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Orthopedic surgery increases atherosclerotic lesions and necrotic core area in *ApoE-/-* mice

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Abstract

Background and aims: Observational studies show a peak incidence of cardiovascular events after major surgery. For example, the risk of myocardial infarction increases 25-fold early after hip replacement. The acuteness of this increased risk suggests abrupt enhancement in plaque vulnerability, which may be related to intra-plaque inflammation, thinner fibrous cap and/or necrotic core expansion. We hypothesized that acute systemic inflammation following major orthopedic surgery induces such changes.

Methods: *ApoE*$^{-/-}$ mice were fed a western diet for 10 weeks. Thereafter, half the mice underwent mid-shaft femur osteotomy followed by realignment with an intramedullary K-wire, to mimic major orthopedic surgery. Mice were sacrificed 5 or 15 days post-surgery (n=22) or post-saline injection (n=13). Serum amyloid A (SAA) was measured as a marker of systemic inflammation. Paraffin embedded slides of the aortic root were stained to measure total plaque area and to quantify fibrosis, calcification, necrotic core, and inflammatory cells.

Results: Surgery mice showed a pronounced elevation of serum amyloid A (SAA) and developed increased plaque and necrotic core area already at 5 days, which reached significance at 15 days (p=0.019; p=0.004 for plaque and necrotic core, respectively). Macrophage and lymphocyte density significantly decreased in the surgery group compared to the control group at 15 days (p=0.037; p=0.024, respectively). The density of neutrophils and mast cells remained unchanged.

Conclusions: Major orthopedic surgery in *ApoE-/-* mice triggers a systemic inflammatory response. Atherosclerotic plaque area is enlarged after surgery mainly due to an increase of the necrotic core. The role of intra-plaque inflammation in this response to surgical injury remains to be fully elucidated.

Introduction

Observational epidemiological studies have confirmed what experienced clinicians suspected for many years: cardiovascular events can be triggered by a variety of common non-cardiovascular clinical conditions, particularly those that are associated with systemic inflammation [1-3]. One of the best documented examples of non-infectious systemic inflammation causing an acute increase in cardiovascular risk is major surgery. A recent study indicated that the risk of myocardial infarction in patients undergoing total hip or knee replacement is increased by no less than 25 fold, mainly during the first days to weeks post-surgery [2]. This phenomenon is hardly investigated and the mechanisms which underlie this postoperative risk remain elusive.

The acuteness of the increased postoperative cardiovascular risk suggests an effect on atherosclerotic plaque stability [4]. Several studies indeed show that at least half of the cases of myocardial infarction following non-cardiac surgery originate from rupture of unstable plaques [5-7]. Why plaques could become more prone to rupture briefly after surgery is unclear. Plaque instability is not only related to factors such as fibrous cap strength and intra-plaque hemorrhage, but also to the necrotic core area which, in turn, are all influenced by intra-plaque inflammation [8-11].

Plaque area was shown to increase in mice after a single injection of serum amyloid A peptide, a marker and mediator of inflammation[12] . A recent study addressing the combined effects of major blood loss and surgery reported increased plaque size growth as well as increased plaque vulnerability already after 72 hours [7]. Major orthopedic surgery is known to cause massive systemic inflammation [13]. We therefore hypothesized that acute systemic inflammation caused by major orthopedic surgery could rapidly enhance plaque vulnerability by means of increased intra-plaque inflammation, fibrous cap thinning and/or necrotic core expansion, and we addressed this hypothesis in atherosclerosis-prone *ApoE-/-* mice.

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Materials and methods

Mouse model

ApoE-/- mice (9-10 weeks old) were obtained from Charles River Laboratories (Calco Como,Italy) and housed in specific pathogen-free rooms, on a 12-h light–dark cycle. All mice were fed a high-fat diet (0.15 % cholesterol) (4022.83, AB diets, Woerden, the Netherlands) for 10 weeks after which the surgery group underwent a mid-shaft femur osteotomy followed by realignment with an intramedullary K-wire, to mimic major orthopedic surgery. Under general anesthesia (2% isoflurane) and analgesia (0.1 mg/kg buprenorfine), the femur was accessed via a lateral skin incision of the greater trochanter just proximal of the knee joint. The femur was approached using microsurgical scissors and forceps. With a dental mini-cutter, a mid-shaft osteotomy was performed. After that a stainless steel 0.6 mm K-wire (DePuy Synthes, Amersfoort, The Netherlands) was retrogradely introduced into the medulla of the femur until it exited medial and proximal of the greater trochanter. This was done using an electrical rotary system (Model 3000, Dremel, Breda). The osteomy was reduced and the osteotomy was stabilized by normogradely introducing the K-wire into the medulla of the distal femur until seated in the distal metaphysis. The K-wire was cut flush with the trochanter, where after the wound was closed by interrupted sutures (Monocryl 5-0 FS-2, Eticon) for both the femoral fascia and skin. During the operation bleeding was kept to a minimum by using bipolar radiofrequency micro-forceps (Pfizer, Valleylab Force 30). Blood was sampled after a 4-hour fasting period at several time points surrounding surgery/control (t=-1 day, 1 day, 5 days and 15 days). Mice in both operation (n=22) and control group (n=13) were sacrificed 5 or 15 days post-surgery respectively post-saline injection. All procedures were approved by the Animal use and care Committee at the VU University Medical Centre of Amsterdam.

