Title: A brief elevation of serum amyloid A is sufficient to increase atherosclerosis

Joel C. Thompson\textsuperscript{a,b}, Colton Jayne \textsuperscript{a}, Jennifer Thompson\textsuperscript{a}, Patricia G. Wilson\textsuperscript{a}, Meghan H. Yoder\textsuperscript{a}, Nancy Webb\textsuperscript{b,c} and Lisa R. Tannock\textsuperscript{a,c,d} *

\textsuperscript{a} Department of Internal Medicine, Division of Endocrinology and Molecular Medicine, University of Kentucky, Lexington, KY, USA

\textsuperscript{b} Department of Pharmacology and Nutritional Sciences, Division of Nutritional Sciences, University of Kentucky, Lexington, KY, USA

\textsuperscript{c} Barnstable Brown Diabetes and Obesity Research Center, University of Kentucky, Lexington, KY, USA

\textsuperscript{d} Department of Veterans Affairs, Lexington, KY, USA

*Correspondence to: Dr. Lisa R. Tannock,
Associate Professor of Medicine
Chief, Division of Endocrinology and Molecular Medicine
Room 553, Wethington Building
900 S. Limestone
University of Kentucky
Lexington, KY 40536-0200
Tel: 859-323-4933 ext 81415
Fax: 859-257-3646
E-mail: Lisa.Tannock@uky.edu

Running Title: SAA increases atherosclerosis

**Abbreviations:**

SAA, serum amyloid A
APR, acute phase response
\textit{apoe}\textsuperscript{−/−}, apolipoprotein E deficient
\textit{rag1}\textsuperscript{−/−}, recombination activating gene 1
TGF-β, transforming growth factor beta
Abstract

Serum Amyloid A (SAA) has a number of pro-atherogenic effects including induction of vascular proteoglycans. Chronically elevated SAA was recently shown to increase atherosclerosis in mice. The purpose of this study was to determine if a brief increase in SAA similarly increased atherosclerosis in a murine model. \textit{rag1}^{+/-} \times apoe^{-/-} and \textit{apoe}^{-/-} male mice were injected, multiple times or just once respectively, with an adenoviral vector encoding human SAA1 (ad-SAA), or controls and maintained on chow for 12-16 weeks. Mice receiving multiple injections of ad-SAA, in which SAA elevation was sustained, had increased atherosclerosis compared to controls. Strikingly, mice receiving only a single injection of ad-SAA, in which SAA was only briefly elevated, also had increased atherosclerosis compared to controls. Using in vitro studies we demonstrate that SAA treatment leads to increased LDL retention, and that prevention of TGF-\(\beta\) signaling prevents SAA induced increases in LDL retention and SAA induced increases in vascular biglycan content. We propose that SAA increases atherosclerosis development via induction of TGF-\(\beta\), increased vascular biglycan content and increased LDL retention. These data suggest that even short term inflammation with concomitant increase in SAA may increase the risk of developing cardiovascular disease.

Key words:

JLR editorial board: apolipoproteins, extracellular matrix, lipoproteins, proteoglycans, vascular biology

