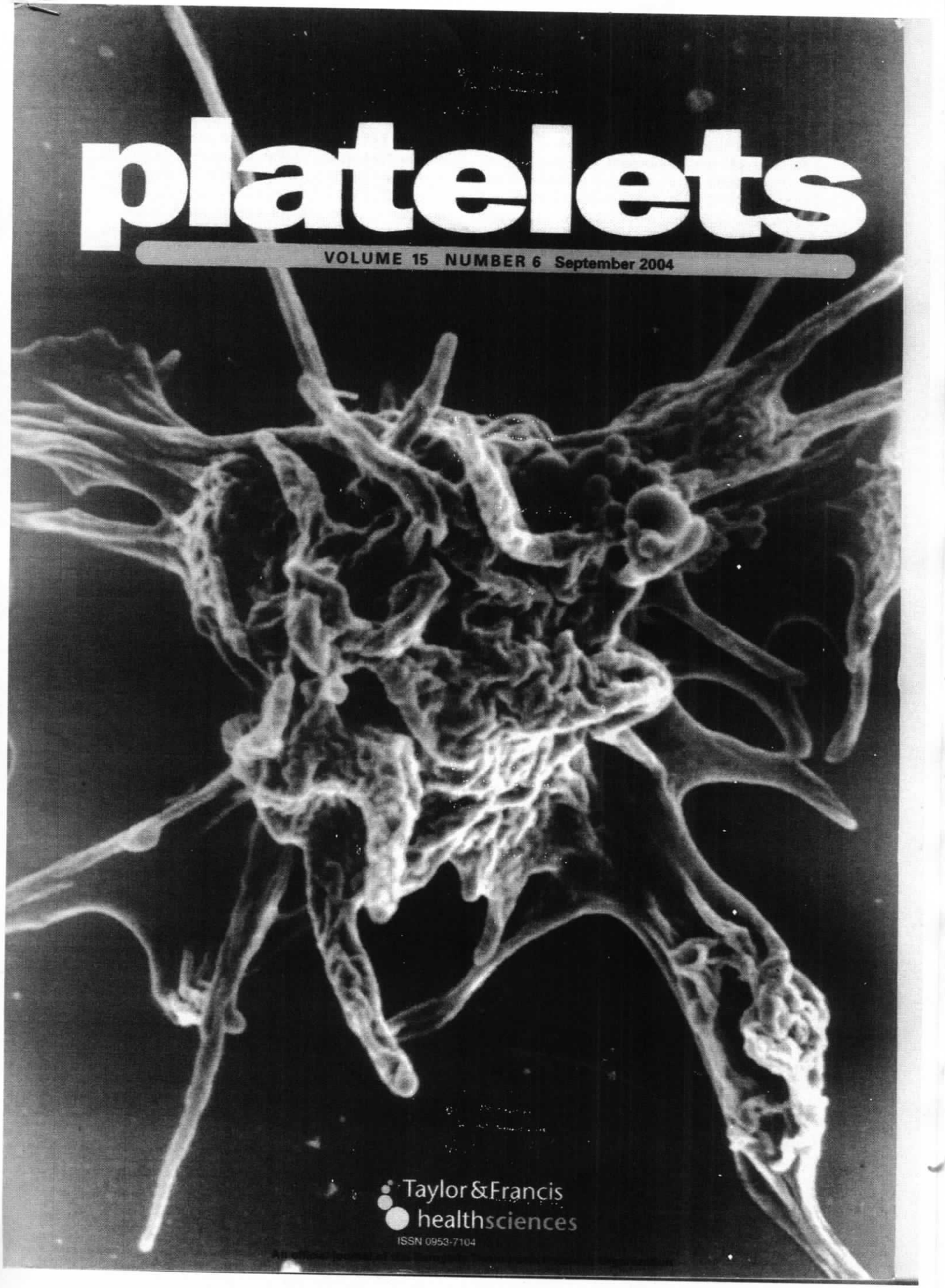
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platelet fibrinogen binding, P-selectin expression, platelet-leucocyte aggregation (PLA) and platelet-neutrophil aggregation (PNA). Additional samples were incubated with the agonist ADP to assess sensitisation of platelets. Results are given in medians with statistical analysis using the Wilcoxon signed rank and Mann Whitney U tests.