Analysis of systemic inflammation and serum lipids

Levels of serum amyloid A (SAA), soluble VCAM-1 (CD106) and soluble E-selectin (CD62E) were measured by ELISA (Life Technologies, Bleiswijk, the Netherlands for SAA and R&D

Systems, Abingdon, UK, for VCAM-1 and E-selectin). Total serum cholesterol and triglyceride levels were measured in serum by commercially available enzymatic assays (cholesterol CHOD-PAP 11491458 and triglycerides GPO-PAP 11488872, Roche, Woerden, the Netherlands). For determination of the lipid profile, pooled plasma per group was fractionated using an ÅKTA fast protein liquid chromatography system (Pharmacia, Roosendaal, the Netherlands). Fractions were collected and assayed for total cholesterol using an enzymatic assay cholesterol CHOD-PAP 11491458 (Roche) and analysed as reported [14].

(Immuno)histochemistry and morphometry

Dissected hearts including the aortic roots were embedded in paraffin after overnight fixation (4 % formalin) for analysis of atherosclerosis. Serial cross-sections (4 μm) directly distal to the valve area of the aortic root were stained with Alcian Blue (indicative of glycosaminoglycans), Elastica van Gieson, Von Kossa and MAC-3, scanned with a PathScan Enabler IV, after which the mean surface area of plaque and the concordant area of fibrosis (EVG), calcification (Von Kossa) and macrophage area (MAC-3) was analysed blindly from each specimen using QuickPHOTO MICRO 3.0 software or in the case of the MAC-3 staining and the fibrous cap thickness[15] with ImageJ software. The necrotic core area was calculated by subtracting the area of fibrosis, calcification and macrophages from the total plaque area. For immunohistochemical analysis, serial cross-sections were dewaxed, rehydrated and incubated in methanol/H_2O_2 (0.3%) for 30 minutes to block endogenous peroxidases. Antigen retrieval was performed using pepsin for 25 minutes at 37 ℃ (Ly-6G and CD206), boiling in Tris-EDTA buffer, pH 9.0 (Caspase-3) or in citrate buffer, pH 6.0, for 15 minutes at 100 ℃ using a microwave (MAC-3 and i NOS). Sections were incubated with normal rabbit serum (1:50 dilution, DAKO, X0902) for 10 minutes at room temperature (RT) and subsequently with the primary antibody using rat-anti-mouse-MAC-3 antibody, detecting macrophages (1:30 dilution, BD Pharmingen, clone M3/84, cat. 553322, overnight, RT) or rat-anti-mouse-Ly6G antibody (detecting neutrophilic granulocytes) (1:200 dilution, BD

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Pharmingen, clone 1Ab, cat. 551459, 1 hour, RT) as appropriate. Thereafter, sections were incubated with biotinylated rabbit-anti-rat (1:300 dilution, DAKO, E0468, 30 minutes)/ Streptavidin-HRP (1:100 dilution, DAKO, P0397, 1 hour). For iNOS staining (to detect type 1 macrophages) the primary antibody polyclonal rabbit-anti-iNOS (1:200 dilution, Abcam, Ab15323, 1 hour, RT) was used and for the Caspase-3 (apoptosis marker) the primary antibody polyclonal Rabbit-anti-Caspase-3 (1:500 dilution, cell signaling technology, cat. 9661-L, 1 hour, RT) was used. Both were followed by incubation with HRP labeled anti-rabbit (100 µl undiluted, DAKO, K4003) for 30 minutes. For the CD206 staining (detecting type 2 macrophages) the primary antibody polyclonal Rabbit-anti-mannose receptor CD206 (1:1000 dilution, Abcam, Ab64693, 1 hour, RT) was used, followed by a swine anti-rabbit HRP secondary antibody (1:100 dilution, DAKO P0217, 30 minutes, RT). For CD45 staining (detecting lymphocytes), antigen retrieval was performed using citrate buffer, pH 6.0, for 15 minutes at 100^0C using a microwave. Sections were incubated in water (97℃) for 30 minutes and subsequently incubated in methanol/H_2O_2 (0.3%) for 30 minutes. Afterwards, sections were incubated with rat-anti-mouse-CD45 antibody (1:50 dilution, BD Pharmingen, cat. 550539, overnight, 4℃), rinsed in PBS, incuba ted with rabbit-anti-rat-HRP (1:50 dilution, DAKO, P0450) for 30 minutes. All staining's were visualized with 3,3-diamino-benzidine-tetrahydrochloride/H_2O_2(DAB, 0.1 mg/ml, 0.02% H_2O_2) for 10 minutes and counterstained with haematoxylin. Mast cells were stained with Toluidine Blue 1%. MAC-3 area was quantified as percentage MAC-3 positive staining of the total plaque area, using an automated macro in ImageJ software. Numbers of Ly-6G and CD45 positive cells were manually quantified per surface area of the atherosclerotic plaque as cells/μm^2. Immunoscoring was performed by R.L and W.W.F. and agreement was reached between both observers.

