysis of data normality (Kolmogorov-Smirnov's test) and homoscedasticity (Bartlett's test). Results were expressed as the mean + standard error (SEM) throughout the manuscript. Differences were considered significant at $p < 0.05$.



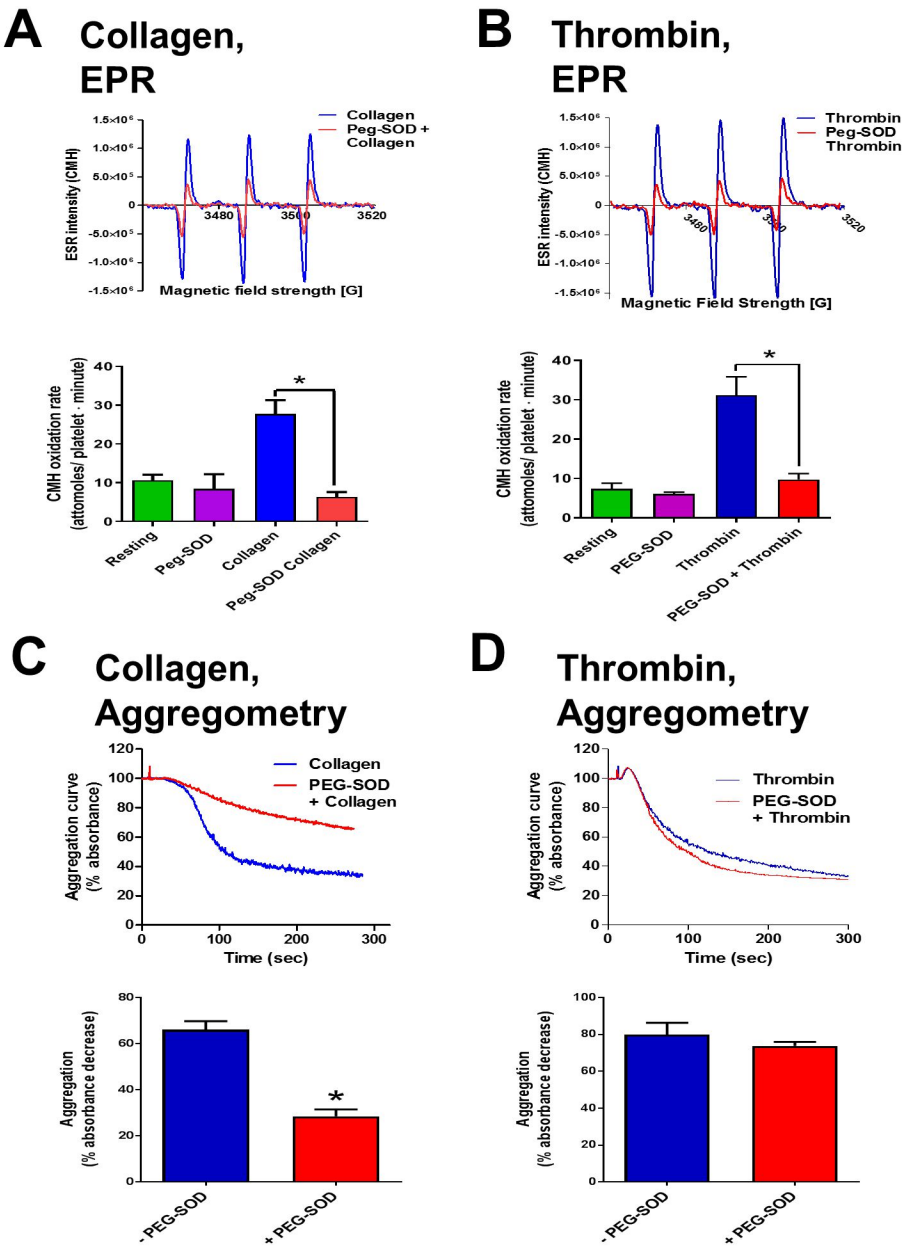

**Supplementary Figure 1: Schematic diagram of the EPR/turbidimetry technique for the detection of oxygen radical generation and activation in human platelets.** (A) CMH oxidation (predominantly intracellular) into CM•. (B) Chemical structure and EPR properties of CMH and CM•. (C) Schematics of the assay, with platelet isolation from whole blood, stimulation in the presence of CMH, classical turbidimetry and simultaneous EPR analysis of platelet supernatant for CMH oxidation by EPR.



