Genetic Associations of Bone Mineral Density with Aortic Stenosis: a Mendelian Randomization Study

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Abstract

Background: Aortic stenosis (AS), the most common form of heart valve disease in the developed world, is characterized by ectopic calcification of valve leaflets and therefore may share common genetic mechanisms with osteoporosis. Identifying common genetic pathways between osteoporosis and AS could point to novel therapies that impede ectopic calcification and slow the progression of AS. Accordingly, we sought to determine whether common genetic variants that predispose to osteoporosis may be associated with AS, individually or when combined in a genetic risk score (GRS), to provide evidence for a causal link between osteoporosis and AS using a Mendelian randomization (MR) approach.

Methods and Results: In a large case-control study of AS (n = 44703, 3469 AS cases, mean age (SD) = 69.7 (8.4), 50.8% women), self-reported presence of osteoporosis was associated with AS (OR = 1.25, 95% CI: 1.13-1.39, $p < 0.0001$). After adjustment for age, sex, BMI, hypertension, smoking, dyslipidemia, and diabetes, the association between osteoporosis and AS remained significant (OR = 1.17, 95% CI 1.04-1.32, $p = 0.012$). We identified 60 common independent single nucleotide polymorphisms known to associate with lower bone mineral density at $p < 5.0 \times 10^{-8}$. When combined into an osteoporosis GRS, the GRS was not associated with AS in age and sex-adjusted analyses (OR= 1.00, 95% CI: 0.99-1.01, $p = 0.78$).

Conclusion: In this large-scale case-control study, osteoporosis is strongly associated with aortic stenosis. However, our genetic analyses do not support a causal association and suggest that confounding and/or other biases likely explain these observational results.

Résumé

Contexte: La sténose aortique (SA), la forme la plus courante de valvulopathie dans les pays développés, est caractérisée par la calcification ectopique des feuillets de la valve et pourrait partager des mécanismes génétiques communs avec l'ostéoporose. L'identification de voies génétiques communes entre l'ostéoporose et la SA pourrait mener vers de nouvelles thérapies qui freineraient la calcification ectopique, ralentissant ainsi la progression de la SA. Par conséquent, nous avons cherché à déterminer si des variants génétiques qui prédisposent à l'ostéoporose pourraient également être associés avec la SA, individuellement ou lorsque combinés dans un score de risque génétique (*genetic risk score*, ou GRS), pour confirmer un lien causal entre l'ostéoporose et la SA, en utilisant une approche de randomisation mendélienne (RM).

Méthodes et Résultats: En utilisant une vaste étude cas-témoins de patients atteints de sténose aortiques (n = 44703, 3469 cas de SA, âge moyen (SD) = 69.7 (8.4), 50.8% de femmes), l'ostéoporose autodéclarée était associée avec la SA (OR = 1.25, 95% CI: 1.13-1.39, $p < 0.0001$). Après avoir ajusté pour l'âge, le sexe, l'indice de masse corporelle, l'hypertension et le tabagisme, l'association entre la SA et l'ostéoporose est restée significative (OR = 1.17, 95% CI 1.04-1.32, $p = 0.012$). Nous avons identifié 60 polymorphismes nucléotidiques (SNPs) indépendants et communs connus pour leur association avec une densité minérale osseuse plus faible à $p < 5.0 \times 10^{-8}$. Lorsque ces SNPs sont combinés dans un GRS pour l'ostéoporose, le GRS n'était pas associé avec la SA lorsque les analyses étaient ajustées pour l'âge et le sexe (OR= 1.00, 95% CI: 0.99-1.01, $p = 0.78$).

Conclusion: Dans cette vaste étude cas-témoins, l'ostéoporose était fortement associée à la sténose aortique. Cependant, nos analyses génétiques n'appuient pas un lien causal entre les deux et suggèrent qu'un facteur confusionnel et/ou d'autres biais expliquent ces résultats.

Acknowledgements

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Chapter I: Introduction to Aortic Stenosis

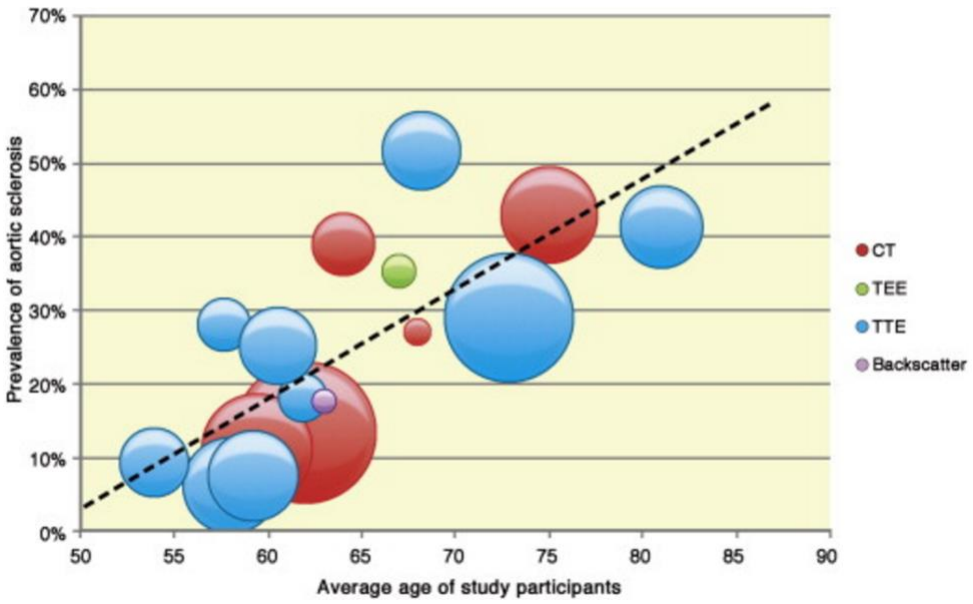
1.1 Aortic stenosis: Epidemiology

Aortic stenosis (AS) is the narrowing of the exit of the heart's left ventricle causing left ventricular outflow obstruction. The term AS is commonly attributed to aortic valve stenosis since subvalvular or supravalvular disease are less common. In AS, the normally thin and elastic leaflets of the aortic valve become thick, calcified, and rigid. Thus, causing obstruction of the blood flow through the valve. The early symptoms of AS are dyspnea and dizziness upon exercise; while the later clinical signs are heart failure, angina, and syncope[1]. The main causes of AS are congenital bicuspid or unicuspid aortic valves and subsequent calcification, rheumatic valve disease, and calcific aortic valve disease (CAVD).

In North America and Europe, CAVD is the most common cause of AS[2], affecting over 5 million people[3]. The term CAVD includes a broad range of clinical presentations from aortic sclerosis, asymptomatic thickening and calcification of aortic leaflet to severe AS. In a systematic review and meta-analysis of 22 reports, Coffey et al. found that aortic sclerosis prevalence increases with age from 9% in a study with a mean age of 54 years to 42% when mean age was 81 years (Figure 1). Every year about 1.9% of patients with aortic sclerosis progress to aortic stenosis[4]. The prevalence of AS increases with age from 1.3% at ages 60-69 to 9.8% at ages 80-89[5]. As a result, due to the aging population in the United States, the prevalence of AS is estimated to double by 2040[6]. While there have been profound advancements in understanding of the molecular processes underlying the disease, none have translated into clinical treatment for the prevention of AS. Surgical interventions such as valvuloplasty or more commonly aortic valve replacement

either surgical or transcatheter, although effective, are associated with high costs, procedural risks, substantial morbidity, and can only be performed in the later stages of disease. Thus, patients often suffer from a decreased qualify of life until symptoms become severe enough to require surgery[7]. A better understanding of the pathophysiology of AS could lead to new treatments, that would prevent the need for valve replacement.

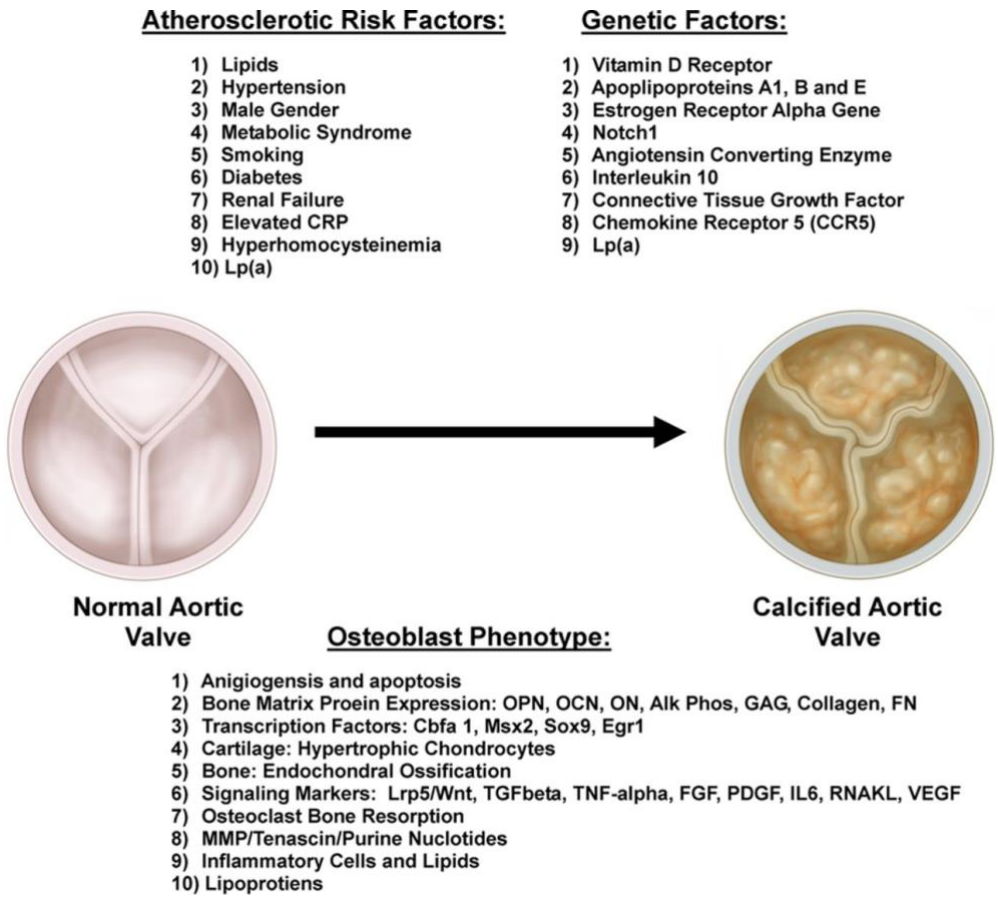
Figure 1. Prevalence of aortic sclerosis increases with age. There is a 1.5% (95% CI: [0.75%-2.25]) increase in prevalence of aortic sclerosis for each year increase in mean age ($p = 0.0007$, $r^2 = 0.549$). CT = computed tomography; TEE = transesophageal echocardiography; TTE = transthoracic echocardiography. Colors and size of circles reflect type of study and population size[4] (from Coffey et al. with permission from Elsevier.)



1.1.1 Risk factors for aortic stenosis:

Aortic stenosis shares several clinical factors with atherosclerosis including age, male gender, lipoprotein(a) [Lp(a)], low-density lipoprotein cholesterol (LDL-C), hypertension, and smoking. However, only about half of patients with severe aortic stenosis also have coronary artery disease[8]. Additionally, these common vascular risk factors are not good predictors of aortic stenosis[9]. Therefore, atherosclerosis and CAVD appear to be distinct processes, albeit with some overlap, and a host of additional factors contribute to the initiation and progression of CAVD.

Figure 2. Risk factors shared between CAVD and atherosclerosis, genetic factors involved in CAVD, pathways implicated in osteogenic transition and disease progression (with permission from Dr. Rajamannan)[10].



1.2 Aortic stenosis: Pathophysiology

In the cardiac cycle, throughout an average lifetime, the aortic valve opens and closes over three billion times[6]. Thus, CAVD was previously thought to be a simple degenerative consequence of "wear and tear" due to the stress and strain exerted on the aortic valve (Figure 3). The fact that CAVD associates with age had supported the belief that CAVD was a passive result of tissue damage due to aging. However, with the growing understanding of the underlying mechanisms of CAVD in past two decades the disease is now characterized by a combination of molecular processes leading to fibrosis and mineralization of the aortic valve[11]. Specifically, the pathology of CAVD has been divided into two phases beginning with an early initiation phase of lipid deposition, injury, and inflammation and continuing with a progression phase of osteogenic signaling[12] (figure 4.).

