Raised Soluble P-Selectin Moderately Accelerates Atherosclerotic Plaque Progression

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Abstract
Soluble P-selectin (sP-selectin), a biomarker of inflammatory related pathologies including cardiovascular and peripheral vascular diseases, also has pro-atherosclerotic effects including the ability to increase leukocyte recruitment and modulate thrombotic responses in vivo. The current study explores its role in progressing atherosclerotic plaque disease. 

Introduction
Cardiovascular disease (CVD) remains the largest cause of death in the world [1]. A vital mechanism for the development of an unstable atherosclerotic plaque is through increased vascular leukocyte recruitment, which is part of the inflammation hypothesis of atherosclerosis in CVD [2]. Critically, inflammatory plaques lead to extracellular matrix remodelling and vascular smooth muscle cell (VSMC) destruction [2]. These characteristics have been utilised experimentally by generating a VSMC inducible knockout mouse, the SM22α-hDTR Apoe−/− mice placed on a high fat diet (HFD) were given daily injections of recombinant dimeric murine P-selectin (22.5 μg/kg/day) for 8 or 16 weeks. Saline or sE-selectin injections were used as negative controls. In order to assess the role of sP-selectin on atherothrombosis an experimental plaque remodelling murine model, with SM22α-hDTR Apoe−/− mice on HFD in conjunction with delivery of diphtheria toxin to induce targeted vascular smooth muscle apoptosis, was used. These mice were similarly given daily injections of sP-selectin for 8 or 16 weeks. While plaque mass and aortic lipid content did not change with sP-selectin treatment in Apoe−/− or SM22α-hDTR Apoe−/− mice on HFD, increased plasma MCP-1 and a higher plaque CD45 content in Apoe−/− HFD mice was observed. As well, a significant shift towards a more unstable plaque phenotype in the SM22α-hDTR Apoe−/− HFD mice, with increased macrophage accumulation and lower collagen content, leading to a lower plaque stability index, was observed. These results demonstrate that chronically raised sP-selectin favours progression of an unstable atherosclerotic plaque phenotype.
Materials and Methods

Materials
Recombinant murine dimeric soluble E- and P-selectin-Fc Chimera were purchased from R & D systems (USA). Both recombinant proteins were endotoxin free as analysed by the manufacturer and confirmed in independent limulus assays, giving values below 0.01 EU/ml. Diptheria toxin was from Corynebacterium diphtheriae and purchased from Sigma-Aldrich (USA).

Animals
All procedures and protocols were approved by the AMREP Animal Ethics Committee (project approval number: E/0972/2010/B) and conformed to the Guide for the Care and Use of Laboratory Animals (NIH).

Male Apoe$^{−/−}$ mice on the C57BL/6 background and male SM22α-hDTR Apoe$^{−/−}$ were bred and housed at the Precinct Animal Centre, Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia. SM22α-hDTR Apoe$^{−/−}$ mice were created based on the published model described by Clarke et al [3]. Briefly, the mouse SM22α promoter (−445 to +88 relative to transcriptional start site) was cloned into the pSTEC-1/2 vector upstream of the mDTR-eGFP sequence. DNA was prepared for injection of the vector into oocytes and implantation and generation of chimeric progenitors was provided by the Biomedical Institute (Australia). SM22α-hDTR mice were born at the expected frequency and developed normally. SM22α-hDTR were subsequently crossed with Apoe$^{−/−}$ and progeny developed normally with no difference in phenotype from Apoe$^{−/−}$. From 6 weeks of age mice received daily sub-cutaneous (s.c.) injections of either vehicle (saline), sE-selectin (22.5 μg/kg/day) or sP-selectin (22.5 μg/kg/day) and were fed high fat diet (HFD) containing 0.15% cholesterol and 22% fat (Speciality Feeds, Western Australia) for 8 or 16 weeks. The dose of selectins was chosen on the basis of raising plasma sP-selectin by approximately 150 ng/ml, which is observed in human pathologies, most notably in cardiovascular and peripheral vascular disease, as previously described [15]. SM22α-hDTR Apoe$^{−/−}$ mice and their Apoe$^{−/−}$ mice controls were injected with Diptheria toxin (5 ng/g; three times weekly) for the last three weeks of the HFD.

