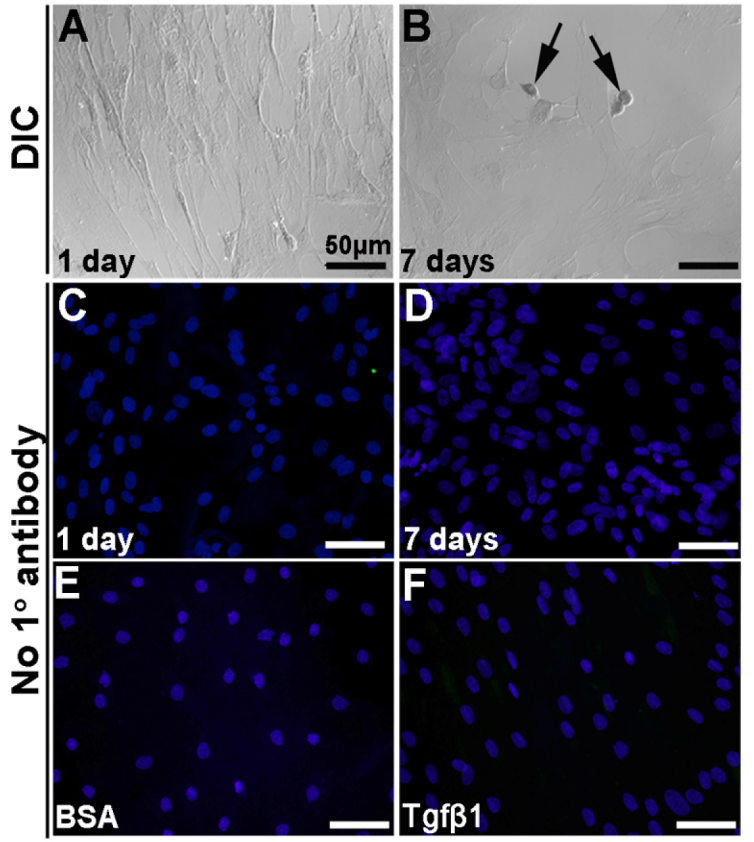
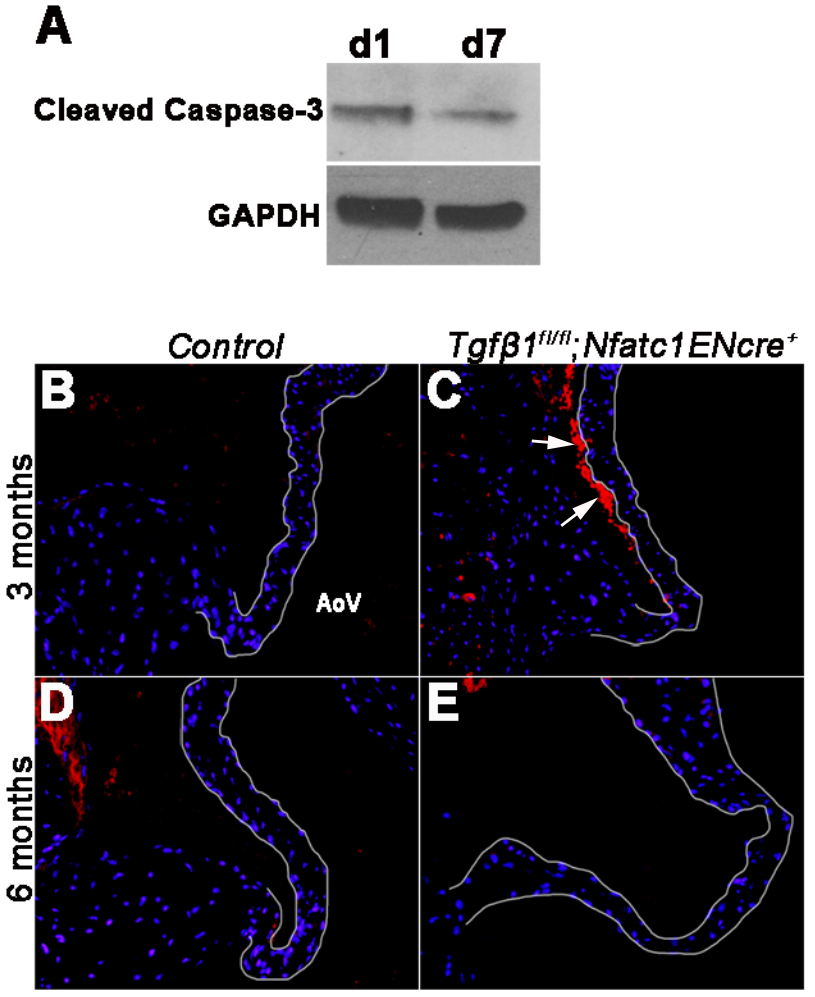