Figure 3. Stress due to hemodynamic flow across the aortic valve during the cardiac cycle[13] (with permission from Dr. Rajamannan).

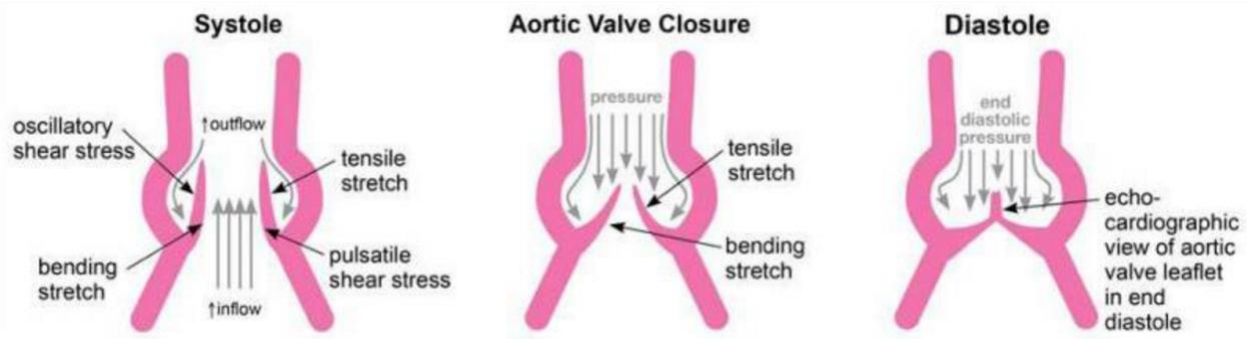
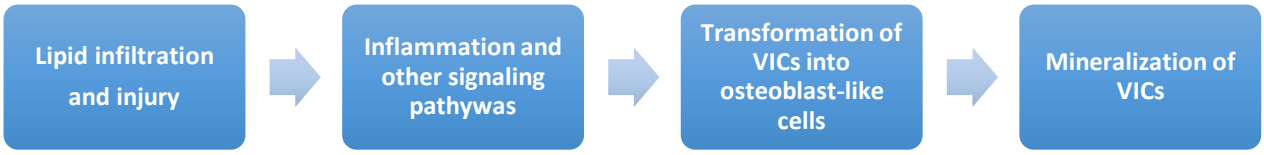


Figure 4. Main players in CAVD pathophysiology. Lipid infiltration and build up along with injury promotes inflammation. Inflammatory cell signaling along with other signaling pathways such as phosphate signaling, and release of pro-osteogenic factors derive the mineralization of valve interstitial cells (VICs).



1.2.1 Is there a role for hemodynamics in CAVD?

To answer this question, we can leverage the outcome of hemodynamic changes that happen in patients with bicuspid aortic valve (BAV). Normally, the aortic valve has three leaflets or cusps; however, in about 1-2% of the population, the aortic valve has only two leaflets at birth[14]. Patients with bicuspid aortic valves are known to develop CAVD earlier in life and with a much higher incidence[15]. Conti et al. used a mathematical modelling approach to show that bicuspid aortic valves undergo higher stress compared with normal tricuspid valves[16]. As a result, it has been hypothesized that the hemodynamic strain on normal valves is simply magnified in BAV and leads to more rapid CAVD progression. However, whether the higher mechanical stress on bicuspid valves is the only cause for the faster CAVD progression is not entirely known. It is possible that genetic variations that lead to BAV are also responsible for the rapid progression through cellular changes distinct from those in tricuspid valves[7]. Despite this, endothelial damage due to mechanical stress is believed to be at least in some cases, the initiating injury in CAVD[8].

1.2.2 The initiation phase: Lipids, inflammation, and oxidative stress

Lipids: Lp(a) and LDL-C

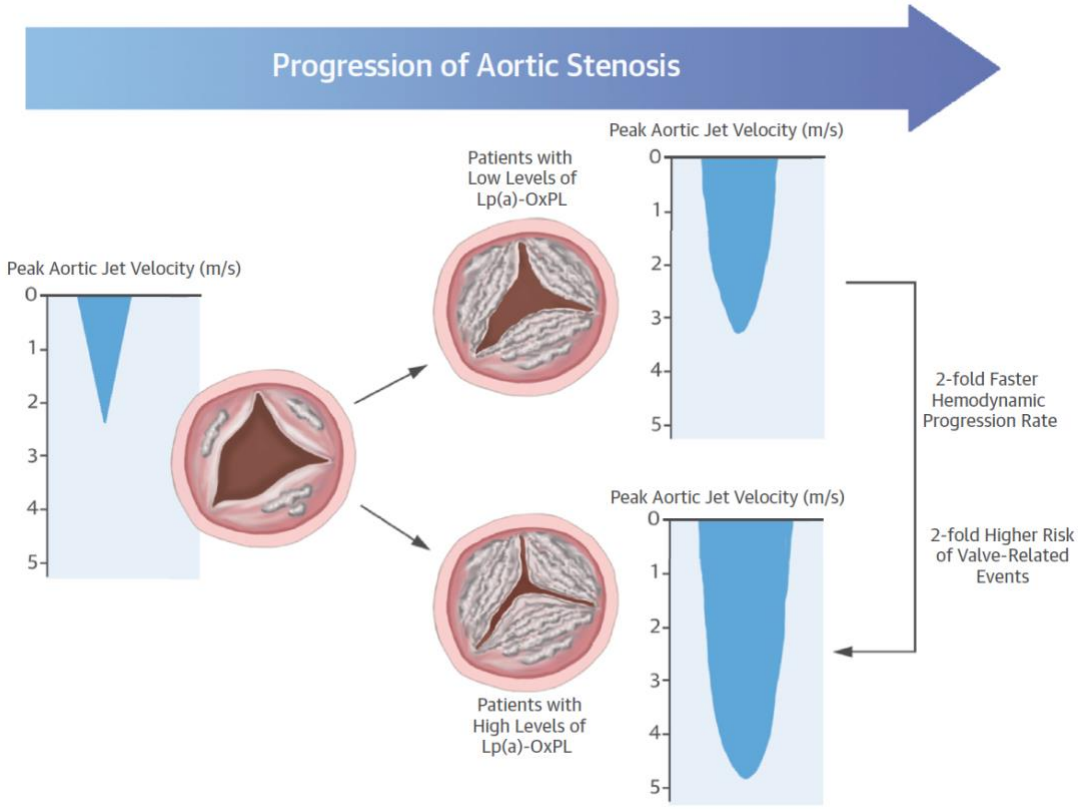
About 40% of patients with AS have elevated Lp(a) levels[17]. Lp(a) levels strongly correlate with CAVD prevalence[18], aortic valve calcification[19] and stenosis[20,21]. Mendelian randomization studies leveraging the polymorphisms in the *LPA* gene that increase serum Lp(a) levels revealed this cholesterol-rich lipoprotein to be likely causally associated with CAVD[22,23]. In a genome-wide association study, a common genetic variant in the LPA gene (rs10455872), which strongly associates with elevated plasma Lp(a) levels, was shown to double the odds of valve calcification at genome wide significance ($p=9 \times 10^{-10}$). Thanassoulis et al. have also demonstrated that lifelong elevations in Lp(a) increases the prevalence of aortic valve calcification in adulthood and causes the development of aortic stenosis[24].

Lp(a) has a diverse role in the pathophysiology of the initiation phase. Firstly, Lp(a) is a major carrier of oxidized phospholipids (oxPLs) in circulation; 85% of lipoprotein-bound oxPL is carried on Lp(a)[25]. OxPLs bind to pattern recognition receptors and trigger the innate immune system mediating a pro-inflammatory and pro-oxidative response in the surrounding tissue[26,27]. Secondly, Lp(a) consists of a molecule of apolipoprotein(a) bound to a molecule of apolipoproteinB with a disulfide bridge[28]. The apo(a) molecule of Lp(a) possesses lysine binding sites allowing Lp(a) to bind damaged endothelium[29]. Because of the stress exerted on the aortic valve throughout the cardiac cycle, the leaflets are more likely to be damaged, thus attracting Lp(a) carrying oxPLs, a process that is more pronounced in individuals with higher plasma Lp(a) levels[30]. In endoplasmic reticulum-stressed macrophages, Lp(a) and its associated oxPL and apo(a) were found to induce oxidative stress and apoptosis, a similar process to that is seen in the initiation

phase of CAVD[31]. Thirdly, Bouchareb et al. show that Lp(a) also transports Autotaxin (ATX or ectonucleotide phosphodiesterase 2) to the aortic valve. ATX is present at higher levels in stenotic valves and mediates remodeling and inflammation through the NF-κB/IL-6/BMP pathways[32]. Finally, in addition to the involvement of Lp(a) in CAVD as a carrier of oxPL and ATX, Lp(a) could be detrimental by delivering cholesterol to the aortic valve. Once deposited in the valve leaflets, cholesterol associates physically with hydroxyapatite crystals and promotes calcification[33].

The pivotal role of Lp(a) in CAVD is also illustrated by Capoulade et al. They measured both Lp(a) and OxPL-apoB levels in 220 patients with mild to moderate aortic stenosis and followed them up for an average of 3.5 ± 1.2 years. Their observational study showed that, the progression of AS is significantly faster in patients in the top tertiles of both Lp(a) levels ($p = 0.005$) and OxPL-apoB levels ($p = 0.01$). Additionally, patients in the top Lp(a) tertile had higher odds of rapid AS progression defined as yearly peak velocity increase greater than 0.20 m/s/year (OR: 2.1; 95% CI: (1.2,3.8); $p = 0.009$). Being in top tertiles of Lp(a) or OxPL-apoB levels remained an independent predictor for faster AS progression rate ($\beta = 0.21 \pm 0.04$, $p \leq 0.02$) after adjustments for covariates (age, sex, hypertension, smoking, metabolic syndrome, blood pressure, statin use, LDL-C, apoB, creatinine, bicuspid aortic valve status, aortic valve calcification score, baseline V_{peak}, and valvuloarterial impedance). Their survival analysis also revealed that, after adjustment for age, sex, and baseline AS severity, patients in the third Lp(a) tertile had double the risk of aortic valve replacement or cardiac death compared to those in the first and second Lp(a) tertiles (HR = 2.0, 95%CI:(1.1-3.7), $p = 0.02$)[17]. Based on these results, it is now increasingly believed that Lp(a) and OxPLs are crucial in AS progression.

Figure 5. Plasma Lp(a) and OxPL are important in CAVD. Patients with high Lp(a) and OxPL have two times faster CAVD progression rate (illustrated by higher peak aortic jet velocity) compared to those with lower Lp(a) and OxPL plasma levels (from Capoulade et al.[17] with permission from Elsevier).



No study to date had distinguished whether the association between high Lp(a) and CAVD is due to direct effects of Lp(a) or the higher serum OxPL levels. The recent *in vitro* work by Yu et al. illustrates, for the first time, a direct causal role for Lp(a) in human VIC calcification and elucidates its mechanism of action[34]. Using radio-labeled Lp(a), they showed that Lp(a) is rapidly taken up by human VICs. Additionally, RT-PCR for *LPA* mRNA in stenotic aortic valves and cultured VICs showed that *LPA* mRNA is also locally expressed in the valve. Immunofluorescence localized Lp(a) expression to intracellular vesicles and the endoplasmic reticulum. They

investigated the apoptosis, differentiation, and gene and protein expression profile of VICs from non-calcified human aortic valves upon short term (up to 48 hours) or prolonged (3 weeks) incubation with 50 mg/ml Lp(a). They used anti-OxPL E06 antibody (a murine monoclonal IgM against the phosphocholine of OxPLs) staining to demonstrate that the purified Lp(a) medium has no detectable levels of OxPLs. Short term incubation with purified Lp(a) increased cell proliferation ($p = 0.013$), intracellular ($p = 0.003$) and medium ($p = 0.0001$) ALP activity and phosphate levels ($p = 0.0001$). Prolonged incubation resulted in calcium nodule formation, increased calcium deposition ($p = 0.001$) and caspase-3/7 activity ($p = 0.0003$), and decreased cellular proliferation shown by MTT assay ($p<0.01$). Interestingly, when comparing to mineralization induced by LDL, Lp(a) incubation lead to significantly higher human VIC mineralization levels ($p = 0.006$). Short term incubation with Lp(a) increased phosphorylation of MAPK38, MAPK kinase 3/6, and GSK3a/b; while pharmacological inhibition of MAPK38 or GSK3a /b significantly reduced ALP activity and calcium deposition. Lp(a) treatment significantly increased mRNA expression of osteocalcin, osterix, RUNX2, MSX2, WNT-3a/5b, and BMP-2/4. Upon prolonged Lp(a) incubation, there was an increase in expression of ALPL, BMP-2, WNT11, oxidized LDL receptor 1 (LOX-1). Taken together, endogenously expressed and internalized Lp(a) activate kinases known to induce osteoblastic differentiation, cellular remodelling, and apoptosis. Given the direct causal role of Lp(a) in the pathophysiology of CAVD, investigating the efficacy of serum Lp(a) reducing drugs such as of PCSK9 inhibitors or niacin as treatment for AS is warranted. Furthermore, targeted-Lp(a) drugs or downstream kinase or Wnt inhibitors may be promising in preventing or retarding CAVD progression. Indeed, the EAVaLL trial (Early Aortic Valve Lipoprotein(a) Lowering) a pilot, randomized controlled trial of Lipoprotein(a) lowering using extended release Niacin as prevention for CAVD is currently underway[35].