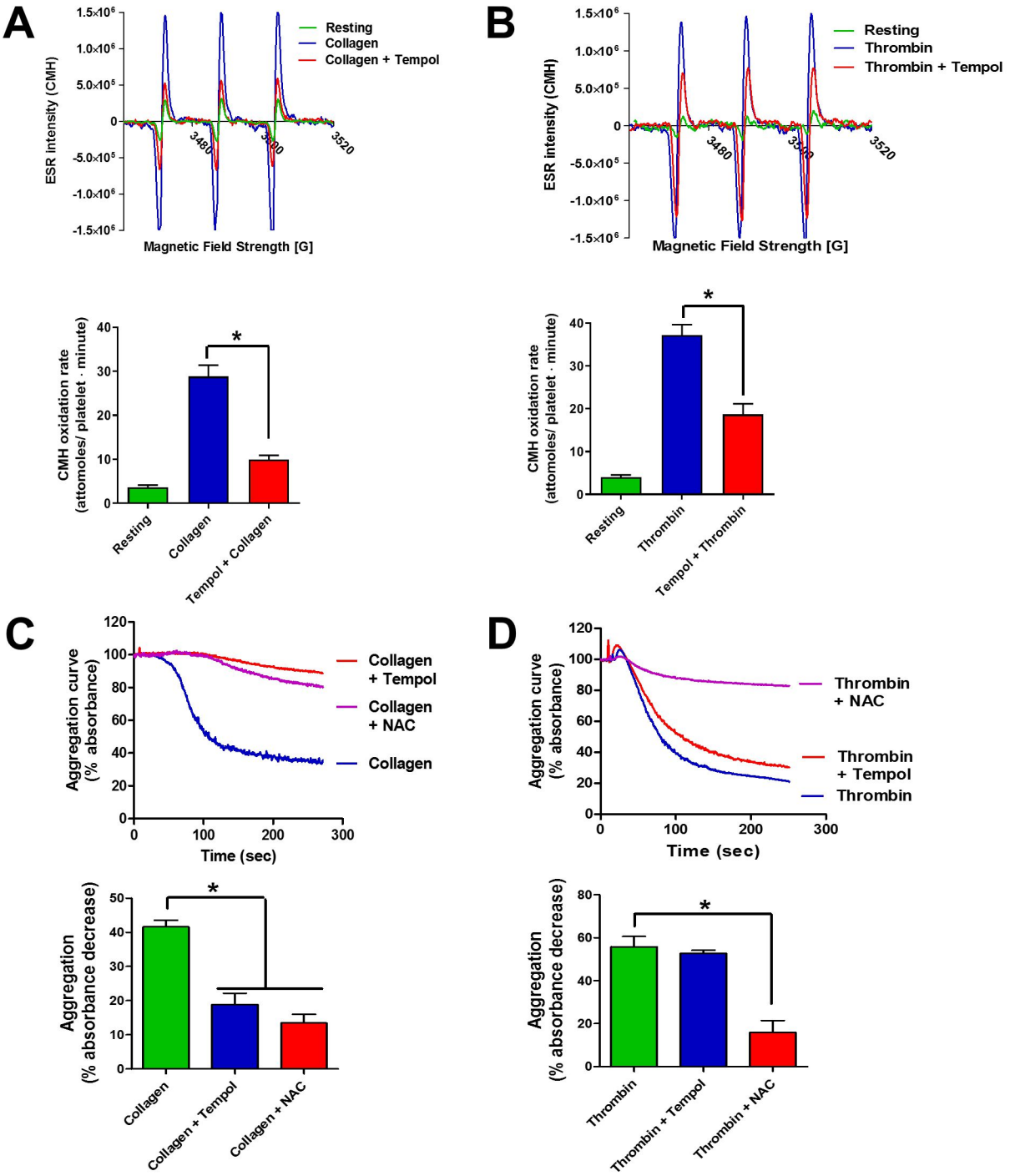

**Supplementary Figure 2: Calibration curve and CMH oxidation rate calculations.** (A) Representative example of the concentration response curve for CM• (300nM to 10μM). (B) Calibration curve EPR intensity vs CM• concentration.