Statistics

Statistical analysis was performed with SPSS (20.0 for Windows, SPSS Inc.). To test for differences in plaque area, necrotic core area and inflammatory cells between groups, an

independent sample t-test was used when the data were normally distributed, or a Mann–Whitney U test if not. All data are presented as median and interquartile range. Results were considered statistically significant if the two-sided *p*-value was <0.05.

Results

Surgery causes acute systemic inflammation

To determine whether major orthopedic surgery was indeed accompanied by an acute systemic inflammatory response, we measured serum SAA levels at different time points (t=-1 day, 4 hours, 1 day, 5 days and, in the late-sacrifice group, after 15 days) (Fig. 1A). SAA levels had increased 4 hours after surgery, increased by almost 100 fold after 24 hours, and returned to baseline levels within 5 days. In contrast, SAA values in the control group remained low and stable. Endothelial inflammation markers (VCAM-1 and E-selectin) did not increase in response to trauma (Fig. 1BC).

Surgery transiently decreases body weight and alters lipid profile

Mice in the surgery group had weight loss (6.6%) at 5 days compared to baseline, whereas control mice gained weight (5.8%). Serum cholesterol and triglyceride levels were measured at t=-1 day, 1 day, 5 days and, in the late-sacrifice group, after 15 days. We observed a transient lipid-lowering effect of surgery. This effect was relatively small for cholesterol (Fig. 1D), but more pronounced for triglycerides (Fig. 1E). The lipoprotein profile for cholesterol distribution in VLDL, LDL and HDL-sized particles showed a slight reduction in VLDL which was paralleled by an equally small increase in LDL from t=5 days onward (Fig. 1F-I).

Surgery does not increase the content of inflammatory cells within atherosclerotic lesions

We observed a significant reduction (*p*=0.037) of the macrophage area relative to the total atherosclerotic plaque area between the surgery and control group at t=15 days (Fig. 2B). Subtyping of typical type 1 macrophages (M1 - iNOS positive area) and type 2 macrophages (M2 - CD206 positive area) relative to total macrophage area (MAC-3 positive area) was

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performed (Fig. 2D,F). At t=5 days, a significantly lower M1 fraction was observed in the surgery group 44% [33-54] *versus* 55% [47-87] in the control group; p=0.028) which increased significantly to 80% [66-121] (p=0.007) at 15 days. The relative density of M2 macrophages was not affected significantly. No significant differences were found between surgery and control mice in the number of neutrophilic granulocytes (Ly-6g) and mast cells (toluidine blue) (Fig. 2H and J). We did, however, observe a significant reduction in lymphocyte density (Fig. 2L) 15 days post-surgery as compared to the control group (p=0.024).

Atherosclerotic plaque and necrotic core area increase after surgery

Aortic root sections of all experimental groups showed advanced atherosclerotic lesions infiltrated by macrophages and lymphocytes, with a thin fibrous cap (Fig. 3A-D). To assess the effect of surgery, we measured total plaque area in the root, and evaluated the relative contribution of different plaque components. At t=5 days, a non-significant 5.5% higher plaque area was observed in the surgery group (481 x 10^3 μm^2 [387-558] *versus* 456 x 10^3 μm^2 [394-491] in the control group; p=0.297). This difference became more pronounced and statistically significant at t=15 days; (519 x 10^3 μm^2 [485-634] in operated mice *versus* 387 x 10^3 μm^2 [331-464] in control mice, p=0.019; Fig. 3E). This plaque enlargement was mainly attributable to a 160% increase in the necrotic core area (257 x 10^3 μm^2 [151-315] in operated mice *versus* 99.0 x 10^3 μm^2 [71.2-155] in control mice, p=0.004; Fig. 3F). The area of the other plaque compartments (fibrosis, macrophages, calcification and apoptosis) did not significantly contribute to the increase in plaque area (Fig. 4). Also, we found no evidence of fibrous cap thinning at t=5 days (28.7 [22.4-42.3] μm in operated mice *versus* 19.8 [17.5-37.2] μm in control mice, p=0.3), nor at t=15 days (36.7 [29.6-45.2] μm in operated mice versus 24.5 [18.7-42.3] μm in control mice, p=0.6). In addition, we assessed old intra-plaque hemorrhage but only found small non-significant areas of Fe-positivity that had only a very minor contribution to the total area and that did not differ between groups (data not shown). Finally, in the roots of all mice (surgery and control) Alcian Blue positive staining was

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observed both in the necrotic core area and in the other regions of the plaque, with no differences between the groups.