Histology
The proximal aorta from within the heart was dissected and sectioned into sinus root, arch, thoracic and abdominal areas and embedded in OCT compound and snap frozen with isopentane chilled by liquid nitrogen. 6–10 μm sections of each aortic segment were analysed for lesion size defined as the cross sectional surface area of Oil Red O staining. Within the aortic intima, or by immunohistochemistry to identify CD45 (Pharminogen), CD68 (Serotec) and alpha smooth muscle actin (αSMA) expression (Abcam). Briefly, sections were fixed in cold (−20°C) acetone for 20 min. The sections were then sequentially incubated in 3% hydrogen peroxide in PBS, 10% normal goat/horse serum/PBS and biotin/avidin blocking reagents (Vector Laboratories). Thereafter, the sections were incubated with primary antibodies in normal goat serum. Sections were incubated with the corresponding biotinylated secondary antibodies and detected using the Avidin Biotin staining Complex (Vector Laboratories) with DAB (3,3′-Diaminobenzidine) reagent (Vector Laboratories). DAB treated sections were counterstained with hematoxylin and Scotts tap water. Apoptotic cells were identified by the in situ cell death detection kit dUTP nick end-labelling with peroxidase label (TUNEL-POD) (Roche). Collagen was stained with 0.1% Sirius Red F3BA (Sigma-Aldrich) in isopropanol and lipid with Oil Red O or Sudan-IV. Necrotic core was determined by analysis of Mayer’s Hematoxylin and Eosin stained sections with the unstained cellular regions defined as necrotic, as previously described [18].

Images were taken using an FSX100 Olympus microscope and analysed using Fiji 1.47 h software. Stained areas were expressed as a percentage of total plaque area. Stained cells (eg. TUNEL and CD45) were expressed as a percentage of total plaque cells. All mean data including mean lesion size was calculated from the measurement of cross sections taken from every 60 μm of the first 180 μm of the aortic sinus root proximal to the aortic arch or from every 100 μm of the first 600 μm in the ascending aorta proximal to the heart. Only advanced plaques (defined as those displaying necrotic cores) were analysed in this study. Plaque stability scores were defined as ratio of collagen content over lipid area, as previously described [19].

Sudan En Face Staining
Aortae segments (abdominal, thoracic, arch) were cleaned of peri-aortic fat, stained with Sudan IV and imaged by a light microscope (Motic SMZ-168) connected to a digital camera and computer. Stained areas were quantified using Fiji 1.47 h software and expressed as a percentage of total aorta area.

Plasma Protein/Cytokine/Chemokine Measurements
Blood was collected via cardiac puncture and plasma stored in sodium citrate (129 mM; tubes and analysed for soluble E- and P-selectin levels using ELISA (R & D) and mouse MCP-1 (CCL2), Rantes (CCL5), TNF-α, MIP-1α (CCL3), and IL-1β content using a Bio-Plex assay kit (Bio-Rad CA, U.S.A) according to the manufacturers’ instructions.

Statistical Analysis
Results are expressed as mean ± SEM. Comparisons between groups were carried out using a student’s unpaired t-test or for multiple comparisons, by a one way ANOVA followed by Tukey’s post-hoc test. A value of P<0.05 was considered as statistically significant. At least 6 mice per group were analysed for each data set.

Results
Effect of Treatment on Plasma Soluble Markers in Apoe$^{−/−}$ Mice
Plasma sP-selectin and sE-selectin levels were analysed in all animals. Figure 1A shows that following either 8 or 16 weeks of daily injections of recombinant sP-selectin, circulating plasma levels increased to around 300 ng/ml, which is significantly higher than levels observed in saline or sE-selectin treated animals, and is comparable to human pathologies including CVD and PVD [13,20]. Similarly sE-selectin injections led to increases in plasma levels of sE-selectin as measured by ELISA (figure 1B).

To generate a picture of the overall systemic inflammatory response following treatments, a plasma BioPlex ELISA was performed. sE-selectin treatment significantly increased plasma levels of IL-1β, MCP-1 and MIP-1α (figure 1C), while a significant decrease in RANTES was also noted (figure 1C).