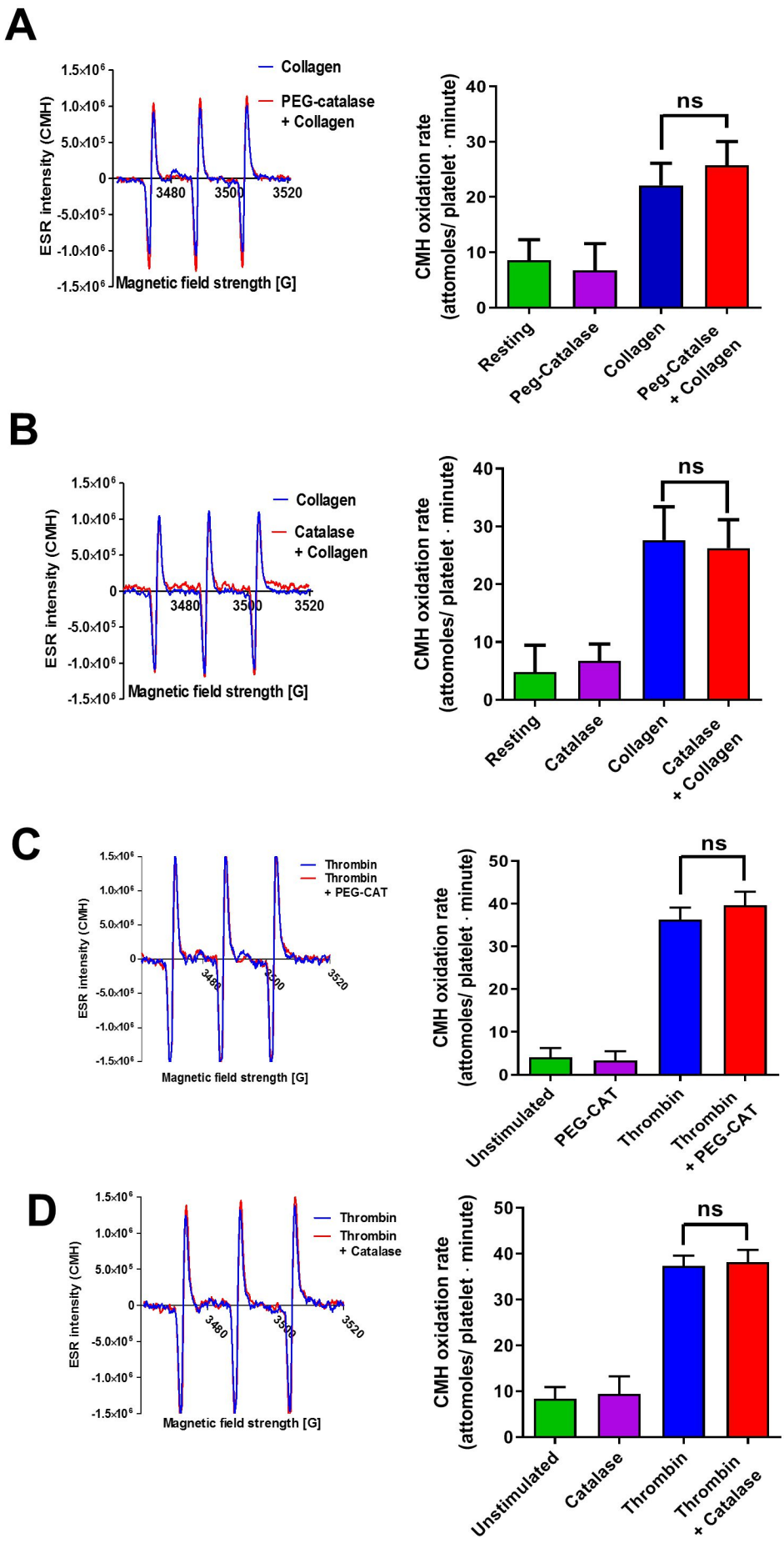

**Supplementary Figure 3.** CRP induced concentration-dependent increases in aggregation and oxygen radical generation rates between 0.3 and 10 µg/ml (A and C, respectively), while treatment with PEG-SOD abolished the EPR response (B) (suggesting superoxide anions are formed) and inhibited platelet aggregation (D) (which is therefore, superoxide anion-dependent). Examples of EPR traces and aggregation curves are representative of 3 independent experiments. Statistical analysis was performed by one-was ANOVA with Bonferroni post-test (A, B and C) or t-test (D). * = $p < 0.05$, compared to resting platelets (A and B, n=3) or negative control (i.e. scrambled peptide) (B and D, n=3).