Discussion

Major orthopedic surgery in *ApoE-/-* mice causes acute systemic inflammation. At 15 days post-surgery, we observed a significant increase of plaque area, mainly due to necrotic core enlargement. Plaque necrosis is a characteristic hallmark of atherosclerotic lesions that causes acute atherothrombotic vascular disease [9-11]. Our finding of a post-surgery increase of necrotic core area may reflect increased plaque vulnerability and could, at least partly, explain the increased incidence of cardiovascular events after major orthopedic surgery.

The surgical procedure caused a marked acute systemic inflammatory response as demonstrated by a rapid increase in SAA, a relatively stable inflammation marker that integrates the signals of proatherogenic cytokines (IL1, TNFα, IL6), many of which may have contributed to the effect on the atherosclerotic leasion[16, 17]. Several studies suggest that SAA itself participates in the pathogenesis of atherosclerosis and a recent study shows that it may contribute to plaque vulnerability [18]. For instance, SAA induces TGF-β, increases vascular biglycan content, and increases LDL retention [19]. SAA also stimulates NF-κB activation and induces the expression of pro-atherosclerotic factors, such as ICAM-1, MCP-1, MMP-9 and tissue factor in macrophages [20]. Lastly, SAA displaces apoA-I from the surface of the HDL particle, thus generating free apoA-I, which is cleared faster by the kidney, thus potentially affecting the anti-atherogenic effect of HDL[21]. In addition to the effects on SAA, we observed a slight effect on circulating lipoproteins (i.e. decrease in VLDL and increase in LDL) in the orthopedic surgery group. Although the shift toward more atherogenic particles is relatively small, it may have contributed to the development of atherosclerosis in the orthopedic surgery group.

We did not find indications of fibrous cap thinning after orthopedic surgery, which besides necrotic core expansion is another hallmark of vulnerable, inflammatory plaque[8, 22]. We

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cannot exclude fibrous cap thinning because it is known to be very heterogeneously distributed, varying between and within lesions[23]. In parallel, we did not find an increased density of inflammatory cells after orthopedic surgery. This may have been caused by the fact that we examined plaques after 5 and 15 days. We cannot exclude inflammatory cell infiltration taking place before these time points and that activated macrophages in particular had already ‘dissolved’ at 5 days, contributing to the necrotic core. Indeed, death of lesional macrophages promotes the formation of plaque necrosis [9], and could thus be compatible with the decreased plaque macrophage content we observed at later time points. Similarly, activation of lymphocytes may cause loss of CD45-positive epitopes and cells can become undetectable by CD45-staining. It thus remains possible that surgery-induced intra-plaque inflammation due to infiltration of immune cells contributed to the observed increase in the necrotic core area (and hence total lesion area).

There is a growing understanding that the balance between pro- and anti-inflammatory macrophages in plaque is dynamic. [24]. We found an increase in the fraction of M1 pro-inflammatory macrophages at 15 days, supporting a pro-inflammatory postoperative plaque milieu.

We have found no differences between the different groups and time points for Caspase-3, an apoptosis marker, and therefore we cannot conclude that apoptosis is a major driver of the necrotic core expansion after surgery. Of note, however, these test also do not exclude a role for apoptosis. Caspase-3 staining is a temporary phenomenon in apoptosis and after some time, Caspase-3 staining in the necrotic core could be lost. This also emphasizes the need for future studies addressing earlier time points and more in-depth characterization of biomarker profile, and inflammatory cell activity in similar experimental designs.

Several related experimental studies merit discussion. Recently, a study in *ApoE-/-* mice addressed the combined effects of major blood loss and surgery, and reported increased plaque size after 72 hours, along with signs of plaque vulnerability [7]. However, this effect appeared to be mainly due to excessive blood loss (20% of body weight; which is substantially more than in our model), rather than surgery itself. As in our study, the density

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of intra-plaque macrophages did not differ, but other inflammatory cell types were not quantified. A few experimental studies addressed the effects of acute systemic inflammation on atherosclerosis. A recent murine model of intra-abdominal sepsis suggested that atherosclerotic plaque area was enhanced by sustained systemic, endothelial and intimal inflammation, and was not explained by infection itself [25]. More specifically, an increase in intra-plaque macrophages was observed 5 months after the induction of sepsis via cecal ligation and puncture. This coincides with a sustained elevated level of IL6, suggesting that this model mimics a chronic inflammatory state, in contrast to our model of transient acute inflammation. No data on macrophage density at earlier time points (nor other inflammatory cell types) were reported. Another study in *ApoE-/-* mice showed that influenza virus infection caused subendothelial infiltration of a heterogeneous population of cells [26]. These cells were however not quantified per type and plaque area was not assessed. Lastly a study again in *ApoE-/-* mice demonstrated that the systemic inflammatory response to myocardial infarction aggravates chronic atherosclerosis [22]. Together with the results described herein, these studies support an important role for systemic inflammation in plaque destabilization and might thereby contribute to increased risk of cardiovascular events.