Free-form: biglycan, TGF-\(\beta\), cardiovascular disease
Introduction

SAA is a family of apoproteins highly expressed during an acute phase response (APR), the body’s initial response to infection or trauma. Humans and mice each have two acute phase isoforms of SAA synthesized primarily in the liver, but also expressed in vascular smooth muscle cells, adipocytes and macrophages (1, 2). During an APR, SAA levels can increase up to 1000-fold during which SAA becomes the primary apoprotein on HDL (3). The increase in SAA during an APR is thought to play a role in normal host response to an immunogenic stimuli; however, diabetes and obesity as well as other chronic inflammatory diseases are characterized by having persistently elevated SAA expression (4, 5). SAA, like C-reactive protein, is a marker of inflammation and predictive of cardiovascular disease (CVD) events (6). The observation that SAA is increased in diabetes and obesity, and that individuals with those diseases have an increased risk of developing CVD led to the question of whether SAA could play a causal role in CVD. SAA has several characteristics that make it potentially atherogenic. It can enhance monocyte recruitment by increasing expression of CCL2 in a formyl peptide receptor- like dependent manner (7), directly stimulate foam cell formation by upregulating lectin-like oxidized low-density lipoprotein receptor 1(8), stimulate chemotaxis of lymphocytes to subcutaneous sites of recombinant SAA injection (9) and facilitate the binding of high density lipoprotein to vascular proteoglycans (10). SAA has been found in atherosclerotic lesions of both LDL receptor and apolipoprotein E deficient mice co-localized with apoB and apoA-I containing lipoproteins (11). SAA mRNA has also been detected in many different cell types in human atherosclerotic lesions (2). Recently Dong et al overexpressed murine SAA1 via a lentiviral vector in apoE−/− mice and demonstrated that modest but sustained elevation of SAA led to increased atherosclerosis through increased inflammatory cell infiltration into the lesion (12).
As outlined in the Response to Retention hypothesis (13) atherosclerosis is thought to be a disease of lipoprotein retention followed by inflammation. In the earliest stages of the disease, lipoproteins such as LDL are retained by extracellular matrix proteoglycans. The small leucine-rich proteoglycan biglycan is the proteoglycan most consistently found co-localized with LDL (14, 15). The retained LDL becomes chemically modified triggering an inflammatory response characterized by the infiltration of macrophages into the subendothelial space leading to foam cell formation. The lesion development progresses ultimately leading to clinically significant complex atheroma (16). We have previously shown that stimulating vascular smooth muscle cells with physiologically relevant doses of SAA resulted in a dose dependent increase in proteoglycan synthesis, especially biglycan. SAA-stimulated vascular smooth muscle cells secreted biglycan with longer glycosaminoglycan side chains and greater affinity for LDL suggesting another potentially pro-atherogenic role for SAA. Interestingly, the increase in biglycan synthesis was ameliorated when the TGF-β inhibitory antibody 1D11 was given concurrently with SAA, suggesting that SAA acts through TGF-β to increase vascular biglycan.

apoε−/− mice injected with an adenoviral vector encoding human SAA1 (ad-SAA) had increased vascular biglycan content. The increase in vascular biglycan content was seen after only a brief increase in SAA(17). Thus, the present study was performed to determine if a brief increase in SAA, such as that seen in an APR, was sufficient to increase atherosclerosis. Here we demonstrate that not only sustained, but also brief increases in SAA caused increased atherosclerosis in a murine model. This novel finding is clinically relevant given that epidemiological data suggests that survivors of intensive hospitalization or injury with a concomitant acute phase response have increased subsequent mortality primarily from cardiovascular disease(18-20).
Materials and Methods

All work was completed in accordance with Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and the University of Kentucky Animal Care and Use Committee guidelines.

Primary vascular smooth muscle cells were isolated from ~3 week old apolipoprotein E (apoE−/−, stock # 002052) mice and grown to confluence without passaging. Cells were incubated with vehicle, sodium chlorate, endotoxin free SAA (25μg/ml) isolated from acute phase murine plasma [(21) kindly provided by Frederick and Maria de Beer, University of Kentucky] ± TGF-β neutralizing antibody 1D11 (10μg/ml, MAB1835, R & D Systems; Minneapolis, MN), control antibody 13C4 (10μg/ml, Custom antibody; GenScript, Piscataway, NJ) or 10mM sodium chlorate (Sigma-Aldrich, St. Louis, MO) (22), or TGF-β (T7039, Sigma-Aldrich, St. Louis, MO) ± sodium chlorate for 24 hours. The cells were washed and then labeled with Alexa-fluor 594 (A10237; Life Technology; Grand Island, NY) labeled LDL for 2 hours at 4°C. Cells were washed twice in cold PBS then fixed in 4% formaldehyde, imaged and quantified using ImageJ software. LDL binding is expressed as Alexa-fluor 594 surface area normalized to DAPI surface area from 5-10 20x microscope fields. Negative controls included wells with no cells and/or no LDL.