LDL-C has also been shown to be causally associated with aortic valve disease. Smith et al. demonstrated that genetic predisposition to increased LDL-C is associated with presence of aortic valve calcium and incidence of aortic stenosis[36]. LDL-C can be oxidized and circulating ox-LDL levels are significantly correlated with plasma LDL-C ($r = 0.40$; $p<0.001$) suggesting that oxLDL-C may play a role. Indeed, ox-LDL is present in stenotic aortic valves and it co-localizes with inflammatory cells (such as macrophages and T-lymphocytes) and calcified areas[37]. Coté et al. show that higher levels of ox-LDL in valves are associated with greater densities of macrophages, white blood cells, and greater expression of TNF-α[38]. Furthermore, they report that plasma ox-LDL levels are independently (adjustments done for age, gender, hypertension and HDL-C) associated with the remodeling score of the aortic valve ($p<0.001$).

Inflammation: TNF-α and IL-6

In their histological and immunohistochemical study comparing normal valves to stenotic valves, Otto et al. found that along with lipid deposition, there was a higher presence of macrophages and T-cell infiltrates, as well as basement membrane disruption in early lesions of stenotic aortic valves (n= 27)[39]. Similarly, in a larger study (n= 285) of excised stenotic aortic valves from patients with CAVD undergoing aortic valve replacement, the histological work of Coté et al. demonstrated chronic inflammatory infiltrates in aortic valve tissues in 28.4% (n=81) of samples[40]. Indeed, a chronic inflammatory infiltrate in the valve was found to be independently associated with the remodeling score of CAVD ($p<0.0001$). Furthermore, in 57 CAVD patients with available clinical data, the density of leukocytes within the aortic valve correlated with the rate of AS progression ($r = 0.25$, $p = 0.05$) and with the levels of TNF-α mRNA ($r = 0.30$, $p =$

0.04). Additionally, the presence of inflammatory infiltrates i.e. leukocytes, macrophages, and T-cells was associated with osseous metaplasia ($p<0.0001$) and neovascularization ($p<0.0001$).

While the process through which inflammation provokes mineralization and remodelling is poorly understood, TNF-α seems to be play an important role. Kaden et al. reported an increase in multiple markers of the osteoblast phenotype upon stimulating cultured human aortic valve myofibroblast cells with TNF-α *in vitro*[41]. Currently it is thought that predominantly macrophages release TNF-α which in turn promotes mineralization by 1) TNF receptor-1 mediated apoptosis through activation of Fas-associated protein and capsase 8 and 3[42] 2) cAMP/PKA mediated pathway which promotes osteoblastic transition[43] and 3) increasing alkaline phosphatase (ALP) by a direct induction of msh homebox 2 (MSX2) expression through the NF-κB pathway[44].

Performing microarray and q-PCR experiments on aortic valve tissues, El Husseini et al. show that compared to normal valves, human stenotic valves have 3-fold more expression of IL-6 protein and 9-fold more expression of IL-6 mRNA ($p < 0.0001$)[45]. They also demonstrated that VICs express IL-6 and upregulate their expression upon exposure to mineralizing medium. Importantly for a transition into the osteogenic phase, they found that IL-6 treatment of VICs increased the expression of osteoblastic and fibrotic genes, such as *BMP2*, which is key in establishing the osteogenic fate of VICs[46] and is highly expressed in calcified aortic valves[47].

Oxidative stress:

Miller et al. have demonstrated a role for oxidative stress in CAVD. They have shown that reactive oxygen species (ROS) levels are increased in calcified regions of human aortic valves[48].

Wada et al. show that 4-HNE, a major product of lipid peroxidation, is expressed in the stenotic valves of patients with severe AS[49]. Expression levels of other oxidant products have also been found to be increased in calcified foci. In animal models, antioxidants such as lipoic acid, decrease progression of AV calcification further demonstrating that oxidative stress is likely important in calcification[50]. In vascular cells, inducing oxidative stress promotes osteoblastic differentiation by increasing alkaline phosphatase and mineralization[51]. Additionally, *in vitro*, oxPLs have been found to induce osteoblastic differentiation in a dose dependent manner in atherosclerotic lesions[52]. OxPLs are also present in valvular lesions and in circulation on apoB containing lipoproteins. Due to the similarity of oxPLs to some pathogenic peptides, they can strongly induce the innate immune system worsening the inflammation in the valve[53,54]. In endothelial cells, oxPL increase the expression of chemo-attractants and cell adhesion molecules and recruit inflammatory cells, playing a critical role in the initiation phase of CAVD[55]. Finally, Mathieu et al. hypothesize that high ROS levels in stenotic valves might promote oxidation of lipids; therefore, leading to further inflammation and calcification[56].

1.2.3 The progression phase: osteogenic signaling and differentiation

Notch and Wnt signaling:

Notch1

The role of Notch1 in aortic valve disease was first demonstrated by Garg et al. through an investigation of five generations of a family of European-decent with 11 cases of congenital heart disease[57]. Their genome-wide scan revealed that Notch1 transmembrane receptor mutations lead to bicuspid aortic valve disease (BAV), illustrating the importance of Notch1 in the development of the aortic valve. Indeed, through *in situ* hybridization, they show that Notch1 mRNA is highly expressed in developing aortic valves of mice. Interestingly, certain family members with Notch1 mutations had normal tricuspid aortic valves yet developed severe calcification suggesting a direct effect of calcification in addition to the defect in embryological development. Notch1 mutations have now been found in a French-Canadian population with tricuspid valves were accompanied with AS[58].

The severe calcification in anatomically normal, tricuspid, aortic valves suggests a direct effect of Notch1 on valve calcification. Mechanistically, Notch1 suppresses the activity of Runt-related transcription factor 2 (RUNX2) and bone morphogenetic protein 2 (BMP2) which are pivotal transcription factors in osteoblastic differentiation. Therefore, a loss of function mutation of Notch1 leads to over expression of RUNX2 and BMP2, both of which play a role in CAVD. The detailed mechanism is as follows: Notch1 signaling starts with the binding of jagged and delta like proteins to the Notch receptor thus activating γ-secretase which allows the release of notch intracellular domain (NICD) from the cell membrane. NICD translocates to the nucleus where it interacts with the suppressor of hairless family of proteins, leading to the activation of Hairy family

of repressors; Hairy repressors directly inhibit BMP2 and Runx2 activity. As such, wild type Notch1 signaling leads to inhibition of transcription factors involved in the osteoblastic fate. BMP2 is an important protein for the transition of VICs to the osteoblastic fate. Similarly, RUNX2 is important for the expression of osteoblastic genes such as osteopontin and osteocalcin[59]. BMP2 and RUNX2 are both upregulated in calcified aortic valves[60,61].

Nus et al. further demonstrate the importance of Notch1 and its downstream proteins. After pharmacological inhibition of Notch signaling, they note a reduction in Hey1 expression, and an upregulation in osteogenic markers BMP2, Runx2, alkaline phosphatase (ALP), and osteocalcin[62]. Similarly, recombining binding protein suppressor of hairless (RBPJ) heterozygous mutant mice (which lead to downstream Notch inactivation) develop CAVD[63]. Therefore, defects in Notch signaling plays an important role in aberrant osteogenic differentiation of VICs and subsequently calcification of the aortic valve in addition to its developmental role in BAV.

Figure 5. The normal role of Notch signaling in CAVD. The activation of Notch signaling by its ligands at the cell surface releases NICD. Upon releases, NICD moves to the nucleus and removes the suppression of Hairy repressors. This activation of Hairy repressor genes inhibits osteogenic transition.

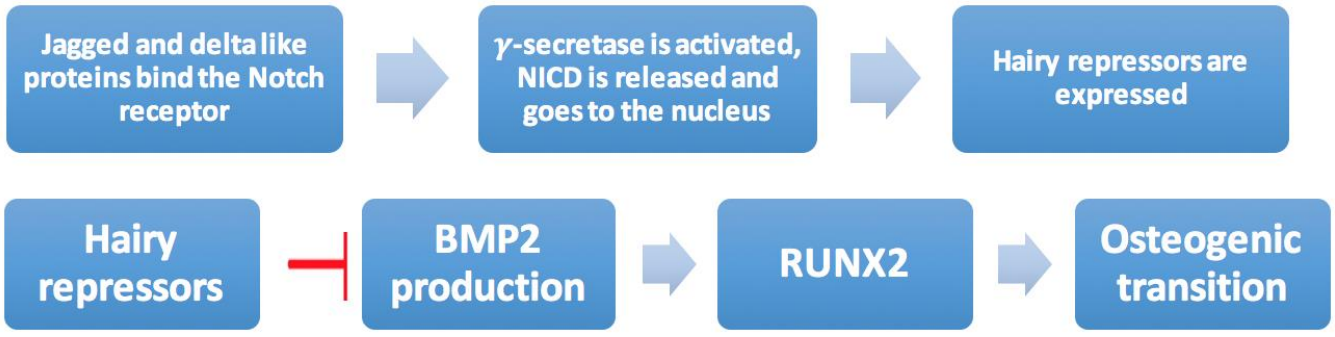
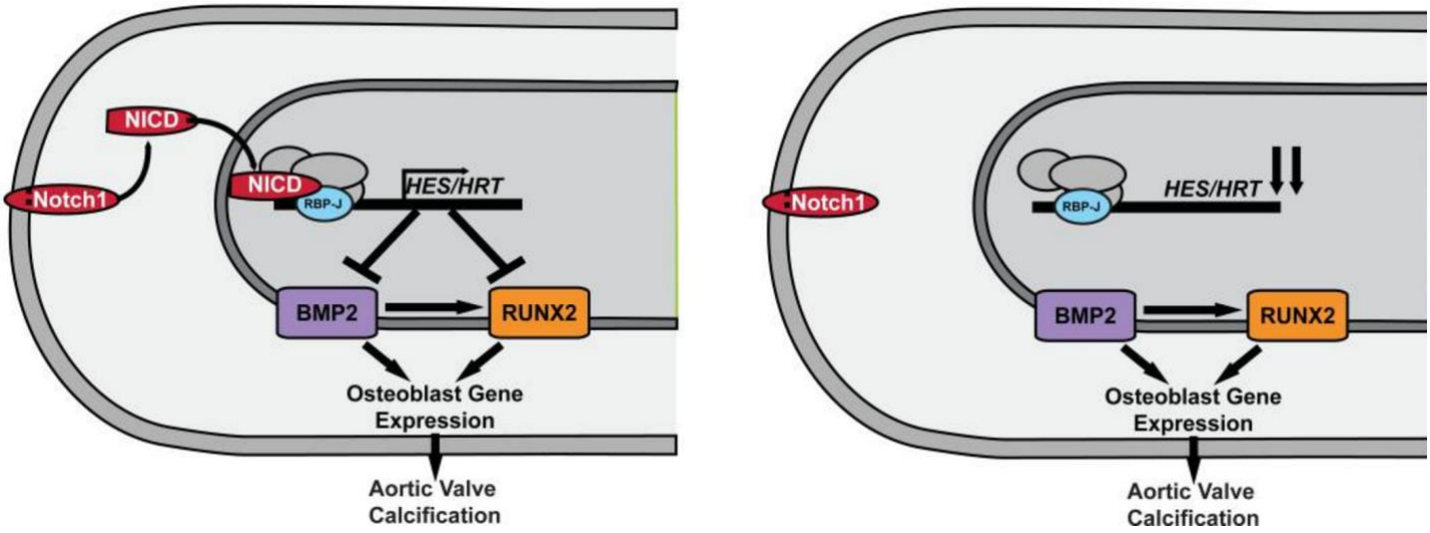


Figure 6. Disruption in Notch signaling is involved with differentiation of VICs to the osteogenic fate and CAVD progression. On the left, with active Notch signaling, NICD binds recombination signaling protein-J (RBP-J). RBP-J complex binds to the promoter of Hairy family of transcriptional repressors. Hairy repressors are expressed and inhibit osteoblastic proteins. On the right, without Notch signaling, the osteoblastic proteins BMP2 and RUNX2 are highly expressed leading to calcification[64] (from Nigam et al. with permission from Elsevier).