Effect of sP-selectin on Apoe$^{−/−}$ Plaque Size and Phenotype
Figure 2 shows plaque size assessed as percentage of Oil-red-O staining in a given field, from daily injections of sP-selectin, or sE-selectin and saline delivered as controls, into HFD fed Apoe$^{−/−}$
mice fed. As expected, aortic sections had significantly greater atherosclerosis in all groups of Apoe2/2 mice from 8 to 16 weeks on HFD (figure 2a). However, there were no significant differences between treatment groups. Plaque mass was also assessed for thoracic, abdominal and arch areas. While arch areas did show significantly increased atherosclerosis compared to either the thoracic or abdominal regions, there were no changes in atherosclerosis between treatment groups at either 8 weeks (data not shown) or 16 weeks of treatment (figure 2b).

Figure 3 shows a trend for an increase in plaque CD45+ leukocytes after 8 weeks of sP-selectin treatment which became highly significant at 16 weeks. A small but significant increase in plaque CD45+ leukocyte content following 16 weeks of sE-selectin administration was also observed, although this was half of that observed following sP-selectin (figure 3).

Effect of sP-selectin on Plasma Inflammatory Markers in SM22α-hDTR Apoe2/−

To further explore the effect of s-P-selectin on plaque phenotype, we applied a published experimental plaque remodelling model [SM22α-hDTR Apoe2/−] [3] in combination with raising plasma sP-selectin over 8 and 16 wks.

To examine if similar changes in plasma inflammatory markers could also be noted in the SM22α-hDTR Apoe2/− model as in Apoe2/− alone (on HFD), we analysed plasma levels of MCP-1, MIP-1α, IL1β and RANTES after sP-selectin or control treatments (saline, sE-selectin) after 16 wks (8 wk data not shown). Similar to Apoe2/− on HFD alone, sP-selectin increased plasma levels of MCP-1 and MIP-1α in this model (figure 4). This was coupled with a decrease in RANTES after sP-selectin treatment (figure 4). There was no difference in IL1β levels (data not shown).

Effect of sP-selectin on Plaque Morphology in SM22α-hDTR Apoe2/−

A preliminary validation group of 16 wk HFD fed SM22α-hDTR Apoe2/− in comparison to Apoe2/− mice showed a significant change in overall plaque mass (figure 5A). There was a significant increase in necrotic core and αSMA content with a decrease in collagen content (figure 5B–D). These data confirm previous findings using the SM22α-hDTR Apoe2/− mouse model, albeit with less significance [3].

We next applied daily injections of sP-selectin, sE-selectin or saline for 8 and 16 weeks. Figure 6 shows that, as in Apoe2/− mice, SM22α-hDTR Apoe2/− mice on a HFD with daily injections of sP-selectin for 16 weeks exhibited no significant change in total plaque mass lipid content (ORO), as compared to control injections using saline or sE-selectin. This was also noted in all regions of the aorta using en-face Sudan IV staining after 16 wk treatment (figure 6B). No change in lipid content was noted after 8 wk treatments (data not shown).

Conversely, sP-selectin treatment led to significantly lower collagen content after 16 weeks (figure 7A) and a significantly
higher CD68+ macrophage accumulation (figure 7B) but not total CD45+ cells in the plaque (figure 7C), compared to saline and E-selectin injected controls (8 wk data not shown). No change in collagen content was noted after 8 wk treatments (data not shown) and there was no effect of treatment on αSMA content and apoptotic cells (figure 7D–E) at 16 weeks. Overall a significant reduction in plaque stability score, as assessed by collagen over plaque area, was observed (figure 7F).