For all in vivo studies, apoE−/−, stock # 002052 and recombination activating gene 1 (rag1−/−, stock # 003729) deficient mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). rag1−/− x apoE−/− deficient mice were generated in our breeding colony. Beginning at eight weeks of age, male rag1−/− x apoE−/− mice were injected via the lateral tail vein every 21 days with 10^{11} plaque forming units of an adenoviral vector encoding human SAA1(ad-SAA), a control adenoviral vector (ad-Null) or saline as previously reported (17). These mice
were utilized as the *rag1*<sup>−/−</sup> made them tolerant to repeated viral injections facilitating chronic expression of SAA (*model of sustained elevation in SAA*). Eight week old *apoε*<sup>−/−</sup> male mice, also from Jackson Laboratory, received a single lateral tail vein injection of ad-SAA, ad-Null or saline (*model of brief elevation in SAA*). In some experiments mice were concurrently injected with 1D11 or 13C4 (2mg/kg, IP). All studies were performed on male mice housed in specific pathogen free caging systems with 12 hour light/dark cycles and ad lib access to normal rodent chow (Harlan Teklad) and water. After 12 weeks (*rag1*<sup>−/−</sup> × *apoε*<sup>−/−</sup>) or 16 weeks (*apoε*<sup>−/−</sup>) mice were euthanized and tissues collected. Plasma levels of human SAA and murine SAA were measured by species specific ELISA kits from Anogen (EL10015; Ontario, Canada) and Life Technologies (KMA0021; Grand Island, NY) respectively. Atherosclerosis was measured on the aortic intimal surface, the aortic root and the brachiocephalic artery as previously described (23). Aortic root sections from ad-SAA, ad-Null and saline groups were double labeled for biglycan (AF2667; R & D Systems, Minneapolis, MN) and apoB (K23300R; BioDesign International, Saco, ME); apoB and perlecan ( MAB458, Millipore, Billerica, MA); biglycan and SAA (kindly provided by the De beer laboratory, University of Kentucky, USA); or isotype controls and visualized using confocal or fluorescent microscopy as previously described (24). Aortas and/or carotid arteries were collected from *apoε*<sup>−/−</sup> mice and analyzed for vascular biglycan content by western blot for biglycan or actin and analyzed by densitometry using imageJ software (NIH). Total plasma cholesterol, triglycerides, TGF-β, and lipoprotein distribution by fast performance liquid chromatography were measured as previously described (24). Statistical differences were assessed by one-way ANOVA to assess effects of treatment with ad-SAA, ad-Null or saline; or ad-SAA and 1D11 or 13C4. P<0.05 was considered statistically significant.
Results

The response to retention hypothesis states that lipoprotein retention by vascular matrix proteoglycans is an initiating event in atherosclerosis (16). To determine if SAA increased the binding of apoB containing lipoproteins to vascular matrix, primary apoe<sup>−/−</sup> vascular smooth muscle cells (VSMC) were incubated with vehicle or SAA for 24 hours followed by incubation with Alexa-594 labeled LDL for two hours. Our previous work revealed that SAA acted through TGF-β to stimulate biglycan synthesis in VSMCs (17). To determine if SAA acted via TGF-β, parallel wells were treated with TGF-β, the TGF-β neutralizing antibody 1D11 or irrelevant control antibody 13C4. Proteoglycans interact with apoB containing lipoproteins via ionic interactions between positively charged residues on apoB and negatively charged sulfate ions on the proteoglycan glycosaminoglycan side chains (25). To determine if the LDL binding was the direct result of proteoglycan/lipoprotein interaction, apoe<sup>−/−</sup> VSMCs were treated with the sulfate biosynthesis inhibitor sodium chlorate, resulting in glycosaminoglycan side chains with a neutral charge thereby inhibiting the proteoglycan/lipoprotein interaction. Cells treated with SAA or TGF-β, known to stimulate proteoglycan synthesis, had increased LDL retention compared to vehicle treated cells (P<0.001; Fig. 1), but the presence of sodium chlorate attenuated this effect, implying the increased LDL binding was due to increased proteoglycan synthesis and/or sulfation. When cells were co-incubated with SAA and the TGF-β inhibitory antibody 1D11, LDL binding was reduced compared to SAA alone, but there was no effect of the control antibody 13C4; further supporting the role for TGF-β in SAA mediated lipid retention.