Results: Median age was 65 years. Both cohorts demonstrated an exercise-induced rise in platelet fibrinogen binding (patients: pre-exercise 0.99% to post-exercise 1.7% $p<0.002$) and P-selectin expression (patients: pre-exercise 0.68% to post-exercise 1.22% $p<0.002$). Similarly, PLA and PNA levels rose after exercise in both cohorts (patients PLA: pre-exercise 6.7% to post-exercise 9.2% $p<0.002$; patients PNA: pre-exercise 6.8% to post-exercise 9.1% $p<0.002$). While there was no difference in pre-exercise levels between cohorts, PAD patients had significantly higher 10 μM ADP-stimulated levels of PNA post-exercise: 30.2% compared to controls 17.8%($p=0.038$).

Conclusions: Platelet fibrinogen binding, P-selectin expression, and levels of PLA and PNA increase after exercise in both PAD patients and controls. However patients demonstrated increased sensitisation of platelets after exercise compared to controls.

PHOSPHORYLATION OF PLATELET NITRIC OXIDE SYNTHASE CONTROLS ENZYME ACTIVITY

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Nitric Oxide Synthase (NOS) is an important regulatory enzyme that catalyses the production of nitric oxide (NO) from the amino acid L-arginine. Platelet-derived NO plays a key role in retarding platelet accumulation at sites of vascular injury. NOS in blood platelets has putatively been identified as the endothelial NOS (eNOS) isoform. The regulation of eNOS in endothelial cells is well characterized. After stimulation of endothelial cells, eNOS activity is regulated by intracellular calcium concentrations ($[Ca^{2+}]_i$) and the phosphorylation and dephosphorylation of key serine and threonine residues. However, the regulation of platelet NOS is uncharacterised. We have used bovine platelets to investigate the influence of protein phosphorylation on NOS activity.

Here we demonstrate that eNOS activity in platelets is independent of phosphorylation of the key serine and threonine residues identified in endothelial cells. Stimulation of platelets with collagen, collagen related peptide (CRP) or thrombin induced NO synthesis as evidenced by increased cytosolic cGMP. eNOS was detected in bovine platelets by immunoprecipitation and immunoblotting. In contrast to eNOS in bovine endothelial cells, platelet eNOS was not phosphorylated at serine^{1177} following stimulation of the platelets with collagen, thrombin and CRP. Phosphorylation of eNOS at serine^{1177} in endothelial cells is associated with an increase in the activity of the enzyme. However, we have found that platelet eNOS is tyrosine phosphorylated on unknown residues and parallels enzyme activity. Tyrosine phosphorylation was independent of aggregation and extracellular $[Ca^{2+}]$.

These results suggest that platelet eNOS may be regulated in a novel and distinct manner from that in endothelial cells.

KINETIC ANALYSIS OF CD62P EXPRESSION IN CANINE PLATELET ACTIVATION BY FLOW CYTOMETRY

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Background: Canine platelet activation has been associated with many cardiovascular, infectious, metabolic and auto-immune disorders, few of which have been well studied. Previous methods used to detect platelet activation (aggregometry, radioimmunoassay, etc.) suffer from several drawbacks. Flow cytometry is a sensitive and rapid research tool for the study by quantitation of the surface expression of several membrane receptors. Determination of the expression of these receptors and the modulation in their expression after stimulation by agonists in canine platelets could represent not only an unique and clinically applicabile test for the detection of activated platelets but also a reference method to evaluate the utility of automated tests developed to detect activated platelets as part of complete blood count (CBC).

