of VIIa/TF in TF-overexpressing VIC were assessed by westernblotting (incubation with FVIIa at 50 ng/mL for 30 min) in presence or absence of anti-TF antibody. Results are expressed as median (interquartile range).
Results: VIC were defined as CD31(-) and CD68(-). TF and PAR-2 relative mRNA expressions were significantly higher in fibrocalcific vs normal valves (3.2 (1.5-.8) vs 0.18 (0.02-0.29), $p<0.05$; 1.15 (0.48-1.62) vs 0.16 (0.02-0.22), $p<0.05$; respectively). Normal VIC constitutively expressed TF (relative mRNA 4.1 (2.1-21.2); activity 36.2 (13.2-101.3) pg/mL) as well as PAR-2 (mRNA and protein by flow cytometry). TF expression was significantly increased in fibrocalcific VIC (relative mRNA 22.8 (9.8-33.8), p=0.05; activity 153 (71.5-247.0) pg/mL, $p<0.05$) when compared to normal VIC. Following IL-1beta stimulation of VIC, TF expression was significantly upregulated compared to unstimulated cells (relative mRNA 15.4 (12.9-28.2) fold increase, $p<0.05$; activity 2.7 (2.2-5.5) fold increase, $p<0.05$). ERK and Smad2 signalling pathways were upregulated in VIIa stimulated TF-overexpressing VIC (pERK/ERK 3.5 (2.9-4.8) fold increase; pSmad2/Smad2/3 1.8 (1.2-1.8) fold increase), an upregulation blunted by VIC preincubation with an anti-TF antibody.
Conclusions: Our results demonstrate the implication of TF/VIIa/PAR-2 axis in VIC commitment to fibrocalcific aortic valve disease. Modulation of this pathway may represent a new therapeutic target for early medical treatment of AVS.

P3908 | BENCH
Simulating early calcific aortic valve disease within novel in vitro 3D tissue platform

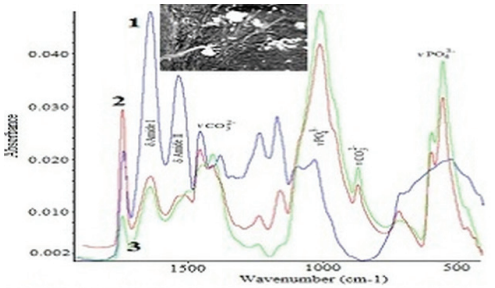
J. Hjortnaes[1], G. Gamci-Unal[1], C. Goettsch[1], K. Scherer[1], L. Lax[1], F.J. Schoen[1], J. Kluin[2], A. Khademhosseini[1], E. Aikawa[1]. *[1]Brigham and Women's Hospital, Boston, United States of America; [2]University Medical Center Utrecht, Utrecht, Netherlands*

Purpose: Calcific aortic valve disease (CAVD) is characterized by progressive nodular valve calcification, stiffening and eventually stenosis. However, the mechanisms underlying early disease progression remain unknown. Particularly the onset of osteoblastic valvular interstitial cell (VIC) differentiation is insufficiently understood. This lack of understanding can be attributed to the absence of physiologically accurate disease models. In this study we use a novel in vitro three-dimensional (3D) tissue platform based on valve extracellular matrix (ECM) components, to study the effect of pathophysiologically relevant conditions on the behavior of VICs in a 3D model of early CAVD.
Methods and results: Porcine aortic VICs were encapsulated in 3D hydrogels composed of hyaluronic acid and gelatin (a denatured form of collagen). When cultured in normal growth media (NM) for 21 days, VICs remained quiescent, characterized by minimal α-Smooth Muscle Actin (αSMA) expression. However, when cultured in osteogenic media (OM), VICs differentiated into activated myofibroblasts with abundant expression of αSMA, similar to diseased valves. Of note, when cultured in OM, initial αSMA expression gradually decreased while Runx2, a key transcription factor of osteoblast differentiation, increased. In addition, constructs exposed to OM formed calcific nodules similar to CAVD. This mineralization was characterized by alkaline phosphatase (ALP) activity peak at day 12 (1.96 ± 0.11 U, $p<0.05$)) and calcium deposition peak at day 16 (11.9 μg/mL, $p<0.05$). Elaborating on the potential of our tissue-like platform to be used as a disease model, we further stimulated the constructs with Tumor Necrosis Factor-α (TNFα). Stimulation of VIC-laden constructs grown in OM with TNFα showed an increase in the amount of calcified noduli compared to unstimulated constructs (69 ± 7 vs 47 ± 4, $p<0.05$). In addition, TNFα inhibited αSMA expression ($p<0.05$) while promoting Runx2 expression ($p<0.05$), suggesting that our tissue model can mimic inflammation-dependent calcification conditions.
Conclusion: This study demonstrated that VICs encapsulated in hyaluronic acid and gelatin hydrogels remain quiescent until osteogenic stimulation, which causes VICs to differentiate into myofibroblastic cells followed by osteoblastic differentiation, leading to calcium deposition. This process was catalyzed by stimulation with TNFα. Thus, we have shown the ability to simulate key events in vitro that might occur during early CAVD in vivo. This model could be used not only to examine mechanisms of CAVD but also as a screening system to study the effects of drugs.