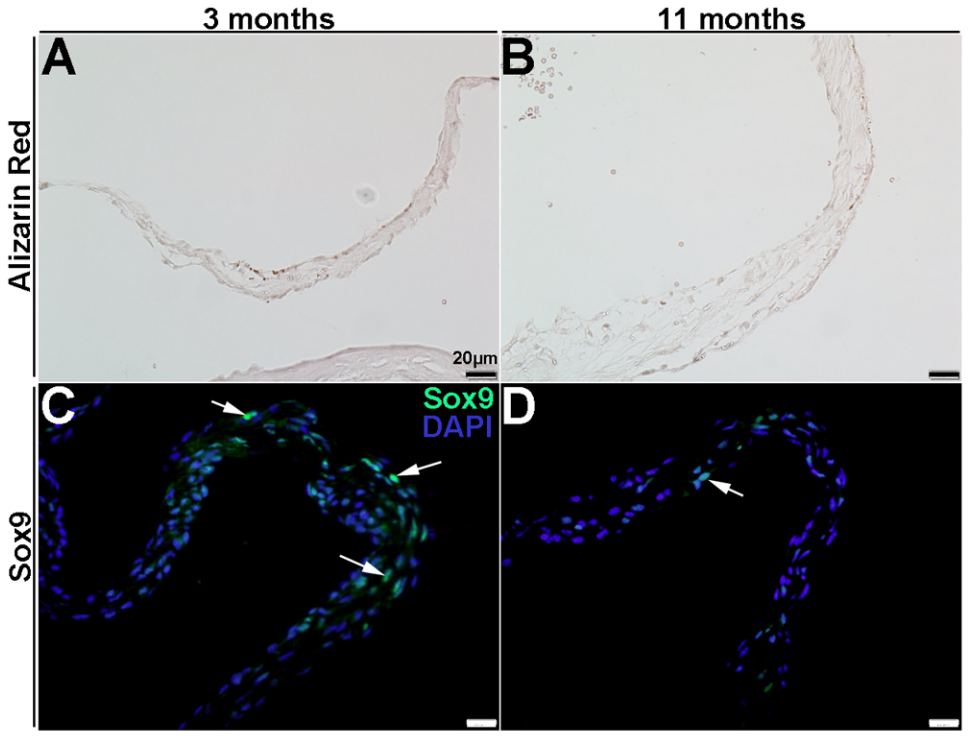