Wnt

Wnt3 is an osteoblast differentiation marker. Immunohistochemistry of human aortic valves shows that the expression of Wnt3 is upregulated in calcified aortic valves compared to normal valves[65]. In the canonical Wnt pathway, without Wnt ligand, β-catenin forms a complex with Axin, APC, GSK3 and CK1 rendering it cytoplasmic; β-catenin is then phosphorylated to be marked for proteosomal degradation. As a result, Wnt target genes are repressed by TCF-

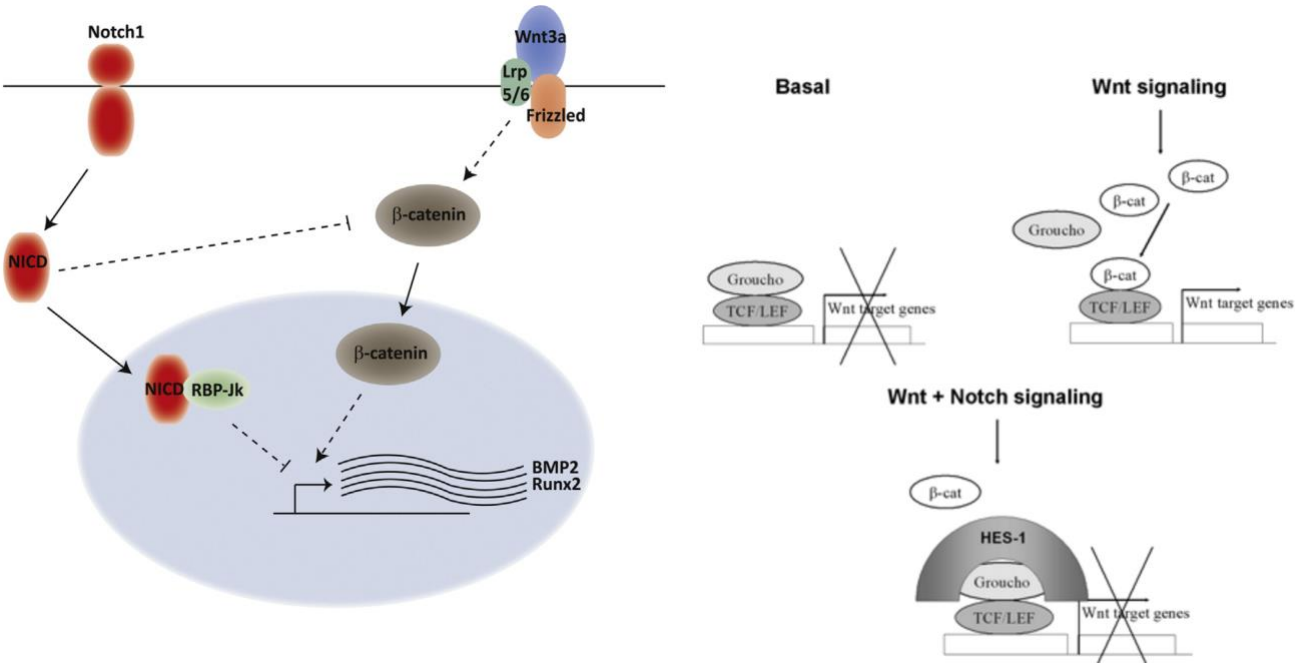
TLE/Groucho complex and HDACs. In the presence of Wnt, a receptor complex of Frizzled, Wnt, and LRP5/6 is formed at the cell membrane. Dishevelled is recruited, LRP5/6 is phosphorylated and Axin joins the complex at the cell membrane. Thus, phosphorylation and degradation of β-catenin is prevented, β-catenin builds up in the nucleus where it activates Wnt responsive genes by binding to the TCF co-activator[66]. In the nucleus, β-catenin augments the expression of osteoblastic genes. For example, Wnt signaling by Wnt3a or overexpression of β-catenin stimulates BMP2 transcription[67]. Importantly, Rajamannan show that aortic valve calcification induced by a high cholesterol diet in rabbits is accompanied with an increase in Lrp5/beta-catenin protein levels in the valve[68]. Concordantly, through *in vitro* models with pig valves, they show that treating isolated aortic valve myofibroblasts with LDL leads to an upregulation of Lrp5 receptor, β-catenin, and osteopontin. Osteopontin (bone sialoprotein I) is an extracellular matrix protein that constitutes the organic component of bone. Therefore, the upregulation of Wnt plays a role in calcification of aortic valve through upregulating Wnt response elements such as bone sialoprotein I osteopontin in myofibroblasts.

Notch and Wnt interplay

Notch signaling inhibits the Wnt pathway which would otherwise promote the expression of osteoblastic proteins. Overexpression of Notch has been shown to reduce the levels of cytoplasmic β-catenin and decrease downstream effects of Wnt 3a[69]. In addition, Notch signaling increases HES-1 expression, which interferes with Groucho/TLE transcription complex and prevents the displacement of Groucho/TLE by β-catenin. Notch signaling hinders osteogenic differentiation of VICs and fibrocalcific remodeling whereas Wnt signaling promotes it. Therefore,

a combination of decreased Notch signaling and increased Wnt signaling play a role in osteoblastic differentiation, calcific remodeling, and development of CAVD.

Figure 7. Notch and Wnt interplay in CAVD. Ntoch1 signaling decreases β-catenin levels and interferes with the transcriptional function of β-catenin[70] (with permission from Dr. Mathieu).



Phosphate signaling:

Serum phosphate

Patients with chronic kidney disease, in whom mineral metabolism is markedly disturbed, have a higher prevalence of CAVD compared to their age and gender-matched non-dialysis controls (n=92, 52% vs 4.3%, P = 0.01)[71]. Multiple studies demonstrate that an increase in serum phosphate or local phosphate production are important in mineralization of aortic valve[70]. In 1,938 individuals without clinical cardiovascular disease, higher serum phosphate levels were associated

with increased odds of valvular calcification; Every 0.5 mg/dl increase in serum phosphate associated with higher adjusted odds ratio of aortic valve sclerosis (OR=1.17, 95% CI= [1.04,1.31], p = 0.01), and aortic annular calcification (OR=1.12, 95% CI= [0.99,1.25], p = 0.05)[72]. This association was independent of other factors involved with calcium and phosphate metabolism such as PTH, calcium, and 25-(OH)D. In patients with moderate CKD with phosphate levels within the normal range, every 1 mg/dl increase in serum phosphate concentration was associated with 25% higher prevalence of aortic valve calcification (P = 0.16)[73]. In an *in vitro* study of isolated VICs from explanted valves (n=12), Mathieu et al. showed that only upon supplementation of the culture with beta-glycerophosphate, calcified nodules expressing osteonectin and ALP could be formed[74].

El Husseini et al. provide compelling results delineating the mechanistic role of phosphate in CAVD. They find that the phosphate transporter SLC20A1 (Pit1) is expressed at higher levels in CAVD tissues compared to normal valves; and that Pit1 levels are upregulated in VICs upon treatment with mineralizing medium (Pi 2 mM) ($p<0.0001$). Treatment with a Pi transporter inhibitor (PFA) inhibited the phosphate induced Pit1 upregulation and averted the phosphate induced calcification of VICs and expression of osseous proteins (osteopontin, osteonectin, osteocalcin, ALP, and Runx2)[75]. They propose a mechanism in which cellular entry of Pi reduces Akt-1, destroys mitochondrial membrane potential, releases cytochrome c in the cytosol, and results in apoptosis and mineralization of VICs.

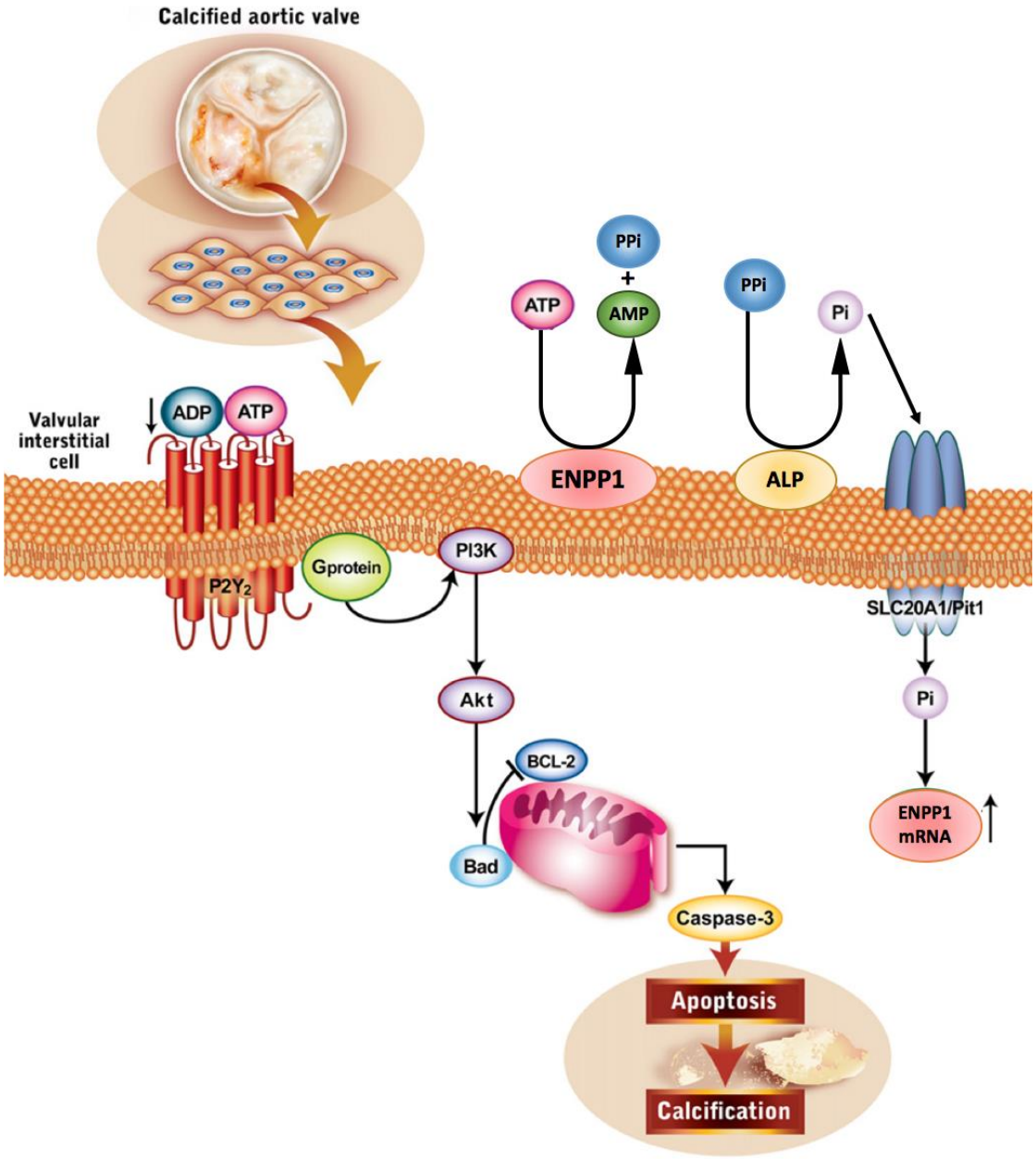
Ectonucleotidases

Ectonucleotidases are plasma membrane bound enzymes that break down secreted nucleotides to phosphate (Pi), pyrophosphate (PPi), and nucleosides[76]. Warfarin treated rats have mineral deposition in aortic valve and develop aortic stenosis; however, in warfarin treated rats, early pharmacological inhibition of ectonucleotidases (administration of ARL67156) prevents the development of aortic stenosis by preventing aortic cusp mineralization[77]. The amount of von Kossa stain (stain for mineral deposition) was significantly lower in warfarin and ARL67156 treated rats compared with warfarin treated rats highlighting the importance of Ectonucleotidases in aortic valve calcification. Furthermore, a specific ectonucleotidase, ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is expressed at high levels in mineralizing VIC cultures[78]. The ENPP1 mRNA is increased 3.9 times in stenotic valves compared to controls ($p = 0.007$). In addition, ENPP enzymatic activity in stenotic valves correlates with valvular calcium content ($r = 0.49$; $p=0.03$). Correspondingly, a single nucleotide polymorphism (rs9402349) in intron 9 of the ENPP1 gene which associates with higher ENPP1 mRNA ($p = 0.0012$) levels results in an increase in calcium concentration in stenotic valves ($p = 0.04$). Moreover, artificial ENPP1 overexpression *in vitro* leads to mineralization of VIC cultures[75].