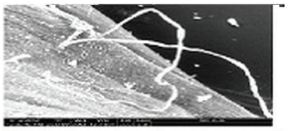
P3909 | BENCH
Pathogenesis of aortic valve stenosis: a spectroscopic study

I. Mamarelis[1], K. Pissaridi[2], E. Koutoulakis[1], V. Dritsa[2], C.H. Kotoulas[1], V. Mamareli[3], J. Anastassopoulou[2]. *[1]Army General Hospital of Athens, Athens, Greece; [2]National Technical university of Athens, Chemical Engineering Department, Radiation Chemistry and Bi, ATHENS, Greece; [3]University of Patras, Patras, Greece*

Purpose: Pathogenesis of aortic valve stenosis is suggesting as a multifunctional process and is associated with age and other risk factors, but the real mechanism of the valve calcification remains still unknown. Herewith, we investigate the changes in infrared spectra of aortic valve tissues in order to characterize the mineral deposition and understand the mechanism of aortic valve mineralization and stenosis.
Methods: 10 samples of calcified aortic valves of patients, who underwent surgical aortic valve replacement due to degenerative aortic valve stenosis. A Nicolet 6700 thermoscientific spectrometer was used to record the IR spectra. The aortic valve surfaces were studied with Scanning Electron Microscopy (SEM-EDAX), without any coating of the samples.
Results: The IR spectra show intensity changes and shifts concerning the peroxidation of tissues, as well as the mineralization. The mineral deposits are consistent of biological and Amorphous Hydroxyapatite (HA). The minerals are developed on cross-link bonds of collagen. Inhibition of stenosis development is suggested from the substitution of calcium with magnesium ions.



FT-IR specta of 1. organic phase, 2 interface 3 individual mineral deposit


SEM of aortic valve. Damaged proteins mineralized of a cross-link bond

Conclusions: The characteristic FT-IR absorption bands of calcified stenotic aortic valves show hyperoxidation of membranes (a pro-inflammation stage), while the mineral deposits are consistent of low crystallinity biological HA ($Ca10(PO4)6(OH)2$), $Ca2HPO4$ and calcium phosphate of phospholipoprotein frangments. SEM-EDAX data show substitution of calcium cations from magnesium cations leading to amorphous HA, preventing thus the aortic valve stenosis. Treatment of these patients with magnesium salts maybe could reduce the progress of aortic valve stenosis.

P3911 | BENCH
Neovascularization of stenotic aortic valve is associated with expression of nuclear factor-kappa B and hypoxia inducible factor-2 alpha

M. Sugahara[1], T. Tsujino[2], H. Akahori[1], Y. Naito[1], H. Sawada[1], M. Fukui[1], M. Ohyanagi[1], M. Mitsuno[1], Y. Miyamoto[1], T. Masuyama[1]. *[1]Hyogo College of Medicine, Nishinomiya, Japan; [2]Hyogo University of Health Sciences, Kobe, Japan*

Background: Valvular calcification of degenerative aortic valve stenosis (AS) shares several features with bone tissue. The process of endochondral ossification requires both the hypertrophic differentiation of chondrocytes (characterized by secretion of collagen X) and the conversion of avascular cartilage tissue into highly vascularized bone tissue (promoted by vascular endothelial growth facter: VEGF). Hypoxia inducible factor-2 (HIF-2) is activated by nuclear factor-Kappa B (NF-KB) and plays a critical role in the expression of collagen X and VEGF. Neoangiogenesis is a prominent feature of stenotic aortic valves, which are originally avascular as cartilage.
Purpose: Objective of this study is to examine whether the NF-KB-HIF-2 pathway is involved in the neoangiogenesis of calcified aortic valve disease.
Methods: We examined 50 specimens of aortic valve leaflets obtained from patients who had undergone aortic valve replacement for degenerative AS. Ten aortic valve leaflets obtained from annulo-aortic ectasia (AAE) patients served as controls. The stenotic valve leaflets were examined by immunohistochemistry to detect NF-KB, HIF-2 alpha, VEGF, vascular endothelial cells, and collagen X. The progression of AS was assessed from 2 serial echo studies performed before operation and separated by at least 180 days. We calculated the annualized changes in the aortic valve area (cm^2) by dividing the temporal changes in the parameters by the number of the days between the studies (ΔAVA, cm^2/year) which was evaluated by serial echocardiography before operation.
Results: NF-KB and HIF-2 alpha were detected in the leaflets from patients with AS but not with AAE. They were expressed in the area adjacent to massive calcified lesion, and HIF-2 alpha was colocalized with NF-KB. HIF-1 alpha was not detected in the valves from both patients with AS and AAE. VEGF, neoangiogenesis, and collagen X located in the area where HIF-2 alpha was expressed ($p<0.0001$, $p<0.0001$, $p<0.0001$). The progression of AS positively correlated with VEGF and neoangiogenesis (p=0.018 and 0.002, respectively).
Conclusions: NF-KB-HIF-2 pathway was expressed in calcified aortic valves and associated with increased neoangiogenesis and expression of VEGF and collagen X. This signaling pathway may play important roles in the pathophysiology of AS.