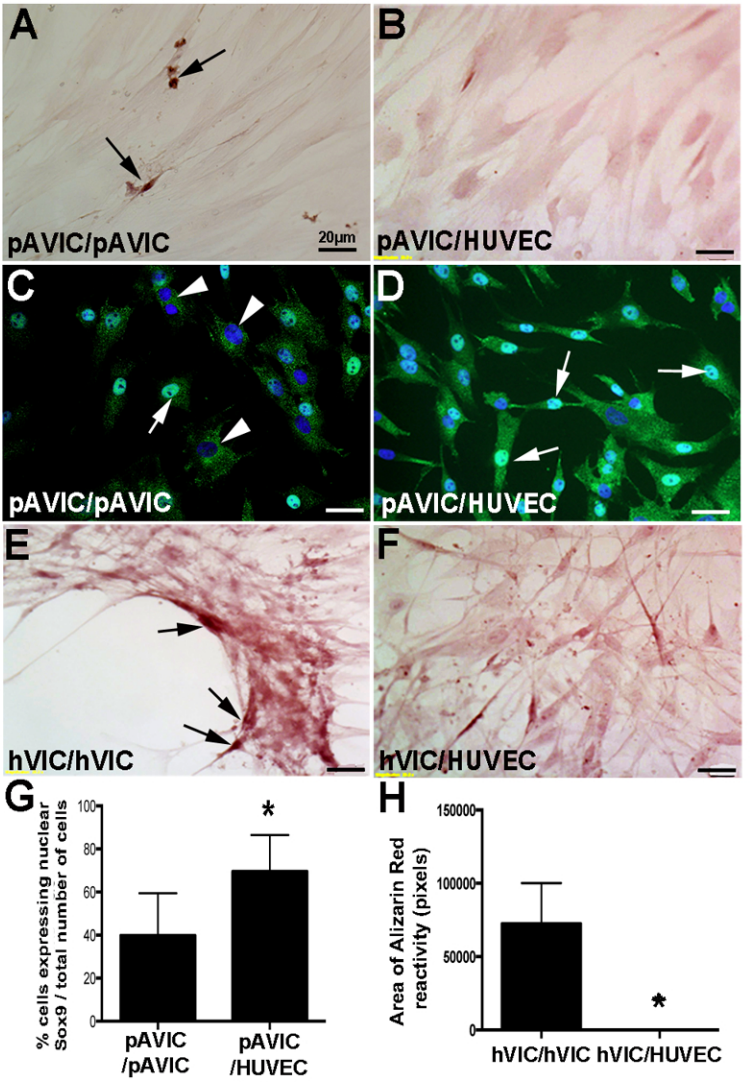

**Supplemental Figure I. Negative controls for pAVIC assays.** (A, B) DIC images of pAVICs following 1 (A) and 7 (B) days of culture of glass. Arrows denote calcific nodules. (C-F) No primary antibody reactions in pAVICs treated for 1 (C) and 7 (D) days, or treated with BSA (E) or TGFβ1 (F).