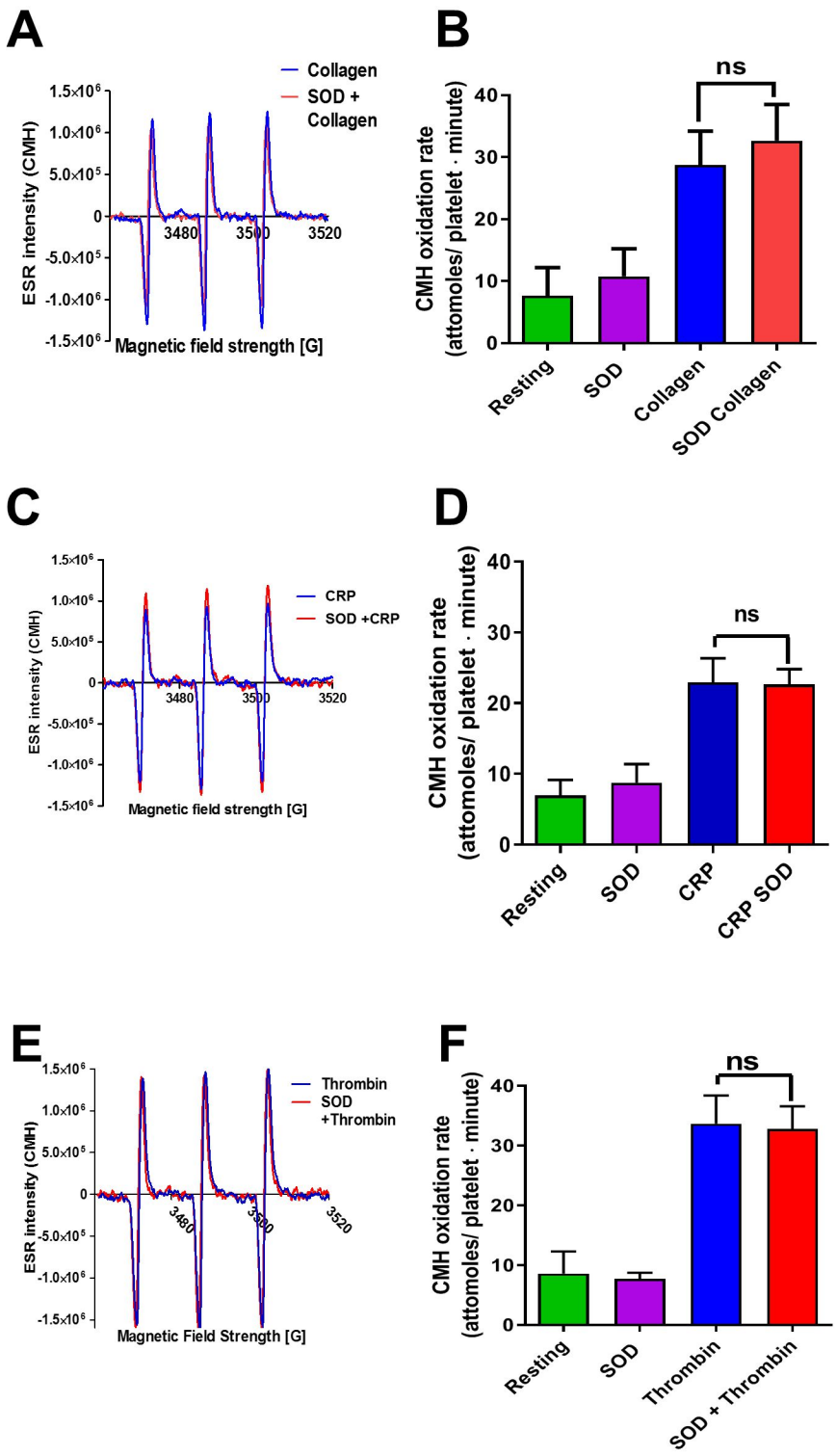

**Supplementary Figure 4: Superoxide anions are the oxygen radicals generated in response to collagen and thrombin, and are required only for collagen-dependent aggregation.** EPR **(A** and **B)** and aggregation analysis **(C** and **D)** of human platelets stimulated with collagen or thrombin in the presence or absence of 100 unit/ml of PEG-SOD are shown, with representative examples (top) and quantification (bottom). 10 µg/ml collagen or 0.1 unit/ml thrombin were utilised to stimulate platelets. Aggregation curves up to 5 minutes are shown, while EPR resonance readings were taken after 10 minutes of stimulation. Examples of EPR traces and aggregation curves are representative of 4 independent experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test for EPR and by unpaired t-test for aggregation (* = $p < 0.05$, compared to stimulated platelets in the absence of PEG-SOD, n=3 for A and n=4, for B, C and D).