Ectonucleotidases play a pivotal role in CAVD via a direct and an indirect mechanism (figure. 8). Directly, ENPP1 breaks down the ATP released by VICs into AMP and PPi; subsequently, ALP breaks down PPi into phosphate which has pro-mineralizing effects extracellularly. Indirectly, ENPP1 plays a role in increasing apoptosis. ENPP1 overexpression decreases extracellular ATP pool which normally functions as a survival signal through the P2Y2

receptor via the PI3K-Akt pathway. Therefore, ENPP1 over-activity reduces extracellular levels of ATP and lowers P2Y2 signaling which leads to lower PI3K-Akt signaling and increase in capsase-3. As a result, depletion of extracellular ATP by ENPP1 leads to apoptosis which is shown to plays an important role in calcification[79]. For example, in vascular smooth muscle cells, apoptosis precedes the onset of calcification[80] and *in vitro*, necrotic and apoptotic cells serve as nidus for calcification[81]. Concordantly, Coté et al. show that the number of apoptotic cells increases in aortic valves of warfarin treated rats; while treatment with ARL67156 abrogates this increase in apoptotic activity[77].

Figure 8 (next page). Ectonucleotidases and CAVD. ENPP1 breaks down ATP to AMP and pyrophosphate; ALP then breaks down pyrophosphate to phosphate (Pi). Pi enters VICs through Pit1 transporter and promotes ENPP1 expression. The increase in ENPP1 depletes extracellular ATP levels leading to a reduction P2Y2 signaling and an increase in apoptosis. Calcium and other cellular content released after cell death are pro-mineralizing and contribute to CAVD. (Modified from Mathieu et al)[76].


Calcified aortic valve
Valvular interstitial cell
ADP
ATP
P2Y2
Gprotein
PI3K
Akt
BCL-2
Bad
PPi
+
AMP
ATP
ENPP1
PPi
Pi
ALP
SLC20A1/Pit1
Pi
ENPP1 mRNA
Caspase-3
Apoptosis
Calcification

The renin angiotensin system (RAS)

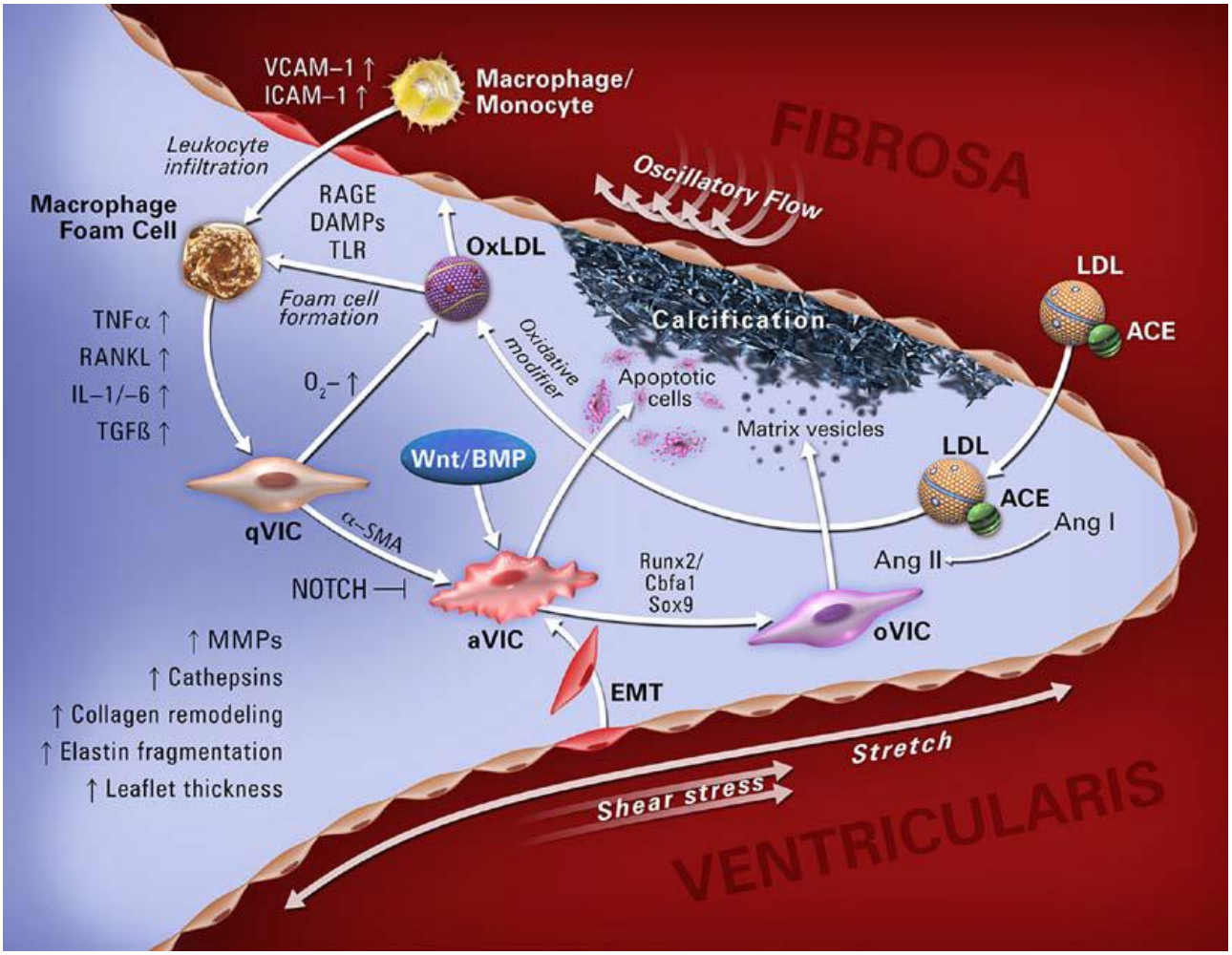
Coté et al. show that in patients with hypertension, plasma angiotensin II level is associated with IL-6 expression in stenotic valves ($r = 0.51$; $p = 0.03$)[82]. In addition, they report that plasma angiotensin II levels are also associated with inflammation and remodelling scores of aortic valves. Similarly, O'Brien et al. show that AVC progression was significantly lower for patients who were on angiotensin-converting-enzyme inhibitors (OR = 0.29, 95%CI: [0.11-0.75], $p = 0.01$)[83]. Angiotensin converting enzyme (ACE) is present and upregulated in calcific aortic valves. It is thought that ACE may be carried to the valve by LDL.

Fujisaka et al. further demonstrate the role of RAS in CAVD by administering different doses of angiotensin II to hyperlipidemic (ApoE knock down) mice for 4 weeks[84]. They found that high-dose Angiotensin II administration leads to endothelial injury and significant aortic valve thickening compared to controls (about 30 μm more increase in thickness, $p < 0.05$). Angiotensin II type 1 (AT_1) receptor blocker abrogated this effect, demonstrating that Angiotensin II mediates its pro-fibrotic effects through this receptor. As a result, it is thought that over-activation of RAS is implicated in CAVD; such that ACE carried to the valve by LDL, converts angiotensin I to angiotensin II which then mediates pro-fibrotic effects through the AT_1 receptor. These results suggest that RAS may have local, as opposed to systemic effects (e.g. hypertension) and point to ACEIs and ARBs as possible therapeutic agents for CAVD, however, these have not yet been investigated in clinical trials.

While it might be apparent that mechanisms in AS are unique and occur in isolation at the valve, efforts to prevent disease progression by modifying factors involved with AS

pathophysiology (for example lipids) have been unsuccessful. In the next chapter, we will discuss how CAVD could be possibly the result of a systemic imbalance of demineralization versus mineralization and demonstrate the striking parallels in mechanisms of skeletal bone turnover and calcific aortic valve disease.

Figure 9. Summary of mechanisms in CAVD[6]. (Reproduced from Yutzey et al. with permission from Wolters Kluwer Health, Inc).



Chapter II: The Boney Aortic Valve: Links between Osteoporosis and AS

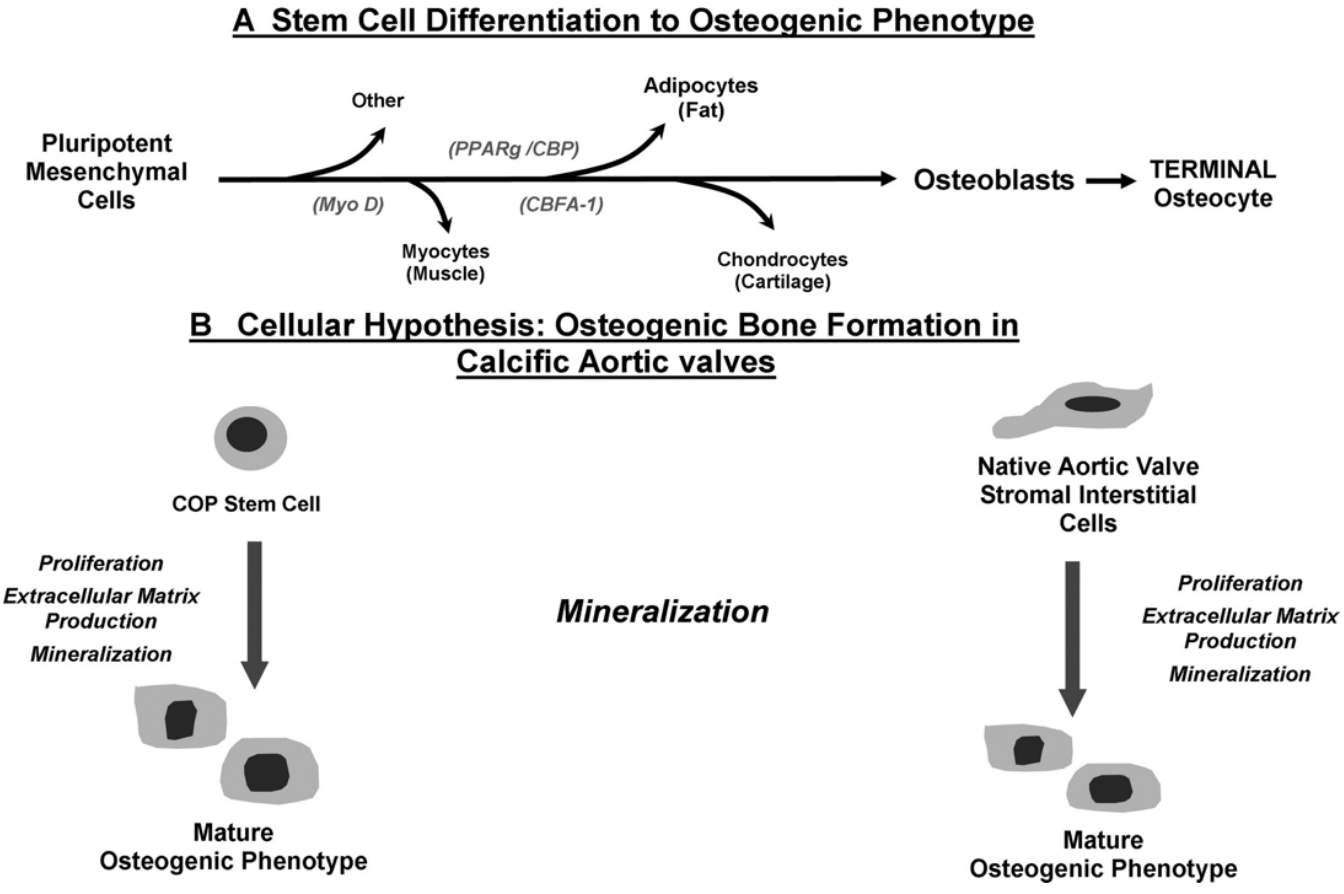
Mechanical stress and subsequent lipid infiltration are important for the initiation of CAVD. However, mounting evidence seems to suggest that once the disease is initiated, calcification is key for disease progression. Mechanisms reminiscent of skeletal bone formation drive the CAVD progression. At this late stage, lipid lowering appears to be an ineffective intervention. Four lipid-lowering randomized control trails (n = 2344) in patients with mild to moderate AS, with substantial calcification of the valve, have failed to show benefit from statin therapy in CAVD progression[85]. Indeed, when the disease transitions to the progression phase, it is believed that inflammation and lipid infiltration are no longer the main players[86].