Methods: Blood was collected from $n=6$ healthy dogs that had received no drug therapy for at least 30 days. Platelet rich plasma (PRP) was obtained by centrifugation at 300 *g* for 15 minutes. In experiments for platelet activation PRP was stimulated with different concentrations of adenosine-5'diphosphate (ADP) 0–15 μM for different times (0–600 seconds) followed or not by fixation with paraformaldehyde (1% final concentration). Immunoflurescence analysis of CD61 (direct) and CD62P (indirect) was performed by a FACS Calibur Flow Cytometer (Becton

Dickinson) to evaluate the expression of these surface receptors and, in particular, to evaluate kinetic of expression of CD62 due to ADP activation. Data analysis was performed by the Paint a Gate program.
Results: A dose and time-dependent activation was detected after ADP stimulation of PRP. A significant ($P < 0.01$) mean intensity of fluorescence (MFI) increase was seen between control and ADP-stimulated platelets. Moreover, significant differences were seen between ADP 2,5 *vs* ADP 7,5 and ADP 15 µM ($P < 0.01$) and between 30 seconds *vs* 180 and 600 seconds ($P < 0.01$). In addition, an activating effect was suspected and showed for the fixing solution.
Conclusions: ADP acts on the expression of CD62 on canine platelet membrane. Dose and time-dependent changes in CD62 expression are consistent with similar trends previously described for platelet aggregation. Partial activation induced by the fixing solution probably represents a limit for this kind of studies, because it modifies the agonist activity on CD62. In any event, flow cytometry studies during platelets induced activation can represent an useful tool to detect platelet function and to elucidate the mechanisms by which several canine disease leads to haemostatic disorders.

A PEPTIDE TOOLKIT TO LOCATE PLATELET-BINDING MOTIFS IN COLLAGEN III

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Background: The interaction of collagen with its diverse ligands defines its unique role as a structure which supports the integrity of higher organisms and as a bioactive material which regulates cell behaviour. Synthesis of peptides that assemble in triple-helical form has provided insight into the interaction of collagen with a subset of cellular receptors, the collagen-binding integrins, α1-, α2- and α11-β1, and with a crucial platelet activatory receptor, Glycoprotein (GP) VI. Here, we develop the use of triple helical peptides by synthesising the whole of a blood vessel wall collagen, Type III, for use to identify regions that interact with the platelet surface.
Methods: The col domain of collagen III was synthesised as a series of 57 over-lapping peptides (the Toolkit) using Fmoc chemistry. Peptides were purified by HPLC, verified by MS and shown to be triple-helical. Cells or proteins interact with the Toolkit in 96-well plates. Bound protein targets are detected immunologically, or captured cells by phosphatase assay of lysates. Binding motifs are located with 9 aa resolution.
Results: Of 35 peptides tested, 10 captured human platelets in static adhesion assays. Adhesion to some was fully Mg^{2+}-dependent, typical of an integrin-mediated process. Others showed entirely cation-independent adhesion, which represents novel collagen-binding activity, whereas some showed both cation-independent and -dependent adhesion, mediated by the fibrinogen receptor, typical of platelet activation e.g. through GpVI.
Conclusions: The Toolkit can be used to investigate new classes of platelet-collagen interaction, and will be used to isolate and identify the receptors involved, mapping their binding within collagen III. The Collagen III Toolkit will also detect binding motifs within collagens for various extracellular targets, e.g. VWF, fibrinogen or fibrin, and collagen receptors on other cells.

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THE ROLE OF COLLAGEN RECEPTORS IN PLATELET NITRIC OXIDE PRODUCTION

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Platelet-derived nitric oxide (NO) plays a key role in protection against thrombosis, although it is unknown whether platelet adhesion, activation and aggregation are required for the initiation of platelet NO synthesis. The collagen-platelet interaction stimulates NO synthesis, but the mechanism of nitric oxide synthase (NOS) activation after collagen stimulation is unknown. Here we analyse the contribution of the $\alpha_2\beta_1$ and GPVI receptors to platelet NO synthesis.

Here we demonstrate that platelet NO synthesis, using cytosolic cGMP as an index of NO production, is independent of collagen-induced platelet aggregation, but dependent on GPVI-dependent platelet activation. Collagen-stimulated platelet cGMP production was unchanged by the presence of EGTA and/or indomethacin suggesting that NO synthesis was independent of aggregation and thromboxane A_2 respectively. The chelation of intracellular calcium