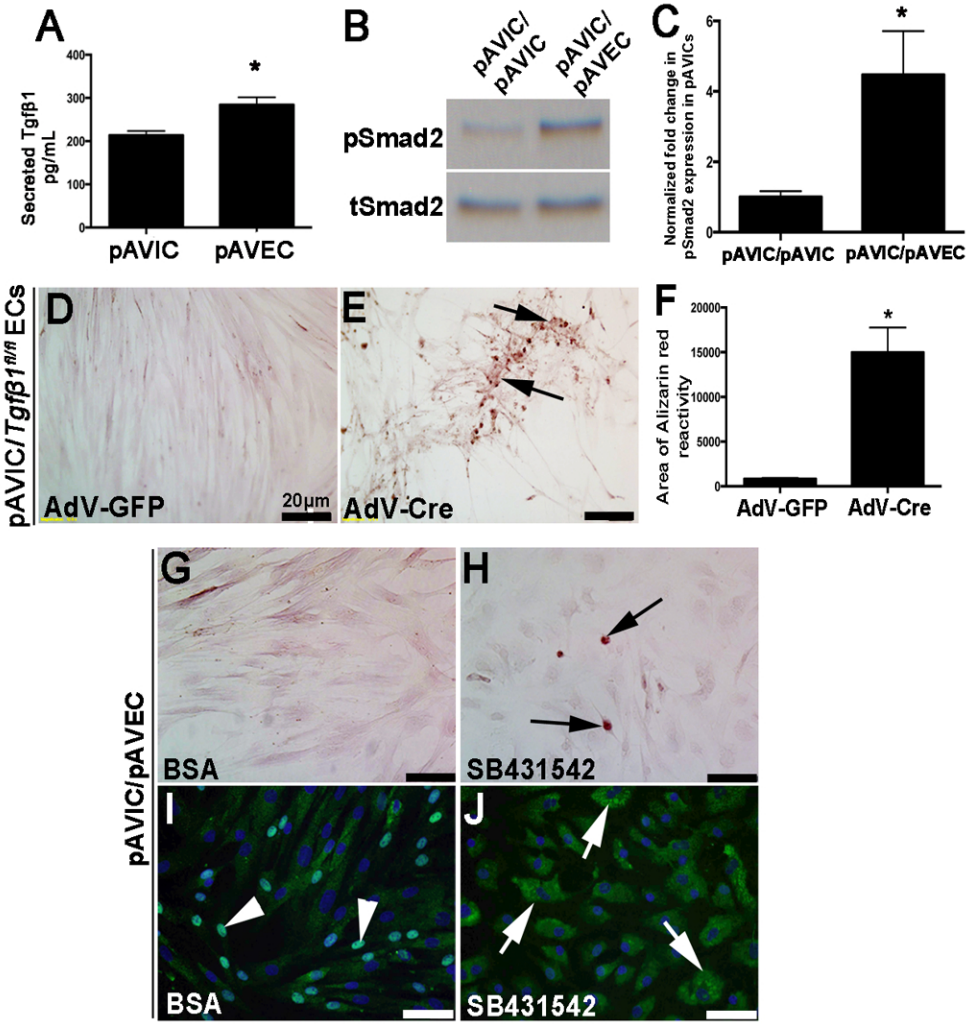

**Supplemental Figure II. Apoptosis is not significantly altered in heart valve calcification assays.** (A) Western blot analysis to show Cleaved Caspase-3 expression in pAVICS cultured for 1, or 7 days. Note that calcification occurs by day 7. (B-E) Immunohistochemistry to detect Cleaved Caspase-3 expression in aortic valve (AoV) leaflets from control (*Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{-}$*) (B, D) and *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* mice. Arrows indicate autofluorescent red blood cells.



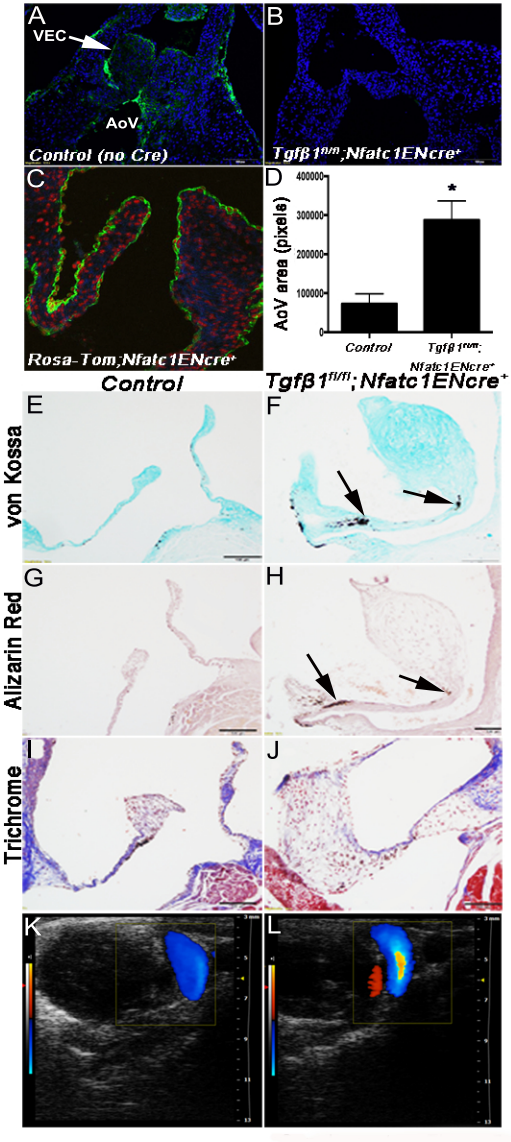

**Supplemental Figure III. Reduced Sox9 expression precedes calcification in hypercholesterolemic mice.** (A, B) Alizarin Red staining revealed undetectable calcification in 3 (A) and 11 (B) month old hypercholesterolemic *Reversa* mice. (C, D) Immunohistochemistry to indicate Sox9 expression in VICs of 3 (C) and 11 (D) month old hypercholesterolemic mice. Note reduced expression in the absence of calcification at 11 months. Representation images shown from n=3.