Discussion

Like all selectins, membrane bound P-selectin has an N-terminal lectin domain, an epidermal growth factor motif, and specifically, nine regulatory protein repeats, a transmembrane section and a short intracytoplasmic tail [6]. After activation of endothelial cells by inflammatory mediators such as TNFα, Weibel–Palade bodies can become rapidly mobilised, which results in P-selectin expression extending approximately 40 nm from the endothelial surface, lasting up to 3 hours depending on the vasculature [6,21]. Similarly, platelets can undergo surface expression of P-selectin upon activation by agonists such as P2 receptor agonists [22]. As P-selectin is a component of the membrane of platelet alpha and dense granules, expression reflects activation [22,23]. P-selectin is a potent adhesion molecule and has been reported to have procoagulant activities by regulating the production of monocyte derived platelet activating factor and tissue factor [24]. Moreover, P-selectin primes monocytes for increased phagocytosis [25]. However, the role of P-selectin in vascular inflammation is complicated by a circulating soluble form, which can arise from...
proteolytic cleavage and direct expression of P-selectin lacking the cytoplasmic domain [26]. Evidence that sP-selectin has a direct role in atherosclerosis has been reported [17]. Studies from our laboratory demonstrate a role for sP-selectin in regulating leukocyte adhesion in patients with peripheral arterial occlusive disease in vivo [14,15]. Pathophysiological concentrations of
sP-selectin engages its ligand, PSGL-1, resulting in Src kinase-dependent Mac-1 (CD11b/CD18) up-regulation and adhesive function [14,15]. Overall, these earlier studies raised the possibility that sP-selectin may promote leukocyte recruitment to sites of vessel wall injury and vascular endothelium in patients with CVD.

We wanted to explore this issue and examine if chronically raised plasma levels of sP-selectin led to direct changes in atherosclerosis progression and/or phenotype in the ApoE−/− experimental mouse model on HFD. Daily injections of 22.5 μg/kg/day of recombinant Fc-chimera sP-selectin over 8 and 16 weeks, led to significantly raised plasma levels of protein which were similar to pathophysiological levels seen in CVD [13,20]. We showed that chronically raising plasma levels of sP-selectin had modest effects on atherosclerosis in ApoE−/− mice on HFD, compared to raised sE-selectin and saline injection controls. When initially examining total plaque mass, we observed no significant change in atherosclerosis over 8 and 16 weeks HFD with sP-selectin, compared to controls. This was disappointing given previous reports using the DeltaCT mouse model which exhibits abnormally high concentrations of plasma sP-selectin [27], leading to increased aortic sinus lesion mass after 16 wks on normal chow [17]. These disparate findings may represent variations in effects due to the structure of sP-selectin, which in the DeltaCT model lacks the cytoplasmic domain [27]. Indeed, the DeltaCT mouse model demonstrates significant increases in procoagulant activity [14,15]. Overall, these earlier studies raised the possibility that sP-selectin may promote leukocyte recruitment to sites of vessel wall injury and vascular endothelium in patients with CVD.

Speculated that chronically raising plasma levels of sP-selectin may alter plaque stability.

In order to test the hypothesis that raised plasma sP-selectin increases plaque vulnerability we employed a plaque destabilising model using the genetically susceptible mouse model, SM22x-hDTR on ApoE−/− and HFD background [3]. Previous work has investigated VSMC apoptosis by generating transgenic mice that express the human diphtheria toxin receptor (hDTR), encoded by heparin-binding EGF-like growth factor from a minimal Tagln (also known as SM22a) promoter [3]. In this model, 50–70% of VSMC apoptosis resulted in normal arteries with no inflammation, thrombosis, remodeling or aneurysm formation [3]. However, the atherosclerotic plaques with VSMC apoptosis in SM22x-hDTR ApoE−/− mice resulted in thinning of the fibrous cap, loss of collagen and intimal inflammation, which are all features of plaque vulnerability [3,28].

VSMCs together with extracellular matrix components comprise the medial layer of adult arteries. VSMC apoptosis can increase as atherosclerotic plaques develop and rupture [29] [28]. After this initial wave of apoptosis, normal medial cell content can be derived several weeks later [30], suggesting that VSMC apoptosis may initiate repopulation. Moreover, VSMC apoptosis may induce calcification, coagulation and pro-inflammatory [31]. Importantly, VSMC apoptosis causes release of IL-1α [32], MCP-1 and IL-8 resulting in infiltration of macrophages [33], as we find in sP-selectin treated SM22x-hDTR ApoE−/− mice.