In early CAVD stages, areas of lipids deposition co-localize with microcalcification areas[39]. It is thought that, these hydroxyapatite microcalcifications are formed by association with cholesterol crystals and/or by apoptotic bodies upon VIC apoptosis[33,12]. These apoptotic bodies are reminiscent of bone matrix vesicles carrying calcium and inorganic phosphate which are required for hydroxyapatite crystal formation and thus skeletal bone formation[87]. In skeletal bone, as hydroxyapatite crystals expand they break the vesicle entering the extracellular space[12]. A similar mechanism may be at work in the early stages of CAVD[88]. Mohler et al. examined the pathology of 256 excised human stenotic aortic valves[89]; they noted fully formed lamellar bone with hematopoietic tissue and active bone remodeling in more than 10% of the valves. They also noted the presence of cells and proteins normally involved is skeletal bone in valve tissue.

Rajamannan et al. compared the components of calcified human aortic valves (n=22) to normal human valves (n=20). Their RT-PCR results show that stenotic valves have increased transcripts of osteopontin, bone sialoprotein, osteocalcin, and Cbfa1 (Runx2), all of which are proteins specific to the osteoblastic fate and involved with skeletal bone formation[90]. Cbfa1 (Runx2), for example, is the key regulator of all osteoblastic genes without which osteoblast differentiation is halted[91]. Similarly, Pohjolainen et al. compare expression levels in 18 human stenotic aortic valves to 5 normal valves. They note an increase in transcripts levels of osteopontin (7.4 times increase, $P < 0.001$) and bone sialoprotein II (5.8 times increase, $P < 0.05$), and osteoprotegerin (1.7 times increase $P < 0.05$) compared to normal valves[92]. Bone sialoprotein[93] and osteopontin[94] both act as a scaffold for the binding of hydroxyapatite and are important for bone formation[95]. While the origins of these phenotypically osteoblastic cells are not clearly known, two hypotheses exist. Osteoblast-like cells in CAVD originate from 1) hematopoietic-derived osteogenic precursor (COP) cell population that differentiate to bone forming osteogenic cells in the valve, and/or 2) the VICs (or myofibroblasts) present in the valve that transdifferentiate into an osteogenic fate[96]. The latter is more commonly accepted. Indeed, many factors are involved in promoting VICs to differentiate to osteoblast-like cells. Pawade et al. note that this transdifferentiation is initially governed by cytokines released from macrophages[97]. It is subsequently governed by Notch and Wnt pathways (see section 1.2.3 notch and Wnt signaling) and receptor activator of nuclear factor kappa B (RANK)/ receptor activator of nuclear factor kappa B ligand (RANKL)/osteoprotegerin (OPG) pathways (see section 1.3.1 RANK/ RANKL/ OPG). Despite the importance of transdifferentiation of VICs as a source for osteogenic cells in CAVD, Egan et al. have shown that osteogenic precursor cells (COPs), a population of a bone marrow-derived type-I-collagen+/CD45+ cells, are present in human stenotic valves[98]. They show

that COPs are present in areas of endochondral ossification and lamellar bone and not in other areas of the valve. Indeed, COP cells have been shown to be capable of expressing Bone morphogenetic protein (BMP) and forming bone *in vivo* suggesting that both mechanisms are likely operational[99].

Figure 10. Cellular origins of osteoblast-like cells within the aortic valve. A) normal differentiation of osteoblasts from mesenchymal cells. B) Two sources of osteoblast-like cells present in stenotic aortic valves (reproduced with permission from Dr. Rajamannan)[100].



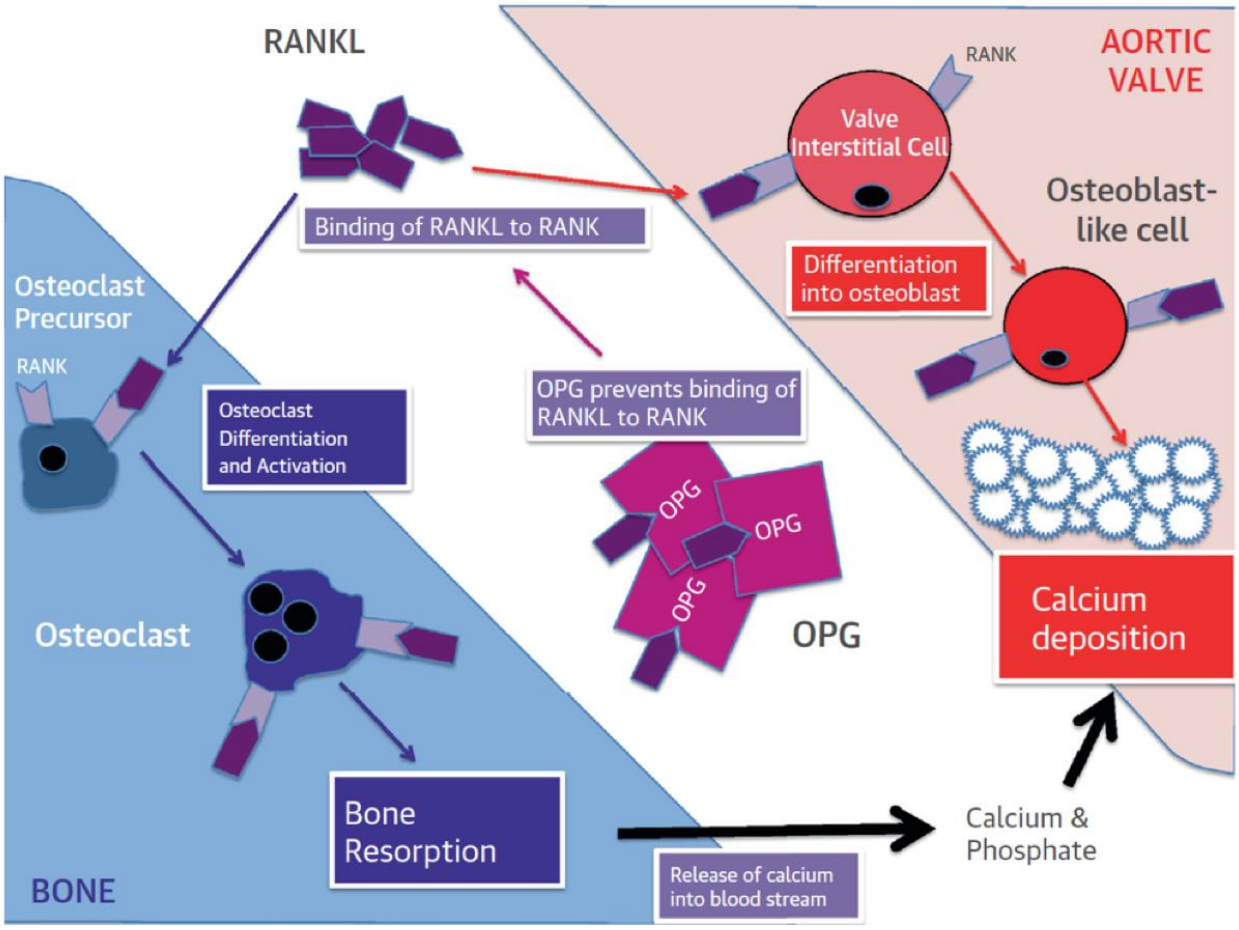
2.1 RANK / RANKL / OPG

In bone physiology, receptor activator of nuclear factor kappa B ligand (RANKL) is a protein released by osteoblasts and regulates osteoclast formation, activity, and survival by binding to receptor activator of nuclear factor kappa B (RANK) expressed on osteoclast cell membranes. Binding of RANKL to RANK on osteoclast precursors leads to cell fusion and formation of multinucleated osteoclasts which then resorb bone. Osteoprotegerin (OPG) also released by osteoblasts binds to RANKL and blocks its ability to bind RANK, lowering bone resorption. Thus, RANK/RANKL/OPG system regulates bone resorption and remodeling in bone[101]. Interestingly, RANKL has the opposite effect on VICs. RANKL induces VICs to differentiate to an osteoblastic phenotype. It results in calcification, and increased expression of alkaline phosphatase, and osteocalcin[102]. Kaden et al. show that RANKL expression is increased in stenotic aortic valves compared to controls. They also show that while OPG is not detectable in stenotic valves, it is expressed in control valves.

Osteoprotegerin-deficient mice provide further evidence for the opposite effect of RANKL in vascular versus bone tissue. OPG knockout mice develop an osteoporotic phenotype and have a higher incidence of fractures; at the same time these mice also develop calcification in the aorta and renal arteries[103]. The reason for this seemingly paradoxical effect of RANKL could perhaps be explained by the cell population present in the tissues. Osteoclast precursor cells predominate in bone; thus, in bone, RANKL mainly induces osteoclast activity and bone resorption. In contrast, due to the lack of osteoclasts in vascular tissue, RANKL mediates its osteoblastic effects on myofibroblast and smooth muscle cells[104]. Therefore, RANK/RANKL/OPG is important in CAVD pathophysiology and may, at least partially, explain the link between CAVD and demineralizing

bone disease. Whether in addition to the differential local molecular mechanisms, increase in bone osteoclastic function leads to bone resorption and increases the systemic availability of calcium and phosphate to promote ectopic mineralization and calcification remains unknown and is an important hypothesis for further investigation.

Figure 11. Differential effect of RANKL-RANK interaction in bone and aortic valve. In bone, it induces osteoclast and leads to bone resorption. In aortic valve, it induces to osteogenic differentiation and calcification. Osteoprotegerin sequesters RANKL and decreases both bone resorption and valve calcification (reproduced from Pawade et al. with permission from Elsevier).