Our study has the following limitations: our goal was to evaluate effects of major (orthopedic) surgery on atherosclerotic plaque. Although we found plaque changes congruent with increased event risk, the study wasn't designed to clarify responsible mechanism(s). Our data are compatible with a role for surgery-induced inflammation, but this requires more in-depth studies. In addition, other, non- or indirect inflammatory effects of surgery could be further explored. For instance, hemodynamic disturbances [27], sympathetic nerve system activation [28] or enhanced platelet activity[29] are potential contributors to atherosclerotic lesion development and increased cardiovascular risk. Furthermore it would be interesting to test our findings in advanced mouse models to study actual plaque complications rather than atherosclerotic plaque development alone [30, 31].

In conclusion, major surgery can cause a strong systemic inflammatory response and lead to an increase in plaque and necrotic core area, contributing to plaque vulnerability. These findings may help explain the high incidence of cardiovascular events following major surgery and, in a broader perspective, other clinical conditions of acute systemic inflammation.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Fig. 1. Major orthopedic surgery transiently increases serum SAA levels and decreases serum lipids. The vertical dotted line represents the timing of surgery. Serum levels of (A) SAA (µg/ml), (B) E-selectin (ng/ml), (C) VCAM-1 (µg/m), (D) cholesterol (mM), (E) triglycerides and (F-H) lipoprotein profile for cholesterol distribution in VLDL, LDL and HDL-sized particles at baseline and at different time points after surgery (n=22) or control (n=13).

Fig. 2. MAC-3 positive area divided by total plaque area (%) at t=15 days post-surgery decreases. (A) MAC-3 positive area (encircled) in the plaque (B) divided by total plaque area (%). (C) iNOS positive area (encircled) in the plaque (D) divided by MAC-3 positive area (%). (E) CD206 positive area (encircled) in the plaque (F) divided by MAC-3 positive area (%). (G) Ly-6g positive cells in the plaque (H) divided by the total plaque area (cells/μm^2). (I) Toluidine blue stained cells in the adventitia (J) divided by the area of adventitia (cells/mm^2). (K) CD45 positive cells in the plaque (L) divided by the total plaque area (cells/μm^2). Arrows point out positive cells. P=plaque; L=lumen; Ad=adventitia. Magnification of A,C,E 10x; magnification G and K 40x; magnification I 50x. All expressed the aortic root of mice sacrificed at t=5 days post-surgery (n=12) or control (n=7) or t=15 days post-surgery (n=10) or control (n=6).

Fig. 3. Major orthopedic surgery increases atherosclerotic plaque and necrotic core area. (A) Representative images of EVG stained cross-sections of the aortic root after 5 days without surgery, 5 days with surgery (B), 15 days without surgery (C), 15 days with surgery (D). All showing 10x magnified advanced atherosclerotic lesions (encircled) with necrotic cores (NC) and areas of fibrosis (F). The arrows indicate the media, the lumen (L) is largely composed of erythrocytes. (E) Plaque area (x103 μm^2) and (F) necrotic core area (x103 μm^2)

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at the aortic root of mice sacrificed at t=5 days post-surgery (n=12) or control (n=7) or t=15 days post-surgery (n=10) or control (n=6) .

Fig. 4. Major orthopedic surgery shows no significant effect on area of fibrosis, macrophages and calcification. Plaque area (x 103 μm^2) of fibrosis based on a EVG staining (A), macrophages based on a MAC-3 staining (B), calcification based on a Von Kossa staining (C) and apoptosis based on a caspase-3 staining (D) at the aortic root of mice sacrificed at t=5 days post-surgery (n=12) or control (n=7) or t=15 days post-surgery (n=10) or control (n=6) (all *p*-values >0.05).