Overall, we would speculate that VSMC apoptosis may lead to evidence of unstable plaque or silent plaque rupture in mouse models of atherosclerosis and that raising plasma sP-selectin levels, would increase the risk of plaque rupture, as suggested by others in the DeltaCT model [17]. As such, we performed a preliminary validation group of 16 wk HFD fed SM22x-hDTR ApoE−/− in comparison to ApoE−/− mice on HFD. The SM22x-hDTR

**Figure 4. Plasma cytokine and chemokine levels after sP-selectin treatment for 16 weeks in SM22x-hDTR ApoE−/− HFD.** Plasma levels of MCP-1, MIP-1α and RANTES after 8 or 16 weeks of daily s.c injections (22.5 μg/kg/day) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). n = 9–15 mice per treatment group. Data represented as Mean (pg/ml) ± SEM where * represents P<0.05 (from saline treatment) as analysed by unpaired students t-test. doi:10.1371/journal.pone.0097422.g004

sP-selectin and Atherosclerotic Plaque
Figure 5. Model validation data. Sections of each aortic segment were analysed for lesion size, necrotic core, \( \alpha \)-SMA and collagen content in Apoe\(^{-/-}\) HFD or SM22hDTR-ApoE\(^{-/-}\) HFD mice. A) lesion size defined as % ORO staining, B) necrotic core defined as % unstained with Mayer’s Hematoxylin and Eosin, C) % \( \alpha \)-SMA content, D) % collagen content. \( n = 6–9 \) mice per treatment group. Data represented as Mean (% stain) \( \pm \) SEM where * represents \( P < 0.05 \) as analysed by unpaired students t-test. Representative images are given for each analysis. Scale bars = 100 \( \mu m \).

doi:10.1371/journal.pone.0097422.g005

Figure 6. Oil-Red-O and en face Sudan IV assessment of plaque area in SM22\(^{a-hDTR}\) ApoE\(^{-/-}\) HFD with sP-selectin treatment for 16 weeks. A) Sections of each aortic segment were analysed for lesion size defined as the cross sectional surface area of % ORO staining within the aortic intima after 16 weeks of daily s.c injections (22.5 \( \mu g/kg/day \)) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). B) En-face Sudan IV staining from indicated aortic sections after 16 weeks of daily s.c injections (22.5 \( \mu g/kg/day \)) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). \( n = 6–9 \) mice per treatment group. Data represented as Mean (%)(\pm SEM).

doi:10.1371/journal.pone.0097422.g006
Apoe/−/− mice showed significant changes in necrotic core, collagen content, increased apoptosis and αSMA content, but was absent of changes in overall plaque mass. These data confirm previous findings of Clarke et al [3], albeit with reduced significance, and demonstrate that SM22α-hDTR ApoE/−/− HFD mice exhibit signs of unstable plaque, leading to an overall decreased plaque stability score, as is noted in human disease [34]. Interestingly, raising plasma sP-selectin in SM22α-hDTR ApoE/−/− HFD mice led to decreased atherosclerotic collagen content and increased CD68+ macrophage plaque infiltration, compared to controls. Moreover, similar to sP-selectin treatment in Apoe/−/− HFD alone, sP-selectin treatment in SM22α-hDTR ApoE/−/− HFD mice elicited increased plasma levels of MCP-1 and MIP-1α.

MCP-1 and MIP-1α are important chemokines involved in monocyte recruitment, which is integral to vascular inflammation and atherosclerosis. The MCP-1 and MIP-1α chemokine axis may be important in regulating recruitment of specific subsets of monocytes with independent effector functions, specifically CCR2highCD14highCD16low and CX3CR1highCD14lowCD16high human monocytes [35–37]. The contribution of these monocyte subsets in atherosclerosis is part of ongoing work by many labs, in which recruitment of CCR2highCD14high monocytes is thought to be particularly important in inflammatory atherosclerosis [35,38]. More work will be needed to understand if soluble P-selectin mediates differences in monocyte subset numbers or recruitment, and macrophage phenotype in atherosclerosis. Further, in light of a recent study showing that inflammatory plaque progression may be regulated by tissue macrophage proliferation independent of monocyte recruitment [39], the role of monocyte recruitment in vulnerable atherosclerosis progression requires further