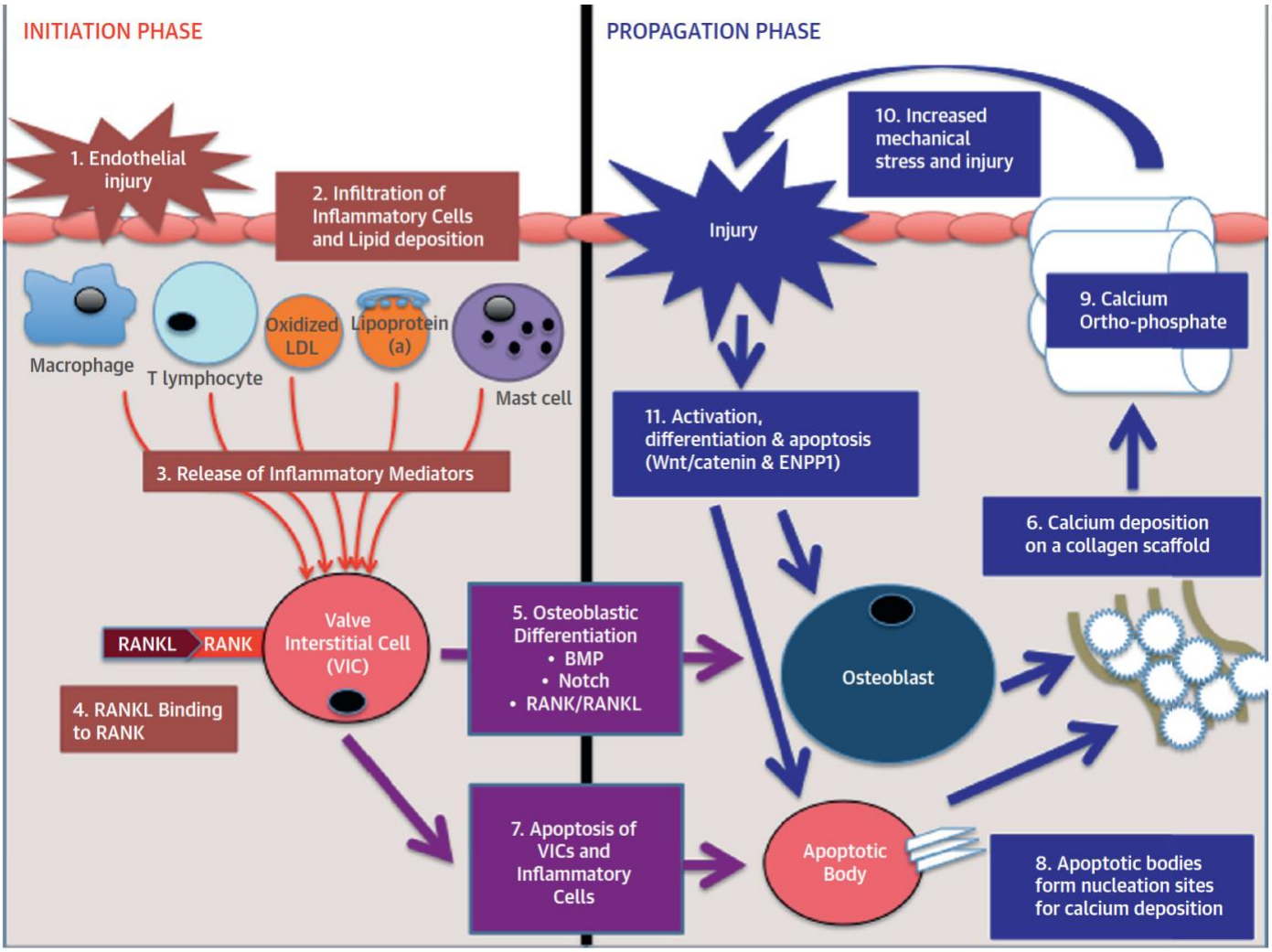
2.2 Inverse association between valvular calcification and osteoporosis—a paradox?

In vivo work of Hjortnaes et al. in mice shows that there is a significant correlation between arterial and valvular calcification and a decrease in bone mineral density (BMD) (r^2 = 0.67 and 0.71)[105]. Similarly, in humans, lower BMD is associated with increased vascular and valvular calcification[106]. Indeed, human diseases of increased bone turnover, such as Paget's disease, which could be a result of RANK mutations, are associated with a higher incidence of aortic stenosis[107]. In the EPIC–Norfolk prospective study of 25,639 men and women, Pfister et al. note an inverse association between bone mineral density and incident aortic stenosis[108]. When adjusted for age, sex, and BMI, each standard deviation increase in broadband ultrasound attenuation of the calcaneus (a quantitative index of increased BMD) decreased the incidence of hospitalization for AS by 20% (p = 0.04). This imbalance of calcium, that is the co-occurrence of low calcium in bone with high calcium deposits in vasculature and the aortic valve has been termed "the calcification paradox." Systemic release of calcium and phosphate upon bone remodeling, and the differential activity of RANK/RANKL in the bone versus the aortic valve provide possible mechanisms that may explain the observed association.

In contrast, Dweck et al. used positron emission and computed tomography techniques in 101 patients with CAVD to measure 18F-sodium fluoride activity (a marker of active tissue calcification) in the aortic valve and thoracic bone. They report no association between the calcification activity at the valve and in the nearby skeletal bone (r^2 = 0.001, p = 0.782). They also report no association between aortic valve calcium scores and BMD (r^2 = 0.000, P = 0.766)[109]. As

a result, they provide evidence against a concurrent relationship between bone demineralization and valve calcification, concluding that calcific mechanisms in CAVD may, in fact, be distinct from the mechanisms in skeletal bone. It is possible that the lack of a concurrent association between BMD and valve calcification in this study stems from the stage of CAVD. It is thought that once CAVD moves onto the progression stage, calcification dominates the pathophysiology. Indeed, Pawade et al. despite describing RANKL-RANK action as an important mechanism in CAVD, explain the progression phase as a "self-perpetuating cycle of calcification and valve injury" and hypothesize that local calcification drives the progression phase (Figure 12). Therefore, is it possible that Dweck et al. measured calcification activity in the aortic valve at a late stage were that it no longer associates with BMD or bone demineralization. Indeed, in their population of 101 CAVD patients the majority, 81 patients, had aortic stenosis characterized by more advanced calcification and only 20 patients had aortic sclerosis, an earlier less severe phenotype. Nevertheless, even if lower BMD truly does associate with CAVD, as most of the literature suggests, no conclusions can be drawn about the direction of the correlation or the causality of the association.

Figure 12. Summary of CAVD Pathology. Once CAVD moves to the progression phase, calcification is key. It promotes more mechanical stress and injury leading to more apoptosis and thus more calcification. As a result, a "self-perpetuating cycle" of calcification, injury, apoptosis, and osteogenic differentiation takes place presumably without the need for lipids or inflammation (reproduced from Pawade et al. with permission from Elsevier)[12].



2.3 Do osteoporosis medications slow AS progression?

Due to the similarities between the pathophysiologic mechanisms in osteoporosis and aortic stenosis, Skolnick et al. investigated whether osteoporosis treatment affects the progression of aortic stenosis. They retrospectively screened echocardiograms and compared the change in aortic valve area (AVA) within one year in patients (n = 18) receiving osteoporosis medication (bisphosphonates, calcitonin, or selective estrogen receptor modulators) with those in patients (n = 37) who did not have osteoporosis medications[110]. AS progression in the osteoporosis treatment

group (0.10 ± 0.18 cm^2/year) was significantly less than that in the control group (0.22 ± 0.22 cm^2/year; $p = 0.025$). Therefore, osteoporosis treatment may slow the rate of CAVD progression. However, the results of this study are difficult to interpret due to the non-randomized nature of the study, the small sample size, and the fact that different classes of osteoporosis medications were used and analyzed as one group. Nonetheless, building on this concept, Pawade and Dweck of the University of Edinburgh have initiated the first randomized control trial (RCT) of osteoporosis medications in AS treatment[111]. Patients (n = 150) will be randomized to either a bisphosphonate (alendronic acid), denosumab, or a placebo; change in aortic valve calcium scores (compared to baseline) will be measured at 6 months and two years of follow up. The results of this RCT will establish whether osteoporosis medications are effective in treating AS. In the following section, we will review previous observational studies that evaluate the association between osteoporosis medications and slowing of the progression of CAVD.

2.3.1 Bisphosphonates and CAVD

Because of the observed association between osteoporosis and low BMD with AS, several studies have evaluated whether medications commonly used for osteoporosis could retard CAVD progression. Bisphosphonates, a common treatment for osteoporosis, are a class of medication that directly promote osteoclast apoptosis and decrease bone resorption through diverse mechanisms[112]. In a cross-sectional analysis of the Multi-Ethnic Study of Atherosclerosis (MESA) cohort, Elmariah et al. show that older women (≥75 years old) who were on bisphosphonates had less valvular calcification compared to those who were not on bisphosphonates (aortic valve ring calcium 38% vs 59%; $p < 0.0001$)[113]. However, no association was observed between bisphosphonate use and calcification in the 65-75 age range and the opposite association was

observed in younger women (55-65 years old) such that bisphosphonate users actually had higher valvular calcification levels. In a smaller study, Sterbakova et al. show that bisphosphonate use associates with slower progression in patients with mild AS[114]. In contrast, Aksoy et al. show that in 801 older women (mean age, 76 ± 7.6 years), bisphosphonate use leads to no significant difference in the rate of change in aortic valve area or peak and mean gradients or survival or freedom from aortic valve replacement[115]. Therefore, current data on the effect of bisphosphonates on aortic stenosis progression remains conflicting.

2.3.2 Denosumab and CAVD

Denosumab, an antiresorptive medication used for osteoporosis treatment, is a monoclonal antibody against RANKL which much like OPG binds to and sequesters RANKL inhibiting its pro-osteoclastic activity. Lerman et al. used porcine VIC models (n = 10) to evaluate the effect of Denosumab on valvular calcification *in vitro*[116,117]. Treating VICs with 3 mM Na3PO4 leads to a 5.2-fold increase in calcification (measured by Alizarin Red staining) in 14 days ($P < 0.001$). However, this calcification is inhibited by treatment with Denosumab. Therefore, Denosumab prevents induced calcium deposition levels *in vitro*. Whether this inhibition of VIC activation is through blocking RANK-RANKL interaction is yet to be elucidated. Furthermore, it is important to evaluate whether these results are externally valid in human aortic valves.

2.3.3 Raloxifene and CAVD

Estrogen has a protective role in bone health by preventing bone resorption. Estrogen upregulates osteoclast's apoptosis, decreases osteoclast's activity, prevents apoptotic death of osteocytes, and has anabolic effects on osteoblasts; thus, explaining the rise in incidence of

osteoporosis in women post-menopause[118,119,120]. The effect on osteoclasts is most prominent; Estrogen increases OPG and decreases RANK. Furthermore, the inflammatory cytokines IL-1, IL-6, TNF-α, granulocyte macrophage colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and prostaglandin-E 2 (PGE 2) which all increase the pre-osteoclast population in bone are inhibited by estrogen[121]. Indeed, a single nucleotide polymorphism in the estrogen receptor alpha gene has been found to be associated with an increased risk of AS [OR = 3.38; 95% CI = (1.13,10.09)], although this has not been subsequently replicated[122].

Raloxifene is an oral selective estrogen receptor modulator (SERM) that has pro-estrogenic actions on bone tissue and diverse activities in other tissues[123]. SERMs, such as tamoxifen, have been shown to slow the progression of atherosclerosis in postmenopausal women with breast cancer by reducing inflammation (lowering CRP), reducing LDL levels, and reducing LDL oxidation[124,125,126]. However, in a large RCT of postmenopausal women (n = 10,101), Barrett-Connor et al. found that although Raloxifene decreased the risks of breast cancer and vertebral fractures, it increased the risk of fatal stroke and venous thromboembolism. There was no significant change in the risk of coronary events[127].

Raloxifene is currently indicated for osteoporosis treatment and vertebral fracture prevention in postmenopausal women[128]. The bone protective mechanisms of raloxifene is thought to mediated by direct anti-apoptotic and anti-oxidant effects to prevent osteoblast apoptosis[129,130]. Interestingly Shuvy et al. found that raloxifene also has protective anti-apoptotic effects in rat aortic valves *in vivo*[131]. They induced aortic valve calcification in rats by a uremic diet (a model for renal failure) and noted that osteopontin levels were lower in the diet and raloxifene-treated

group (3.23-fold decrease) compared with the diet only group. Additionally, raloxifene treatment led to a significant reduction in inflammation in aortic valves; anti-CD68 antibody for macrophages was found in the diet group but not in the diet and raloxifene group. Finally, Raloxifene treatment led to a significant reduction in apoptosis as shown by a significant decrease in the percentage of apoptotic cells and levels of caspase 3 protein. Therefore, raloxifene treatment effectively reduced calcification in rat models of renal failure through decreasing apoptosis and inflammation in the aortic valve. It is not known whether these effects are mediated through the estrogen receptor. It also remains unknown whether these protective effects of raloxifene on the rat aortic valves are at work in human aortic valves. Therefore, the common mechanisms of mineral metabolism in bone and in the aortic valve milieu warrant further investigation.