We previously demonstrated in apoe<sup>−/−</sup> mice that a brief increase in SAA led to increased vascular biglycan content 28 days later (17). To determine if TGF-β is required in vivo for SAA induction of biglycan, apoe<sup>−/−</sup> mice were injected with ad-Null, ad-SAA or ad-SAA concurrently...
with 1D11 or 13C4. Plasma was collected 24 hours after the injections and assayed for human SAA and TGF-β. As expected, mice receiving ad-Null had no detectable SAA or induction of TGF-β. Mice that received ad-SAA alone or in combination with 13C4 had dramatic induction of both SAA and TGF-β. However, mice receiving ad-SAA + 1D11 antibody had the same dramatic increase in plasma SAA observed in the ad-SAA and ad-SAA + 13C4 groups, yet the SAA induced increase in plasma TGF-β was completely inhibited (Figure 2A). Mice receiving ad-SAA or ad-SAA with 13C4 had an increase in vascular biglycan content 28 days after injections compared to ad-Null injected mice; however, 1D11 strikingly attenuated SAA-induced vascular biglycan content (Figure 2B).

To determine if a brief increase in SAA resulted in increased atherosclerosis, eight week old apoe⁻/⁻ male mice received a single injection of ad-SAA, ad-Null or saline and were maintained on normal rodent chow for 16 weeks. The mice receiving ad-SAA had a dramatic increase in human SAA that returned to baseline levels in less than two weeks (Figure 3A). Murine SAA did not differ between groups at any timepoint (Figure 3B). TGF-β increased briefly in ad-SAA-injected mice in concordance with plasma SAA expression, with no changes observed in ad-Null or saline groups (Figure 3C). To determine the persistence of increased vascular wall biglycan induced by increased plasma SAA, carotid arteries were collected 16 weeks after a single injection of ad-SAA, ad-Null or saline and western blot analysis was performed. Remarkably, vascular biglycan content remained increased 16 weeks after a brief increase in plasma SAA (Figure 3D, p<0.05). Despite only the brief increase in human SAA levels, ad-SAA mice developed significantly more atherosclerosis on the aortic intimal surface (Figure 4A, p<0.001), the brachiocephalic artery (Figure 4B, p<0.05) and the aortic root (Figure 4C, p<0.05) compared with ad-Null or saline treated mice. To assess the interaction between vascular biglycan and apoB-containing lipoproteins, aortic root sections from ad-SAA, ad-Null and saline injected mice were double stained for biglycan and apoB, demonstrating co-
localization of biglycan with apoB in atherosclerotic lesions (Figure 4D). Aortic root sections from ad-SAA, ad-Null and saline injected mice were double stained for perlecan and apoB as perlecan is known to be upregulated in murine atherosclerotic lesions (26). No difference in apoB retention was observed between the three groups (Supplemental Figure). Furthermore, murine aortic root sections from ad-SAA, ad-Null and saline injected mice were double stained for biglycan and SAA demonstrating co-localization in mice injected with ad-SAA (Supplemental Figure). The induction of human SAA had no effect on plasma lipids or body weight (Table 1). Lipoprotein distribution was measured using fast performance liquid chromatography with no differences observed between groups (data not shown).