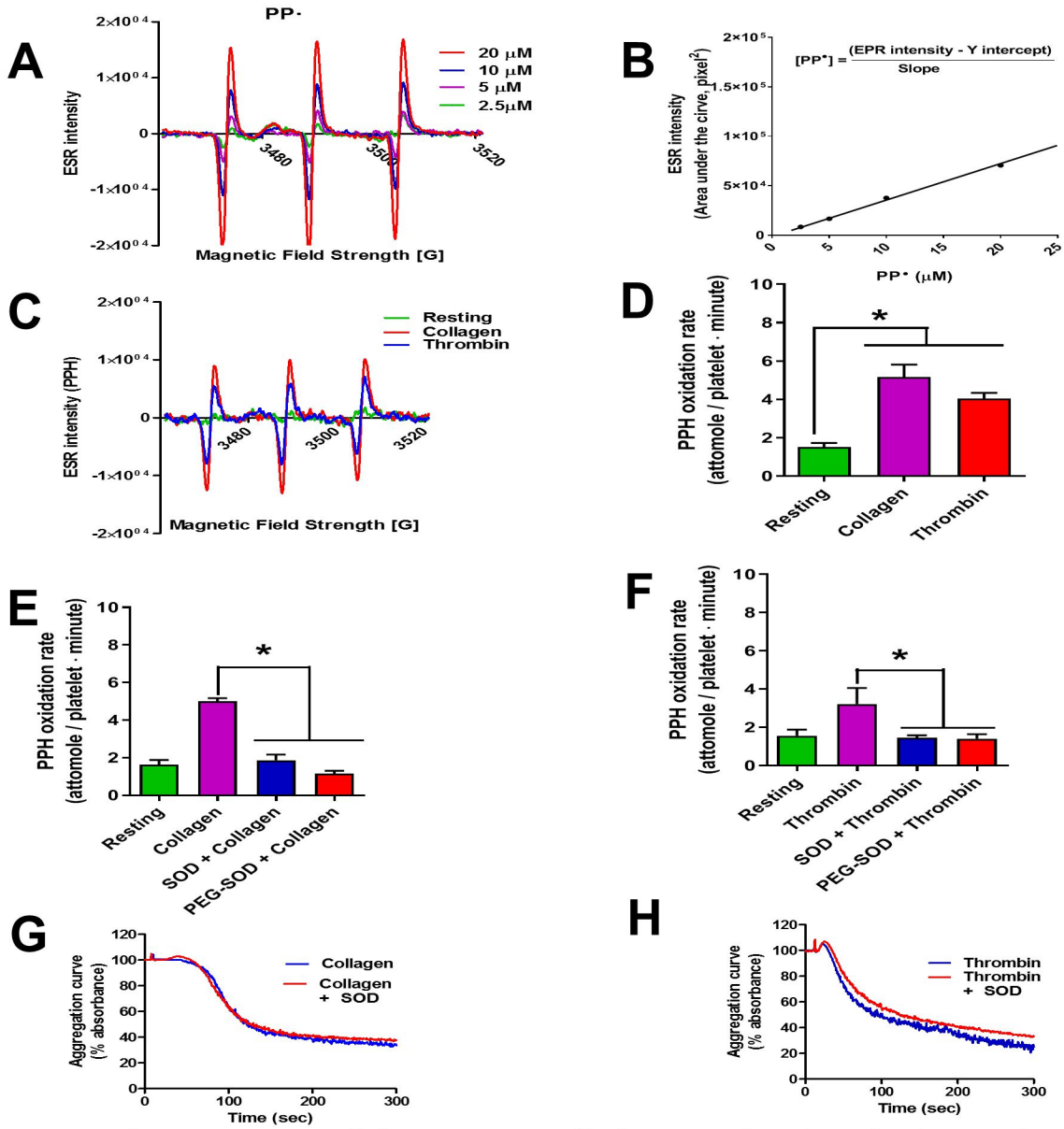

**Supplementary Figure 5: Superoxide anions are the oxygen radicals generated in response to collagen and thrombin, but other oxidant species are required for thrombin-stimulated aggregation. .** EPR (A and C) and aggregation analysis (B and D) of human platelets stimulated with collagen or thrombin in the presence or absence of 10 μM Tempol are shown, with representative examples (top) and quantification (bottom). 10 μg/ml Collagen or 0.1 unit/ml thrombin were utilised to stimulate platelets. For aggregation experiments, 10mM NAC was also tested. Aggregation curves up to 5 minutes is shown, while EPR resonance readings were taken after 10 minutes of stimulation. Examples of EPR traces and aggregation curves are representative of 3 or more independent experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test for EPR (* = $p < 0.05$, compared to stimulated platelets in the absence of inhibitor) (n=3 for A and C and n=4 for B and D).



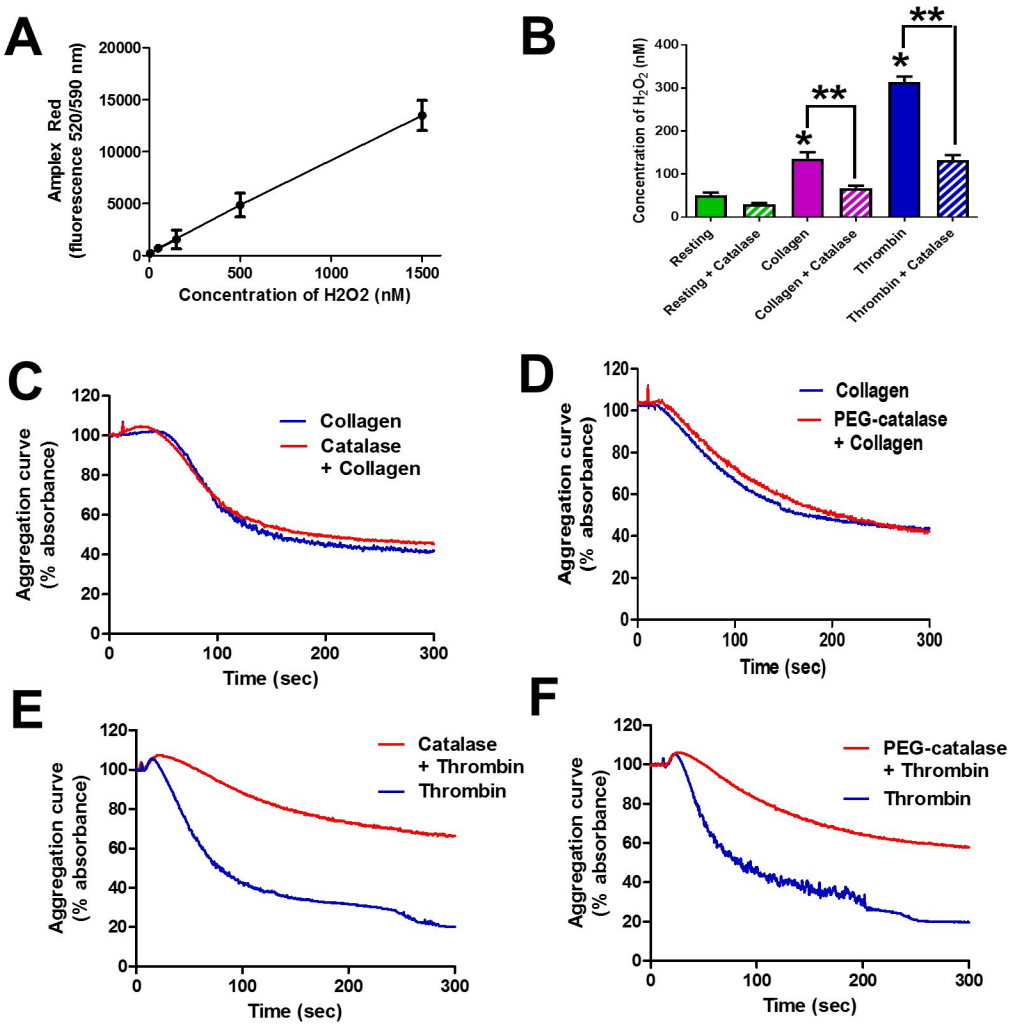

**Supplementary Figure 5. Catalase and PEG-Catalase do not affect CMH-based EPR measurement of oxygen radical formation.** Human platelets stimulated with collagen (A and B) or thrombin (C and D) in the presence or absence of 500 unit/ml of pegylated catalase (CAT, A and C) or 500 unit/ml catalase (CAT, are shown, with representative examples (left) and quantification (right). 10 µg/ml Collagen or 0.1 unit/ml thrombin were utilised to stimulate platelets. EPR resonance readings were taken after 10 minutes of stimulation. Examples of EPR traces and aggregation curves are representative of 3 independent experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test for EPR (n=3 for A, B, C and D).