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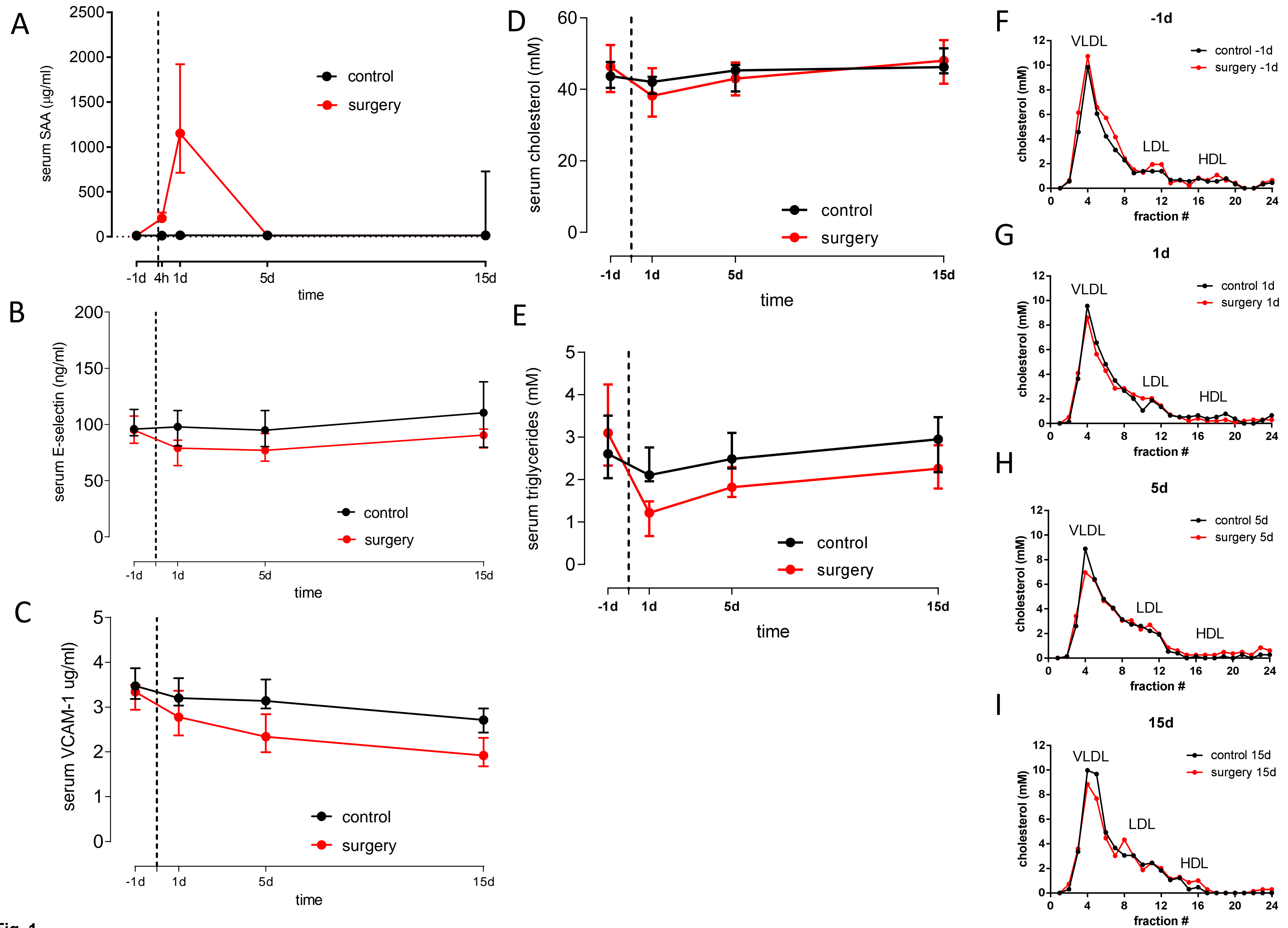