SAA is persistently elevated in a number of chronic inflammatory conditions such as diabetes, obesity, metabolic syndrome, rheumatoid arthritis, etc, all of which are associated with increased cardiovascular disease. To determine the impact of sustained elevations of SAA on atherosclerosis development, eight week old male \textit{rag1 x apo e} \textsuperscript{-/-} mice were injected with ad-SAA, ad-Null or saline once every 21 days and maintained on normal rodent chow for twelve weeks. As immunocompetent mice are intolerant of repeated adenoviral injections, \textit{rag1 x apo e} \textsuperscript{-/-} mice were used. The injection of ad-SAA led to a similar induction of SAA as seen in \textit{apo e} \textsuperscript{-/-} mice, but the plasma SAA elevation was sustained over 12 weeks (Figure 5A). There were no elevations in murine SAA (Figure 5B) in any group at any timepoint. Mice injected with ad-SAA had increased TGF-\(\beta\) compared with ad-Null or saline injected mice throughout the study (Figure 5C). Ad-SAA injected mice had increased atherosclerosis on the aortic intimal surface (Figure 5D, \(p<0.001\)) and within the brachiocephalic artery (Figure 5E, \(p<0.05\)) compared to ad-Null or saline groups. There was a trend towards increased atherosclerosis in the aortic root; however, it did not reach statistical significance (Figure 5F). Similar to the \textit{apo e} \textsuperscript{-/-} model, there were no differences between groups in plasma lipids, body weight (table), or lipoprotein distribution (not shown).
Discussion

We previously demonstrated that SAA increased vascular smooth muscle cell proteoglycan synthesis. Further, proteoglycans synthesized in response to elevated SAA had increased LDL binding affinity (17). We confirmed this in vivo by demonstrating that vascular biglycan content was increased in apoe−/− mice 28 days after ad-SAA. We now demonstrate that SAA’s induction of vascular biglycan content persists up to 16 weeks after a single brief increase in SAA. Strikingly, apoe−/− mice given only a single injection of ad-SAA also had increased atherosclerosis even though SAA levels were back to baseline in less than 10 days. Using in vitro studies we demonstrate that SAA treatment leads to increased LDL retention, and that prevention of TGF-β signaling prevents SAA induced increases in LDL retention and SAA induced increases in vascular biglycan content. We further demonstrate that SAA mediated LDL binding is proteoglycan specific as inhibition of sulfation of the glycosaminoglycan side chains resulted in reduced LDL binding. In the LDL binding studies presented here, the cells were treated with lipid-free murine SAA. There is still debate as to the biological activity of acute SAA in the lipid-free vs. HDL bound states. In vivo SAA is almost exclusively associated with HDL, and SAA displaces apo-AI as the predominant apoprotein on HDL during an acute phase response (3). However, the exact physiological effect of SAA rich acute phase HDL on reverse cholesterol transport is still being debated. Interestingly, in mice lacking both forms of acute SAA, macrophage reverse cholesterol transport was still reduced in LPS induced acute inflammation suggesting that inflammation and not SAA loading alters HDL sterol efflux capacity (27). Furthermore, in previous work we demonstrated that lipid-free, but not HDL bound SAA increased biglycan synthesis in vitro, yet in vivo SAA is found only associated with HDL and still biglycan synthesis increased. This suggests that somewhere within the milieu of the
vessel wall the SAA is delipidated prior to stimulating biglycan synthesis (17). Thus, we propose that SAA increases atherosclerosis development via induction of TGF-β, increased vascular biglycan content and increased LDL retention.

In these murine models, either brief or sustained increases in SAA led to increased atherosclerosis development. In both murine models studied, human SAA was increased with no change in murine SAA implying the absence of any inflammatory response to the adenoviral vectors or experimental manipulation of the mice. Furthermore, injection of ad-SAA led to an increase in TGF-β which correlated with the duration of increased human SAA. In both models increased SAA had no effect on lipids or body weight. Our model of sustained elevation in SAA supports the previous findings from Dong et al (12) that SAA is pro-atherogenic. In that study, murine SAA was overexpressed by lentiviral vector leading to significantly increased atherosclerosis on the aortic intimal surface and the aortic sinus which the authors attributed to up-regulation of vascular adhesion molecules and chemotactic factors capable of increasing leukocyte migration into atherosclerosis susceptible arteries. The study presented here addresses events prior to vascular wall leukocyte infiltration, namely vascular wall proteoglycan synthesis and lipoprotein retention (28). Our data demonstrates that at least one mechanism by which SAA is pro-atherogenic is through the induction of vascular biglycan and subsequent retention of atherogenic lipoproteins. We recently demonstrated correlation between the extent of increased vascular biglycan content and increased atherosclerosis development in the LDL receptor deficient murine model (24), further supporting that increased vascular biglycan content contributes to increased atherosclerosis. The experiments presented here as well as the work by Dong et al utilized the SAA1 isoform specifically. However, acute SAA exists in multiple isoforms and SAA2 has been shown to increase reverse cholesterol transport which would be considered athero-protective (29). Within human and murine physiology, the acute SAAs
increase together and to about the same magnitude after inflammatory insult (1). Diabetic and obese individuals have chronically elevated SAA, both 1 and 2, and a greater risk of developing cardiovascular disease suggesting that the increase in both acute SAAs is predominantly pathologic even if increased SAA2 can increase reverse cholesterol transport (30, 31). However, further studies are needed to explicitly define the role of increased SAA2.