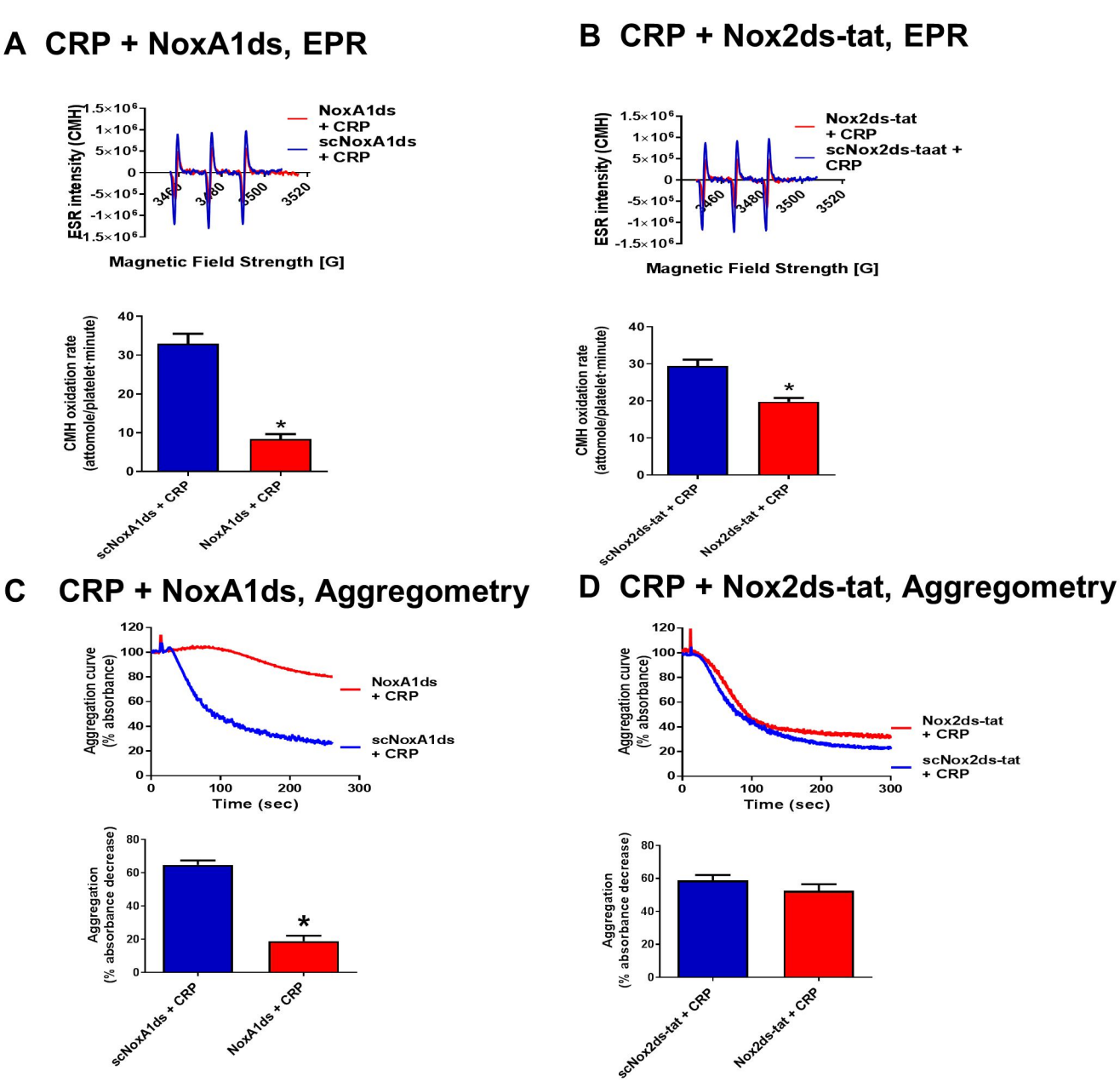

**Supplementary Figure 7. Superoxide dismutase does not affect CMH-based EPT readings suggesting that only intracellular superoxide anion are detected.** Human platelets stimulated with collagen (A and B), CRP (C and D) or thrombin (E and F) in the presence or absence of 100 unit/ml of superoxide dismutase (SOD), with representative examples (A, C and E) and quantification (B, D and F)). 10 µg/ml Collagen, 3 µg/ml CRP or 0.1 unit/ml thrombin were utilised to stimulate platelets. EPR resonance readings were taken after 10 minutes of stimulation. Examples of EPR traces and aggregation curves are representative of 3 or more independent experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test for EPR (n=3 for B and D and n=5 for F).