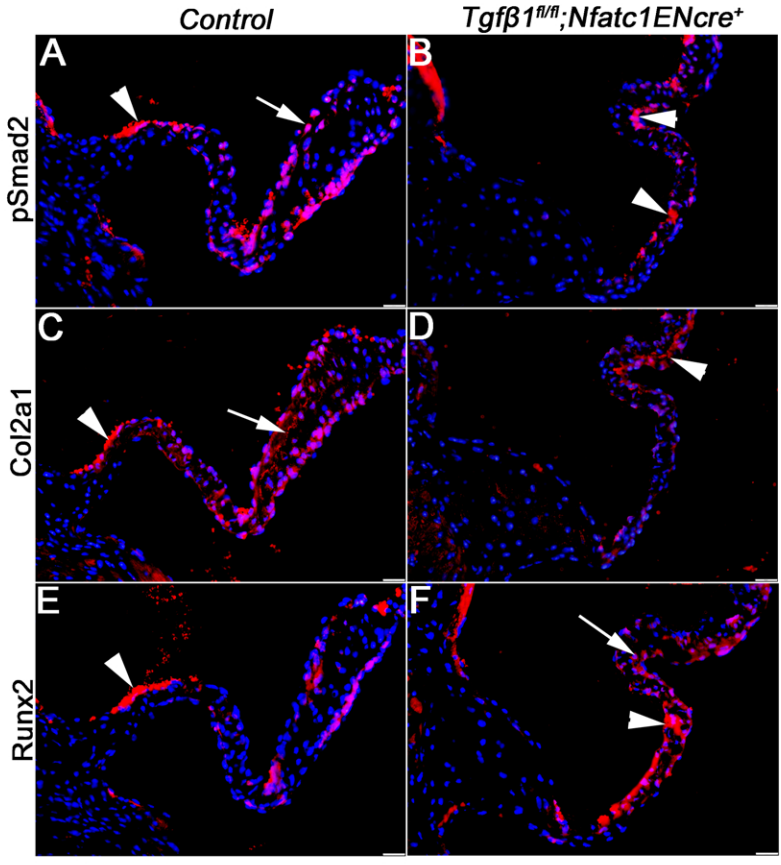

**Supplemental Figure IV. HUVECs attenuate human VIC-mediated calcification in vitro.** (A, B) Alizarin Red reactivity to detect calcific nodule formation and (C, D) Sox9 immunostaining to determine localization in pAVICs co-cultured with pAVICs (A, C) or HUVECs (B, D). (E, F) Alizarin Red staining in human VICs (hVICs) co-cultured with hVICs (E) or HUVECs (F). (G) Quantitation of Sox9 nuclear localization from C, D. (H) Quantitation of Alizarin Red reactivity from E, F. * $p \leq 0.05$ compared to VIC/VIC controls, based on n=4.