Using the rag1 x apoe\textsuperscript{-/-} murine model which is tolerant of repeated adenoviral injections we demonstrate that sustained elevations in SAA increased atherosclerosis to a greater extent than a single brief elevation of SAA. Although direct comparisons of apoe\textsuperscript{-/-} and rag1 x apoe\textsuperscript{-/-} are limited, the literature reports that rag1 or rag2 deficient apoe\textsuperscript{-/-} mice appear to have decreased lesion size compared to their immunocompetent littermates(32, 33). Thus, even with our findings of a 2 fold (aortic intimal surface) and a 5 fold (aortic sinus) increase in atherosclerosis compared to apoe\textsuperscript{-/-} mice, the extent of disease development is still likely underestimated given the relative resistance to the development of atherosclerosis in rag1 x apoe\textsuperscript{-/-} mice. The underestimation of the effect of sustained SAA expression in the rag1 x apoe\textsuperscript{-/-} mice is further exacerbated by the fact that this model was on study for only 12 weeks while the apoe deficient mice were on study for 16 weeks.

In both models presented here, the administration of ad-SAA had the immediate effect of increasing TGF-\(\beta\) levels, which then mirrored the human SAA levels throughout the studies. Inhibition of TGF-\(\beta\) in vascular smooth muscle cells stimulated with SAA attenuated the increase in biglycan synthesis (17). Here, we further demonstrate in apoe\textsuperscript{-/-} mice that concurrent injection of ad-SAA and 1D11 antibodies resulted in no induction of vascular biglycan 28 days after the injection. Thus we propose that SAA is acting through TGF-\(\beta\) to drive an increase in vascular biglycan content, predisposing the vessel wall to increased lipid retention. However, the role of
TGF-β in atherosclerosis is controversial. Mallat et al used a TGF-β inhibitory antibody and demonstrated that suppression of TGF-β led to increased inflammatory cell infiltrate into lesions and increased atherosclerotic lesion area (34). Conversely, Lutgens et al reported that TGF-β inhibition with a soluble receptor resulted in increased inflammatory cell infiltrate, but the atherosclerotic lesion area decreased (35). We have previously shown in an angiotensin (ang) II model of atherosclerosis that inhibition of TGF-β attenuated angII-induced atherosclerotic lesion development; however, there was no change in inflammatory cell content within those lesions (23). Thus, we propose that prevention or attenuation of elevations of TGF-β can limit induction of vascular biglycan, thus limiting atherosclerosis. Further study is necessary to determine if increased TGF-β is necessary and/or required for SAA induced atherosclerosis.