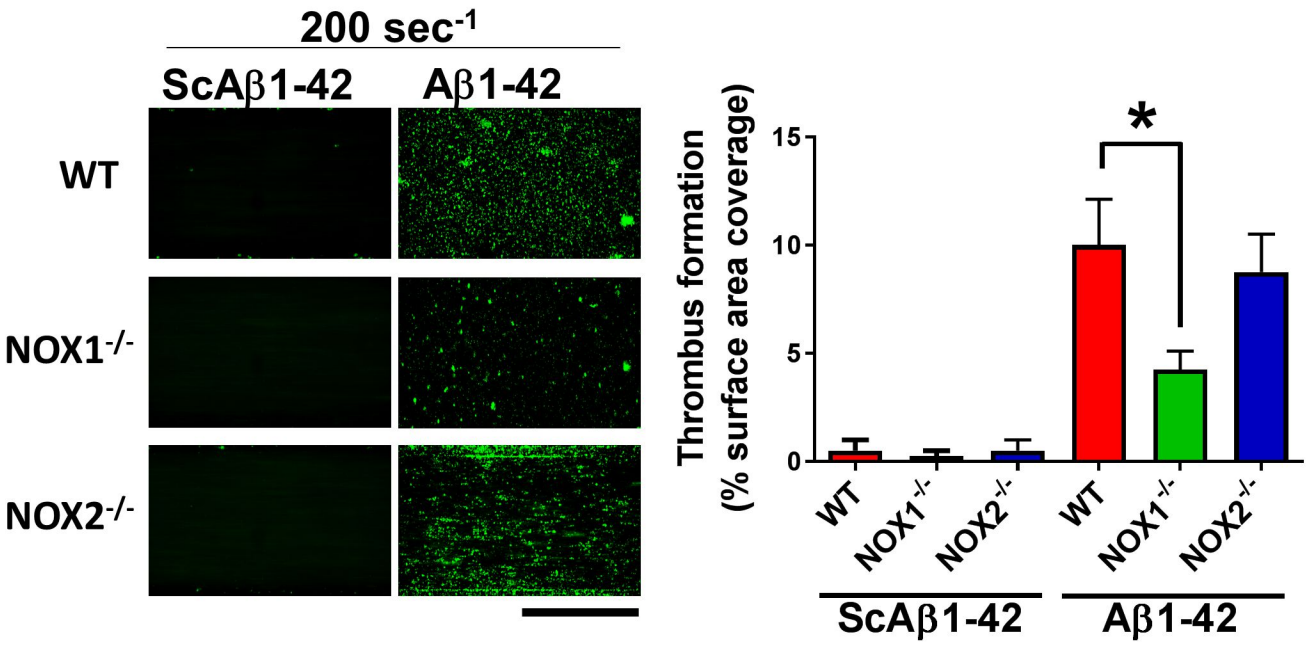

**Supplementary Figure 8: Extracellular oxygen radical generation by platelets activated by physiological stimuli collagen and thrombin.** PPH was utilised for the detection of extracellular oxygen radicals generated by platelets. A concentration response curve to calibrate the essay for generated (A) and a calibration curve was obtained (B). 10 μg/ml collagen or 0.1 unit/ml thrombin led to generation of extracellular oxygen radicals at low rate (<5 attomoles per platelet per minute) (C and D). 100 unit/ml of PEG-SOD or 100 unit/ml of SOD abolished this response suggesting that extracellular $O2^{\bullet-}$ were generated (E and F). Interestingly, the scavenging of extracellular $O2^{\bullet-}$ with SOD did not affect aggregation in response to collagen (G) or thrombin (H). Examples of EPR traces and aggregation curves are representative of 3 or more independent experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test (* = $p < 0.05$, compared to resting platelets, n=3 for F and n=4 for D and E).