Fig. 1

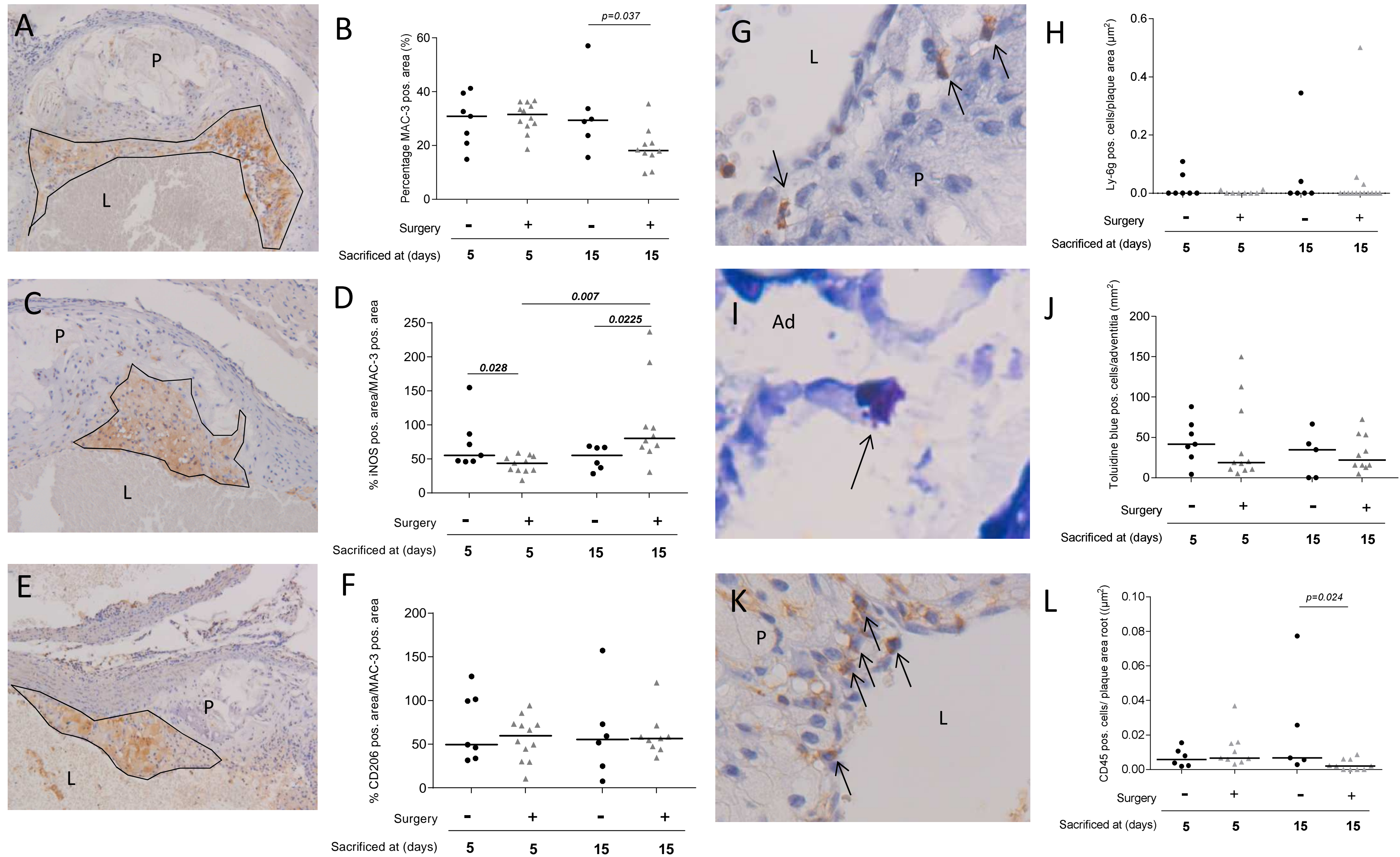

Fig. 2

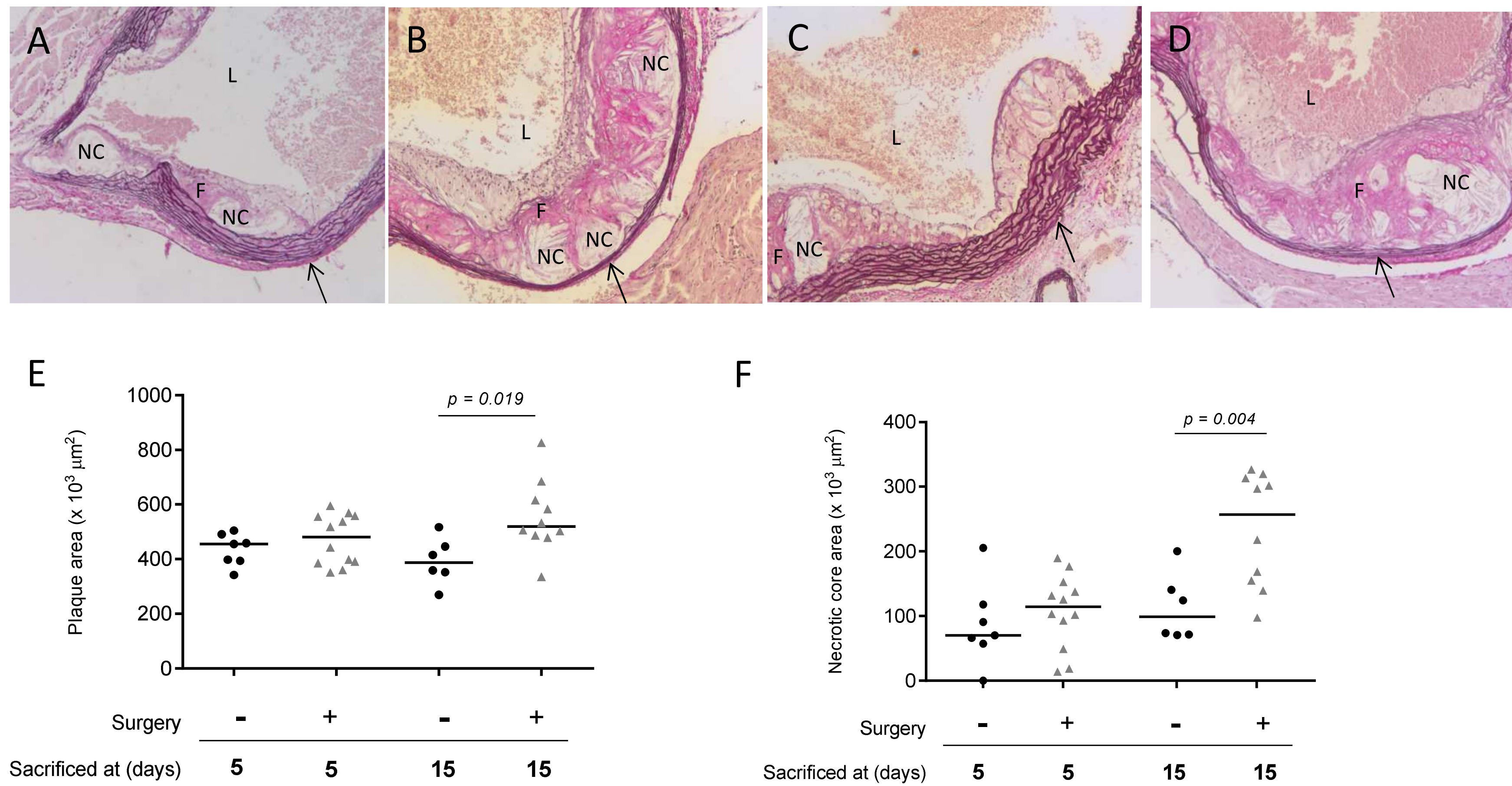

Fig. 3

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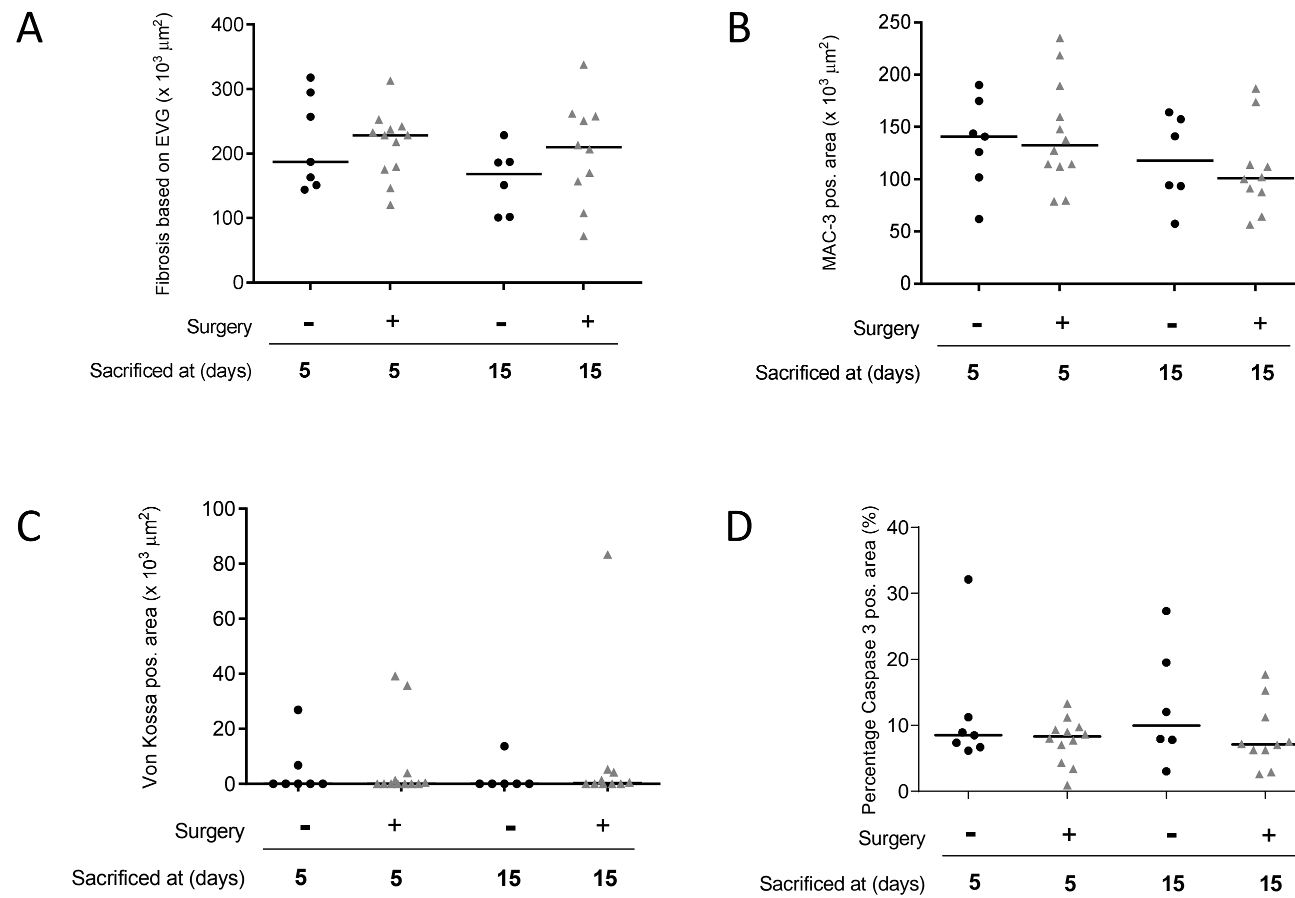

Fig. 4

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Highlights

- We hypothesized that the systemic inflammatory response following major orthopedic surgery causes increased plaque vulnerability expressed as local inflammation, plaque area and/or increased necrotic core area.
- We found that major surgery causes a marked systemic inflammatory response and leads to an increase in plaque and necrotic core area.
- These findings may help explain the high incidence of cardiovascular events following major surgery and, in a broader perspective, other clinical conditions of acute systemic inflammation.

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