We propose that SAA increases susceptibility to early atherosclerosis by directing the pro-atherogenic remodeling of the vessel wall with increased biglycan content resulting in increased lipoprotein retention. In support of this model, the current studies provide evidence that biglycan co-localizes with apoB-containing lipoproteins within the lesion. However, SAA likely contributes to atherosclerosis through multiple mechanisms, including the induction of biglycan presented here. Though we have clearly defined a role for elevated SAA in both brief and sustained models of atherosclerosis, endogenous SAA is not absolutely required for atherosclerosis development. Recently we reported that apoe−/− mice deficient in both SAA1 and SAA2 have no difference in the degree of atherosclerotic lesion formation compared to apoe−/− controls (36). However, the studies investigating deletion of the endogenous acute SAA’s differ in several key aspects compared to the study presented here and the work of Dong et al. The investigation of atherosclerosis in apoe−/− mice lacking acute SAA was investigated after 50 weeks of chow diet or 12 weeks of western diet. We have demonstrated a remarkable effect of elevated SAA to accelerate early atherosclerosis development that may be less
relevant in later stages of the disease. Similarly, Skalen et al reported that mice transgenic for human apoB100 in which the proteoglycan binding domain was mutated had decreased early atherosclerosis (37) but not later atherosclerosis (38) suggesting other mechanisms were responsible for late atherosclerosis to develop. Taken together, the data suggests that atherosclerosis therapies targeted at SAA need only reduce its levels to those observed in a non-inflammatory state and not silence its expression completely.

Our findings have significant implications in westernized societies. It is currently estimated that 347 million people worldwide have diabetes (39) and almost half a billion people are obese (40), with the prevalence of both continuing to rise. These inflammatory diseases are characterized by chronically and significantly elevated plasma SAA among other clinical findings, and individuals with these diseases have an increased risk of developing atherosclerosis (30, 31). Furthermore, acute bouts of inflammation with increased SAA such as seen in patients hospitalized for diseases including sepsis, renal failure, or community acquired pneumonia also confer increased mortality once discharged, often from poor cardiovascular outcomes (18-20). Taken together with our current data on short term increases in SAA, acute but significant bouts of inflammation may need to be considered a risk factor for increased cardiovascular events.

Although elevations in SAA are well validated as a predictor of cardiovascular risk, we propose that beyond indicating risk, elevated plasma SAA plays a causal role in atherosclerosis development and prevention of increased SAA may be a therapeutic target. A number of currently available agents can lower SAA including statins. Individuals with a history of myocardial infarction with elevated SAA are at greater risk for a second cardiovascular event than similar patients with lower SAA; however, treatment with pravastatin reduces this increased risk, perhaps in part by lowering SAA (41). Given the pleiotropic effects of statins it is difficult to dissect the mechanisms by which they lower CVD risk. However it is possible that beyond
lowering LDL cholesterol, statins may have beneficial effects by lowering SAA. Beyond statins, other approaches have been found to lower SAA, such as weight loss (5) and aspirin based therapies (42). However, further clinical research is necessary to validate SAA as a therapeutic target in humans, and to determine if therapies targeted to elevated SAA in humans can be protective.
ACKNOWLEDGEMENTS

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DISCLOSURES

The authors have no conflicts to disclose.
References


Figure 1: SAA stimulated vascular smooth muscle cell secreted matrix proteoglycans from apo<sup>e</sup>-/- mice had increased LDL binding, which was dramatically reduced when TGF-β was inhibited.

VSMCs were treated with vehicle, SAA, or TGF-β for 24 hours, then washed and incubated with Alexa-594 labeled LDL for 2 hours. SAA and TGF-β treated cells had increased LDL binding compared to vehicle treatment. To determine the necessity of TGF-β in SAA mediated LDL retention, VSMCs were treated with SAA and either the TGF-β neutralizing antibody 1D11 or control antibody 13C4. 1D11 prevented the SAA increase in LDL binding; there was no effect of 13C4. To determine the role of proteoglycans in the SAA mediated LDL binding, cells were treated with sodium chlorate, which prevents sulfation of proteoglycan glycosaminoglycan side chains. Sodium chlorate prevented both the SAA and TGF-β dependent increase in LDL binding. LDL binding is expressed as Alexa-fluor 594 surface area normalized to DAPI surface area quantified by fluorescent microscopy using ImageJ software (NIH, USA). Data are presented as mean±SEM from 5-10 20x regions/condition. * p<0.001