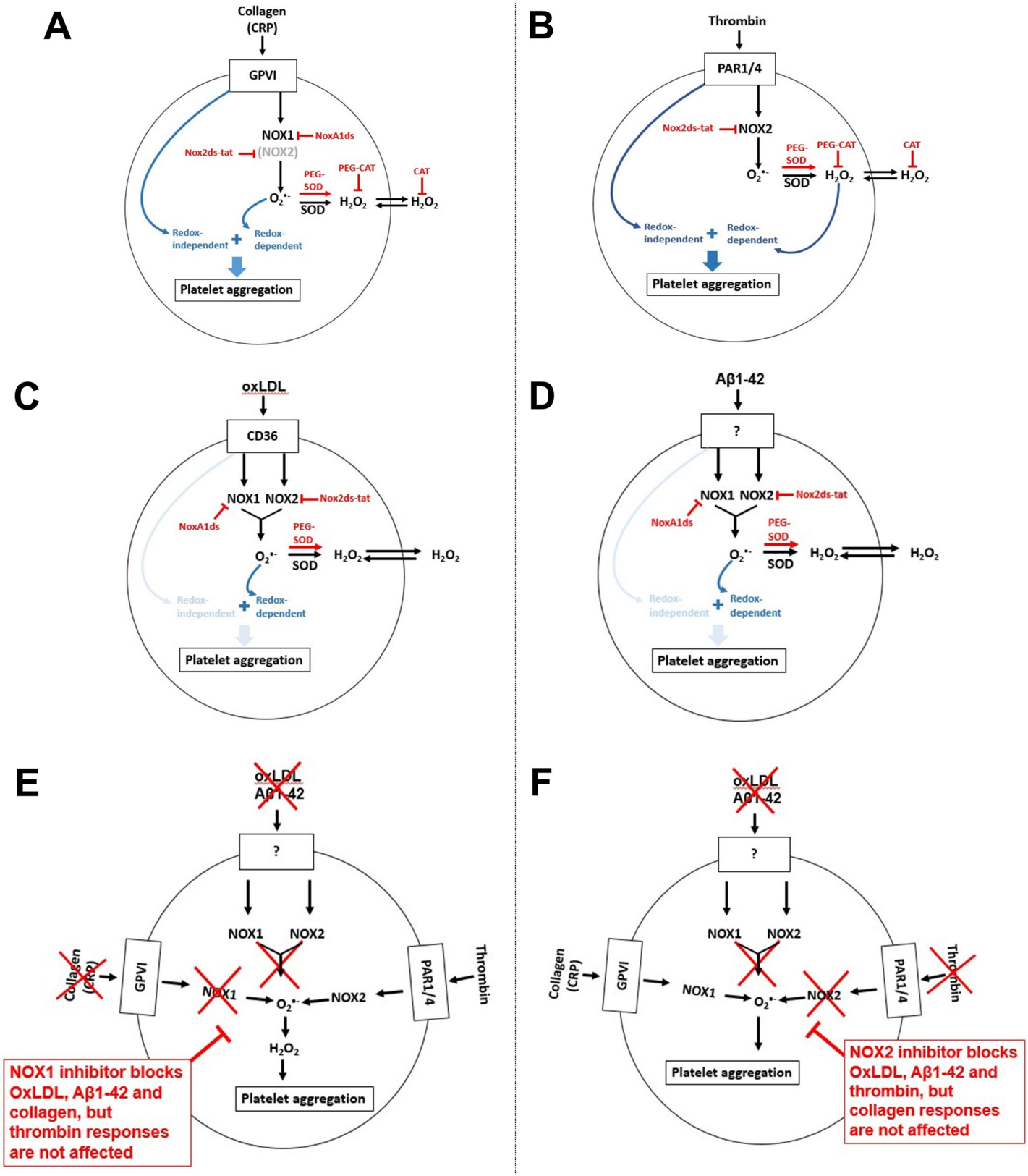

**Supplementary Figure 9: Hydrogen peroxide is formed in response to collagen- and thrombin-dependent stimulation of human platelets, but is required only for thrombin-dependent aggregation.** A reaction mixture containing 100 µM Amplex Red reagent, and 0.2 U/mL HRP was loaded on black 96 well-plates with or without 100 Units/ml catalase. The reaction was started by adding 50 µl of supernatant obtained from resting and stimulated platelets ($2\times10^8$ platelets/ml). After 30 minutes incubation, the fluorescence (excitation 520 nm, emission 590 nm) was measured using a CLARIOstar microplate reader (BMG LABTECH). In (A) the calibration curve obtained plotting the Amplex Red signal corresponding to different hydrogen peroxide concentrations is shown. The calibration curve was utilised to quantify the hydrogen peroxide released by platelets in resting conditions or in the presence of collagen (10 µg/ml) or thrombin (0.1 unit/ml). Where indicated, 500 unit/ml Catalase were added (as a control) (B). Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test for EPR (* = $p < 0.05$, compared to resting platelets, ** = $p < 0.05$ compared to results in the absence of CAT, n=3). Aggregation curves obtained in the same conditions described above are shown in (C-F) and are representative of 3 independent experiments.




**Supplementary Figure 10.** CRP-dependent aggregation NOX1- but not NOX2-dependent. Platelet activation was induced with 3 µg/ml CRP oxygen radical generation was assessed by EPR while aggregation was assessed by turbidimetry. 10 µM of NoxA1ds or Nox2ds-tat were utilised to inhibit NOX1 or NOX2, respectively. The scrambled peptide at the same concentration (scNoxA1ds or scNox2ds-tat) were used as a negative control. Examples of EPR traces and aggregation curves are representative of 3 or more independent experiments. Statistical analysis was performed by t-test (* = $p < 0.05$, compared to negative control, i.e. scrambled peptide, n=3 for A and C and n=5 for B and D).




**Supplementary Figure 11: Platelet adhesion under low shear stress is NOX1- but not NOX2-dependent.** Platelets from wild type, NOX1-/- or NOX2-/- mice were stained with DiOC6 and the Bioflux platform (Fluxion, San Francisco) was utilised to assess the thrombus formation induced by collagen under physiological flow. In A and B, Ibidi Vena8+ flow chambers were coated with 20μM Aβ1-42 or scrambled control peptide (ScAβ1-42). The shear rate utilised was 200 $sec^{-1}$, which leads to single platelet adhesion. Images were taken at 10 minutes of flow and are representative of 4 independent experiments. They were quantified by assessing the surface area coverage by platelets with Image J. Data are representative of 4 independent experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc test (* = $p < 0.05$, n=4).




**Supplementary Figure 12: Models of the redox-dependence regulation of platelets.** The cartoons represent the redox dependence of platelet aggregation in response to collagen (A), thrombin (B), oxLDL (C) or amyloid peptide β 1-42 (D). Selective inhibition of NOX1 would inhibit collagen-, oxLDL- or amyloid peptide β 1-42-dependent response but not thrombin (E), while selective inhibition of NOX2 would inhibit thrombin-, oxLDL- or amyloid peptide β 1-42-dependent response but not collagen (F).

| | EPR | Aggregation |
|---|---|---|
| Thrombin | 0.121 unit/ml | 0.088 unit/ml |
| Collagen | 2.63 µg/ml | 3.45 µg/ml |
| CRP | 0.77 µg/ml | 0.95 µg/ml |

**Supplemental Table 1: Potency table for different agonists as measured by simultaneous EPR and platelet aggregometry**