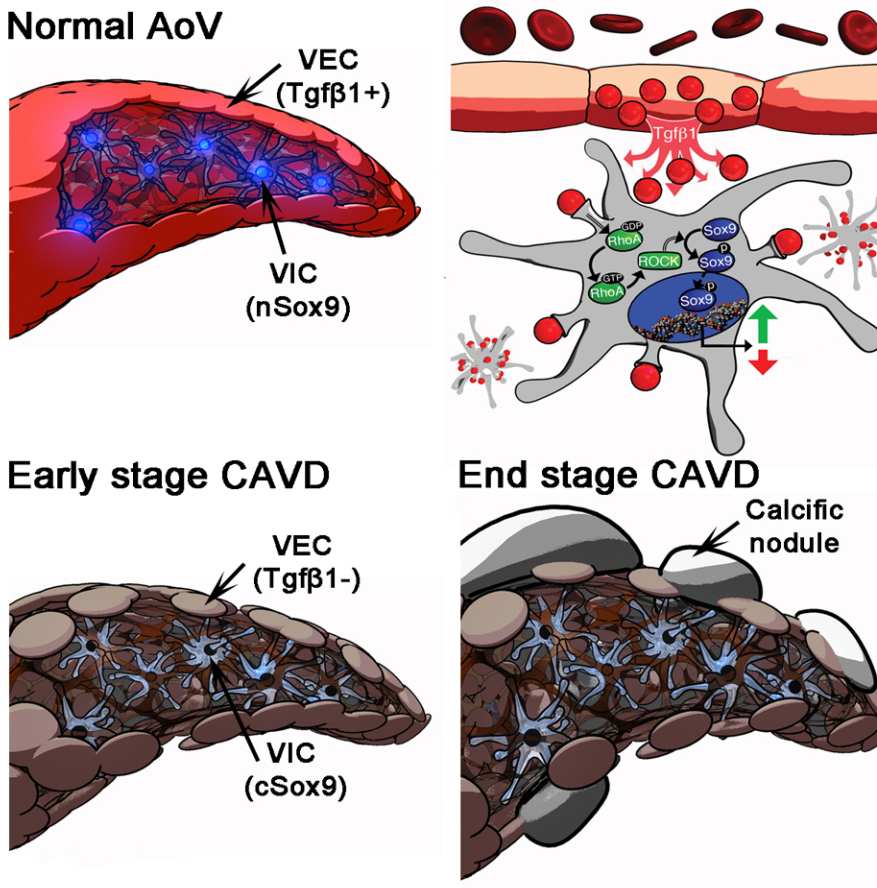

**Supplemental Figure V. Loss of Tgfβ1 in vitro leads to reduced Sox9 expression in VICs and calcific nodule formation.** (A) ELISA analysis of secreted Tgfβ1 in pAVICs and pAVECs, n=4. (B) Western blot to show pSmad2 and tSmad2 expression in pAVICs co-cultured alone, or with pAVECs, based on n=3. (C) Quantitation of B. (D, E) Alizarin Red staining (arrows) in pAVICs cultured in transwell assays with CD31+ murine cardiac endothelial cells (mCECs) isolated from *Tgfβ1*$^{fl/fl}$ mice and treated with AdV-GFP (D) or AdV-Cre (E). Alizarin Red reactivity is quantitated in (F), based on n=3. (G, H) Alizarin Red reactivity to detect calcification on pAVICs co-cultured with pAVECs and treated with BSA (G) or the Tgfβ inhibitor, SB31542 (H). (I, J) Sox9 immunoreactivity in pAVICs cultured as in G, H.




**Supplemental Figure VI. Histological and functional analysis of *Tgfβ1$^{fl/fl}$;Nfatc1ENCre* mice.** (A, B) Immunohistochemistry to show Tgfβ1 expression in VECs of control (*Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{-}$*) (A) and *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* (B) mice. (C) *Rosa-Tomato;Nfatc1ENCre$^{+}$* mice to show *Cre* recombination in aortic VECs. (D) Quantitation of AoV area (2D) in control and *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* mice at 6 months of age. von Kossa (E, F), Alizarin Red (G, H) and Trichrome (I, J) staining of AoVs from 6 month old *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* (F, H, J) and control (E, G, I) mice. Arrows indicate calcific nodule formation. (K, L) Color Doppler imaging to show AoV regurgitation (L, red) in 6 month old *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* mice relative to controls (n=6). AoV, Aortic valve. * $p \leq 0.05$ compared to controls. Representative images are shown from n=3.




**Supplemental Figure VII. Chondrogenic and osteogenic proteins are altered in *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* mice.** Immunohistochemistry to detect changes in pSmad (A, B), Col2a1 (C, D) and Runx2 (E, F) expression in control (*Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{-}$*) (A, C, E) and *Tgfβ1$^{fl/fl}$;Nfatc1ENCre$^{+}$* (B, D, E) mice at 3 months of age. Arrows indicate expression, arrowheads highlight auto fluorescent staining from red blood cells.




**Supplemental Figure VIII. Model representation of the progression of calcific aortic valve disease.** Based on findings from this study we hypothesize that in normal AoVs, Tgfβ1 (red) is expressed in the endothelium to promote nuclear localization of Aox9 expression (blue) in VICs to prevent calcification. This mechanism is potentially regulated by ROCK signaling to promote phosphorylation and nuclear localization of Sox9. As a result, nuclear Sox9 regulates transcription of chondrogenic-like (activate) and osteogenic-like (repress) gene programs to maintain healthy valves. In early stage CAVD prior to the onset of calcific nodule formation, Tgfβ1 expression is reduced in the damaged endothelium (brown), and therefore Sox9 nuclear localization is no longer maintained (diffuse blue). By end stage CAVD, these molecular changes promote VIC-mediated calcific nodule formation (surface of cusp) and stenosis.