Figure 2: Inhibition of TGF-β prevented ad-SAA increase in vascular biglycan content. apo<sup>e</sup>-/- mice were injected with ad-Null, ad-SAA or ad-SAA with the TGF-β neutralizing antibody 1D11 or control antibody 13C4. A. Human SAA and TGF-β were measured in plasma collected 24 hours after injections. 1D11 prevented the ad-SAA induction of TGF-β but had no effects on plasma SAA, N=4-5/group. B. Aortas were collected 28 days after injections and immunoblotted for biglycan (BGN) or actin. 1D11 prevented the ad-SAA induction of vascular biglycan but 13C4 had no effect. Each lane shows protein from an individual mouse representative of 4-5 mice per group.
**Figure 3: Increase in vascular biglycan content after a brief elevation of SAA persists at least 16 weeks.**

Mice were injected with ad-SAA (black circles), ad-Null (open squares) or saline (black triangles) and fed normal rodent chow for 16 weeks. A. Mice receiving a single injection of ad-SAA had a dramatic albeit transient increase in human SAA. B. All groups had a small, transient increase in murine SAA. C. TGF-β was transiently but dramatically increased in ad-SAA mice compared to ad-Null or saline mice. Shown are means ± SEM from n= 2-6 mice/group per timepoint. D. After 16 weeks, carotid arteries were collected and immunoblotted for biglycan (BGN) or actin then analyzed by densitometry using ImageJ software. Each lane shows protein from an individual mouse; densitometry shows means ± SEM for 5-7 mice per group.

**Figure 4: Atherosclerosis is increased in apoε<sup>−/−</sup> mice after only a brief increase in plasma SAA**

Mice were injected with ad-SAA, ad-Null or saline and fed normal rodent chow for 16 weeks. Atherosclerosis was measured at three sites: the aortic intimal surface (A), the brachiocephalic artery (B) and the aortic sinus (C). Atherosclerosis data presented as mean ± SEM, n=3-20/group analyzed by 1-way ANOVA. D. An atherosclerotic lesion in an aortic root from an ad-SAA injected apoε<sup>−/−</sup> mouse was double stained for apoB (green) and biglycan (red). Co-localization is indicated by yellow. Shown is a confocal image magnified 63X, representative of 4 mice. The asterisk indicates the lumen of the aortic sinus. Scale bar 10mm.

**Figure 5: Atherosclerosis was increased after sustained elevation of human SAA in rag1 x apoε<sup>−/−</sup> mice.**

Mice were injected with ad-SAA (black circles), ad-Null (open squares) or saline (black triangles) every 21 days and fed normal rodent chow for 12 weeks. A. Mice receiving ad-SAA
had a significant, persistent elevation of human SAA. B. Murine SAA did not increase nor did it differ between groups throughout the study. C. TGF-β was increased only in mice injected with ad-SAA and remained elevated throughout the study. Shown are means ± SEM from n= 2-6 mice/group per timepoint. D. Ad-SAA injected mice had increased atherosclerosis on the aortic intimal surface (D) and in the brachiocephalic artery (E). There was a trend towards increased atherosclerosis in the aortic root that did not reach significance (F). Data presented as mean±SEM , n of 4-16/group analyzed by 1-way ANOVA.
Table 1: Metabolic parameters known to confer cardiovascular risk did not differ between groups in either study.

*apoε⁻/⁻* mice were injected once, or *rag1 x apoε⁻/⁻* were injected every 21 days with ad-SAA, ad-Null or saline and maintained on normal rodent chow for 16 weeks for *apoε⁻/⁻* or 12 weeks for *rag1 x apoε⁻/⁻*. 

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Figure 1:

Alexa-fluor 549/DAPI area

- Vehicle
- Chlorate
- SAA
- SAA + chlorate
- SAA + 13C4
- SAA + 1D11
- TGF
- TGF + Chlorate

* * *
Figure 2:

A

BGN

Actin

Null SAA+ SAA SAA+ 1D11 13C4

BGN

Actin

Null SAA+ SAA SAA+ 1D11 13C4
Figure 4:

Atherosclerosis (μm²)

Aortic Lesion Area (%)

0 20,000 40,000 60,000 80,000

SAA Null Saline

p<0.05

0 2 4 6 8

SAA Null Saline

p<0.001

Atherosclerosis (μm²)

0 10,000 20,000 30,000 40,000 50,000

SAA Null Saline

p<0.05