Methods Supplement for:

Periodontitis is associated with platelet activation

Papapanagiotou *, Nicu* et al. - "Platelet activation in periodontitis"

Dimitris Papapanagiotou,* Elena A. Nicu,* Sergio Bizzarro, Victor E. A. Gerdes, Joost C. Meijers, Rienk Nieuwland, Ubele van der Velden, and Bruno G. Loos

*These authors contributed equally to the present study.

Methods

Blood collection

In the first study, fasting venous blood samples were obtained without stasis by venipuncture in the antecubital fossa between 8.30 and 11.30 AM. Blood, collected in EDTA, was used to determine leukocyte counts. In addition, a second EDTA-blood tube (10 mL) was centrifuged at 3000 rpm for 10 minutes at room temperature. EDTA plasma was divided in aliquots and stored at -80°C until analysis.

In the second study, fasting venous blood samples were collected by venipuncture of the antecubital fossa, through a 19G butterfly needle (Vygon Nederland BV, Valkenswaard, The Netherlands) without venous stasis. For whole-blood flow cytometry, 0.32% citrate-anticoagulated blood was processed within 5 minutes after collection.

Soluble (s) P-selectin and sCD40 ligand

Plasma levels of sP-selectin and sCD40 ligand were determined by ELISA (R&D systems, Abingdon, UK), according to the manufacturers instructions. EDTA plasma specimens were diluted 1:20. For the sP-selectin ELISA, the diluted samples (in duplicate) together with the peroxidase-conjugated polyclonal antibody against P-selectin were incubated for 1h in a 96-well plate precoated with the anti-human P-selectin monoclonal antibody. The plate was washed three times with 300 μl "Wash buffer". After washing, the "Substrate" was added, followed by a 30min incubation and the reaction was stopped adding "Stop Solution" to each well.

For the sCD40 ligand ELISA, the samples were incubated in duplicate for 2h, at room temperature in a 96-well plate precoated with the anti-human CD40 ligand monoclonal antibody. The plate was washed four times with 400 μl "Wash buffer" and incubated for

another 2h with the polyclonal antibody against sCD40 ligand conjugated with peroxidase. Absorbance values were measured at 450 nm with a multilabel counter (Wallac Victor^2 1420, Perkin-Elmer Life Sciences, Boston, MA). The concentration of each sample was determined by extrapolation from a standard curve estimated from a panel of standards of known concentrations. Reagents marked in quotes were all from the R&D ELISA kits. The intra- and inter-assay coefficients of variation were 5.1 and 6.4%, for sCD40 ligand and 5.1 and 9.9 % for sP-selectin, respectively.

Whole blood flow cytometry

Aliquots of blood (5 μL) were diluted in 30 μL HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 5.6 mM glucose, 20 mM HEPES, 1 mg/ml bovine serum albumin, 3.3 mM NaH_2PO_4, pH 7.4). In addition, the labeling tubes contained 4 μg/mL PerCP-labeled anti-CD61 (5 μL), 4 μg/ml PE-labeled anti-CD62p (5 μL) and 4 μg/mL FITC-labeled PAC-1 (5 μL). To set fluorescence thresholds, 4 μg/mL PE-IgG_1 and 4 μg/mL FITC-IgM isotype control antibodies were used. After mixing and 30 minutes incubation at room temperature in the dark, 2.5 mL HEPES buffer containing formaldehyde (0.2% final concentration) was added. CD61-PerCP and PAC-1 FITC were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA), PE-labeled CD62P from Immunotech (Marseille, France), PE-labelled IgG_1 from Sanquin Reagents (Amsterdam, The Netherlands), and IgM-FITC from Beckman-Coulter (Fullerton, CA, USA). Flow cytometry was performed as described previously [1,2]. After fixation, blood samples were analyzed in a FACScan flow cytometer with CellQuest software (Becton Dickinson). Forward and side scatter were set at logarithmic gain. Platelets were identified by characteristic forward and side scatter, and PerCP fluorescence. Exposure of

platelet activation markers was determined in 5000 platelets. The threshold for platelet activation was arbitrarily set at 1% fluorescence-positive platelet activation with the appropriate control antibody.

Statistical analysis

Means, standard deviations, medians and frequency distributions were calculated. Differences in population background characteristics in both parts of the study were analyzed by t-tests or χ^2 - test (or Fisher's exact test, where needed). To correct for differences in background that were statistically significant between patients and controls in the first study, a general linear model (GLM) was constructed with periodontal condition as factor, and age, educational level, systolic blood pressure, total cholesterol, and triglycerides as co-variates. From the GLM, adjusted means, confidence intervals and P_{GLM}-values were obtained. In the first study, a χ^2-test was performed to test the distribution of controls and periodontitis patients with sP-selectin and sCD40 ligand levels below and above the respective population medians. Partial correlation coefficients between sP-selectin and sCD40 ligand were calculated, both within patients and control groups, correcting for the same co-variates included in the GLM. To analyze a possible effect of periodontal disease severity on the soluble platelet parameters, a stepwise linear regression analysis was conducted, within the patient group in the first study, using sP-selectin or sCD40 ligand as dependent variables and severity of periodontal disease (severe patients were those with ≥7 teeth with ≥50% bone loss), age, gender, ethnicity, smoking status, educational level, body mass index (BMI), systolic blood pressure, total cholesterol, and triglycerides as predictors.

In the second study, the % of cells exposing P-selectin and the % of cells binding PAC-1 showed a skewed distribution (Kolmogorov-Smirnov goodness of fit test $P<0.05$). These data were log transformed before statistical analysis. Subsequently, t-tests were employed for the analysis of platelet activation data. To explore the potential confounding of the non-matched background characteristics on platelet-bound P-selectin and PAC-1 binding, a GLM was constructed using periodontal condition as factor and educational level, BMI, systolic blood pressure, total cholesterol and triglycerides as co-variates. Also for the patients in the second part of the study, correlation coefficients between platelet activation markers and periodontitis severity (number of teeth with ≥50% bone loss) were computed. *P*-values <0.05 were considered statistically significant.

References

[1] Shattil SJ, Motulsky HJ, Insel PA, Flaherty L, Brass LF. Expression of fibrinogen receptors during activation and subsequent desensitization of human platelets by epinephrine. Blood 1986;68:1224-31

[2] Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987;70:307-15