Figure 7. Collagen, CD45+, CD68+, α-SMA and apoptotic cell content and plaque stability scores in SM22α-hDTR ApoE/−/− HFD after sP-selectin treatment for 16 weeks. A) Sections of each aortic segment were analysed for collagen content by immunohistochemistry after 16 weeks of daily s.c injections (22.5 μg/kg/day) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). Representative images are given below. Sections of each aortic segment were analysed for B) CD68+ (representative images shown below) and C) CD45+ cells by immunohistochemistry after 16 weeks of daily s.c injections (22.5 μg/kg/day) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). D) αSMA content or E) apoptotic cell content (% TUNEL) by immunohistochemistry after 16 weeks of daily s.c injections (22.5 μg/kg/day) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). F) Plaque stability scores were defined as ratio of collagen content over lipid area after 16 weeks of daily s.c injections (22.5 μg/kg/day) of vehicle control (saline matched volume; white bar), sP-selectin (black bar) or sE-selectin (checkered bar). n = 6–9 mice per treatment group. Data represented as Mean (% stain or score) ± SEM where * represents P<0.05 (from saline or indicated treatment) as analysed by One-way ANOVA with Tukey’s post-hoc test or unpaired student’s t-test (C, D). Scale bars = 100 μm. doi:10.1371/journal.pone.0097422.g007
investigation. Neovascularisation has been noted as a hallmark of atherogenesis and a mechanism by which monocytes can enter atherosclerotic lesions [40]. We did not analyse neovascularisation in our model as it was beyond the scope of the current study. Future studies are necessary, however, to investigate whether unstable-plaque progression with high levels of sP-selectin promotes neovascularisation and macrophage accumulation.

RANTES is expressed by T-cells, fibroblasts, mesangial cells and platelets [41,42] and when overexpressed in atherosclerosis, can engage chemokine receptors on the endothelium, mediating transmigration of monocytes and lymphocytes into the intima [43,44]. RANTES is also expressed in atherosclerotic plaques [45]. Given this strong association between inflammatory atherosclerosis and RANTES, we would have expected an increase in plasma levels of RANTES after sP-selectin. Unexpectedly, we noted significant decreases in RANTES in our Apoe−/− HFD model. Of interest, recent work in the Atherosclerosis Risk in Communities (ARIC) Carotid MRI study has shown that mean minimum fibrous cap thickness was positively associated with RANTES levels [46]. As the thickness of the fibrous cap dictates the stability of the atherosclerotic plaque [47], it may be that RANTES levels compensate for increased inflammatory burden after sP-selectin treatment. This hypothesis requires further investigation.

Overall, aside from significant decreases in collagen content and significant increases in plaque inflammatory cell infiltrates and plasma inflammatory chemokines, raising plasma levels of sP-selectin in SM22α/dhTTR Apoe−/− HFD model as a model of experimental unstable atherosclerosis only led to modest changes in plaque stability scores, as compared to controls. However, collagen content is a primary characteristic of unstable/vulnerable plaque and strategies that have directly targeted collagen content through either experimental regression studies or therapeutically increasing plaque collagen density have proven to have positive outcomes in experimental models of atherosclerosis [48]. Therefore this reduction in collagen with sP-selectin treatment, coupled with macrophage infiltration and systemic inflammatory chemokines, would lead to a significant increased risk of plaque rupture and warrants further investigation as to whether targeting raised plasma sP-selectin would be an effective therapeutic strategy in unstable CVD.

In conclusion, we have shown in two models of experimental atherosclerosis that raising plasma concentrations of sP-selectin to pathophysiological levels had some effects on plaque phenotype related to characteristics of vulnerable plaque, such as reduced collagen content and increased inflammatory cellular content. We propose that an increase in circulating sP-selectin may not only be a marker of vascular disease, but may also contribute to the inflammatory hypothesis of unstable plaque progression.

Acknowledgments
We wish to acknowledge the technical assistance of Dr Ika Carmichael and Dr Stephen Cody of the Monash Micro-Imaging Department, Baker IDI.

Author Contributions
Conceived and designed the experiments: KW JCD AB NL AA EH PK JJ. Performed the experiments: NL AA KW EH AMJ LF JJ. Analyzed the data: NL KW AA EH JCD LF KA JJ. Contributed reagents/materials/analysis tools: AMJ PK AB. Wrote the paper: KW NL JCD.

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