In summary, the pathophysiology of aortic stenosis is complex involving a confluence of lipid molecules, inflammatory/oxidative mediators, and signaling cascades that tamper with cellular differentiation and apoptosis in a time-sensitive fashion. Certain aspects of AS pathophysiology are reminiscent of mechanisms of bone turnover; for example, the RANK-RANKL-OPG axis at work both in bone and in the aortic valve. Furthermore, disorders of bone metabolism that involve increased bone turnover (Paget's disease) or increased loss of bone mineral (osteoporosis) have been shown in observational studies to associate with aortic stenosis. There could be two provocative explanations for this association: One possibility is that local molecular pathways in bone and valve are unrelated to each other. Alternatively, it is possible that released calcium and phosphate crystals from bone mineral directly causes ectopic calcification in the aortic valve. In order to test these hypotheses, we will evaluate whether there is a causal association between BMD reduction and aortic stenosis. In the next chapter, we will discuss how

a Mendelian randomization study design allows us to test such hypotheses and why we cannot rely on observational studies to draw conclusions on presence or absence of causal associations.

Chapter III: Rationale for a Mendelian Randomization Study Design

As demonstrated in chapter 2, multiple studies have shown associations between reduced BMD and aortic stenosis. Therefore, skeletal bone remodelling might influence the development of CAVD. Indeed, as discussed, the prevalence of calcific valvular and vascular disease is more common among patients with Paget's disease, osteoporosis, and calcium metabolism disorders. Furthermore, common molecular regulatory systems appear to govern calcification in both the aortic valve and bone. Thus far, most of the studies examining associations between BMD and valvular calcification have been retrospective observational studies. Below, we will discuss why making conclusions from observation data can be challenging.

3.1 Disadvantages of observational studies

Most observational studies demonstrate an inverse association between bone mineral density and valvular calcification. However, reverse causation and confounding are important limitations of such observational studies. Observational studies do not establish directionality meaning that we cannot conclude whether lower BMD leads to CAVD or systemic changes due to CAVD lead to a lowering of BMD. Furthermore, even if we had the knowledge that the direction of association is BMD to CAVD, with only observational studies we would not be able to conclude that it is indeed the reduction in BMD that causes CAVD; that is, correlation does not imply causation. It is possible that a "lurking variable" (or confounder) is leading to both a decrease in bone mineral density and CAVD. For example, inflammation could drive both the loss of BMD and the advancement of CAVD. If this is truly the case, no conclusions can be drawn on the direct effect of BMD on AS. The gold-standard for finding whether there is a true causal association

between BMD and AS is a randomized control trial (RCT). Conducting a randomized control trial, however, can be challenging or even unfeasible, in some situations, as we discuss below.

3.2 Challenges of conducting a randomized control trial

A randomized control trial is a true scientific experiment in which participants are randomly assigned to either a treatment group or a control (placebo) group. After this assignment or randomization, the groups of subjects in the trial are assumed to be identical in every way with the only differences being the treatment or intervention they have been assigned. As a result, any differential outcome in the treatment versus the placebo group must have been caused by the intervention. Consequently, RCTs are thought to be the gold-standard for determining medical treatments. One major drawback, however, is that conducting a RCT can be very costly. Johnston et al.'s analysis of the cost effectiveness of RCTs shows that out of 28 RCTs with a total cost of 335 million dollars only four (14%) resulted in cost savings to society. Additionally, in RCTs, there is a need for a large sample size in each group. Indeed, due the difficulty in conducting an RCT with a large enough sample population, external validity (the ability to generalize study results to the whole population) remains a concern[132]. Furthermore, RCTs are time consuming since participants need to be followed long enough for the intervention to lead to a detectable change in outcome. Finally, for ethical or pragmatic reasons certain scientific theories cannot be tested using RCTs. For example, we cannot randomize humans to a demineralizing agent that decreases BMD and follow them up to evaluate whether they develop AS.

Given the limitations of observational studies and challenges in conducting an RCT, whether or not mechanisms involved in osteoporosis cause CAVD remains unknown. Below, we

will describe the concept of the Mendelian randomization (MR) study design and how it could be implemented to conduct a naturally-randomized experiment to evaluate whether there is a causal link between BMD reduction and AS. We will also discuss the advantages and limitations MR studies compared with either observational studies or RCTs. While RCTs are required to provide evidence for implementing new treatments, MR studies can provide mechanisms and biomarkers that causally relate to disease development and accelerate our understanding of candidate interventions to later test in RCTs.

3.3 Mendelian Randomization Study Characteristics

Mendelian randomization refers to the independent assortment of alleles during meiosis at the time of gamete formation. The consequence of this natural phenomenon is that genetic variants are distributed in an almost random fashion among individuals in a population[133]. Importantly, because this natural randomization of genetic variants happens at birth, it is independent of societal, environmental, and behavioral factors that are common confounders in observational epidemiological research. For example, in a retrospective observational study to find an association between BMD and AS, smoking, socioeconomic status, diet, and exercise could confound the association between our variables of interest. However, in a Mendelian randomization study, much like an RCT, every factor is expected to be identical between groups except for the genetic variant that leads to a detectable change in the independent variable of interest, in this case, BMD.

Furthermore, Mendelian randomization addresses the important issue of reverse causality in observational research. If genetic variants that predispose individuals to a lower BMD associate

with AS, then because the distribution of these variants preceded CAVD (i.e. were present at birth), the direction of the causal association is clearly from BMD to CAVD. Importantly, one key assumption for this to hold true is that, there can be no association between the BMD modifying genetic variants and AS through any mechanism independent of BMD[134]. Other imitations of MR and possible solutions will be discussed in further detail in the next section.

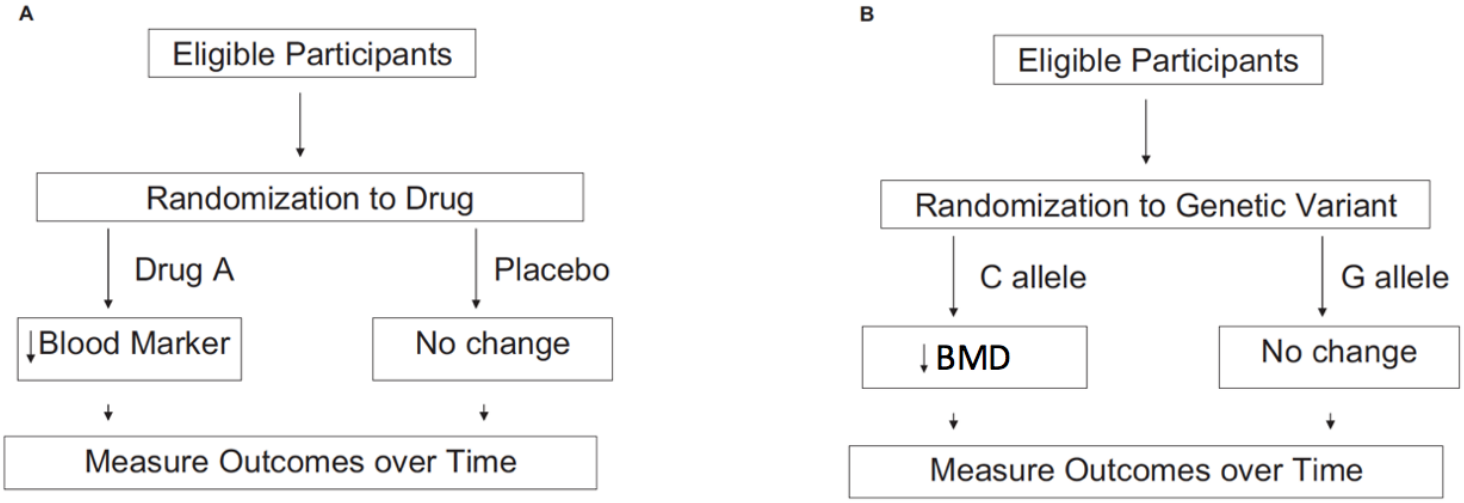
Conducting an MR study offers several other advantages over RCTs. Mendelian randomization studies can be performed using any cohort with available genetic data, as a result larger study populations can be attained without the need to recruit new study participants[135]. Additionally, MR studies can be nested in existing cohorts and are therefore much faster to conduct because there is no need for further follow up of participants; randomization occurred at birth and the study population has been exposed to the variant phenotype of interest (in our study lower BMD) for their lifetime. Finally, while RCTs are limited to one experiment at a time, multiple MR studies can be conducted at the same time in the same study population because there is no need for a true experimental intervention[136].

Conducting a Mendelian randomization study

In an MR study, genetic variation i.e. single nucleotide polymorphisms (SNPs) that modify a phenotype of interest act as exposure variable[137]. Therefore, in our study, SNPs that lead to a reduction in bone mineral density (BMD) will be used as the exposure and the outcome of interest will be the development of aortic stenosis. It is important that the SNPs are robustly associated with the phenotype of interest. Therefore, we used SNPs identified by genome-wide association studies (GWAS) to be significantly associated with modifying BMD levels at genome-wide

significance ($P \leq 5 \times 10^{-8}$)[138]. Once the genetic variants that reduce BMD are identified, individuals can be separated into two categories: those who have the BMD lowering SNPs and those who do not. Consequently, comparing the rates of AS across these two groups can illustrate if a reduction in BMD causes AS. However, an MR study relies on certain assumptions some of which can be difficult to verify. In the following section, we will discuss these assumptions and explain how they may pose limitations to the validity of conclusions drawn from MR studies.

Figure 13. Similarities between a randomized control trial (A) and our Mendelian randomization study (B). In a RCT experimenters randomize participants to two groups with the drug being the only change. In an MR study, participants are randomized at birth to different genetic variants that predisposes them to lower BMD or not (modified from Thanassoulis)[136].

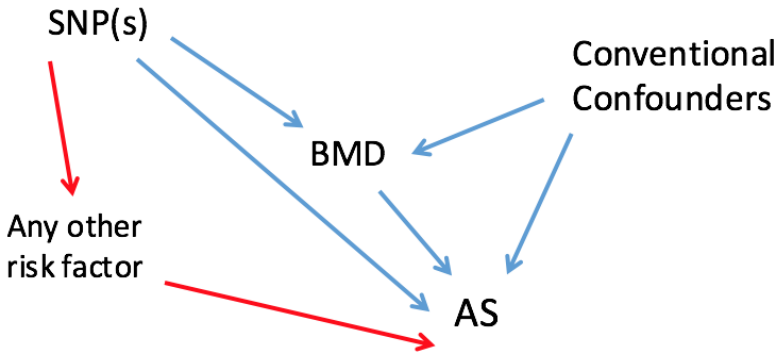


Assumptions and limitations of Mendelian randomization studies

There are several important assumptions for an MR study to be valid. First, the genetic variants that associate with the intermediate factor (BMD) must explain a significant proportion of the variation in BMD. This is not always possible when using single SNPs, which typically have small effects; however, improvements can be made by using multiple alleles (SNPs) that associate with the intermediate variable independently and combining these into a genetic risk score (GRS). Second, the variants selected cannot associate with any other factors or be involved in any other mechanism that can influence the outcome (AS) independently of BMD (Figure 14). This limitation of MR is called genetic pleiotropy; it happens when a variant is involved with modifying multiple phenotypes or multiple biological mechanisms. For example, if a SNP that lowers BMD is being used as proxy for evaluating whether lowering of BMD causes AS, and the SNP, also increases apoptosis in VICs, then our conclusions on the causal role of BMD in AS are overestimated because of the pleiotropic effects of the chosen variant. Unfortunately, our limited knowledge of the SNPs makes it impossible to entirely rule out if they are involved other biologic pathways. However, by using multiple genetic variants that all decrease BMD independently of one another, we can test each variant with AS and examine for consistency across these various SNPs. It is very unlikely that these independent SNPs have pleiotropic effects that all work in the same direction to cause or prevent AS and therefore consistency in associations with several variants helps to exclude pleiotropy[139].

Figure 14. Assumptions of Mendelian randomization (MR). In MR, single nucleotide polymorphisms are used as proxy for the independent variable of interest (BMD). Because the assignment of variants take place randomly at birth, there is no risk for confounding by

conventional confounders of observational research. However, MR relies on the assumption that the SNPs mediate their effect on the outcome (aortic stenosis or AS) through BMD and not through any other mechanism or factor (pleiotropy).



Confounding in MR: Linkage disequilibrium and Population stratification

While Mendelian randomization, via the natural randomization induced at conception, elegantly escapes common or conventional confounders of epidemiological research, it is prone to two other forms of confounding that may occur in genetic epidemiology: confounding by linkage disequilibrium and population stratification (Figure 15)[140].

Linkage disequilibrium: confounding by locus proximity

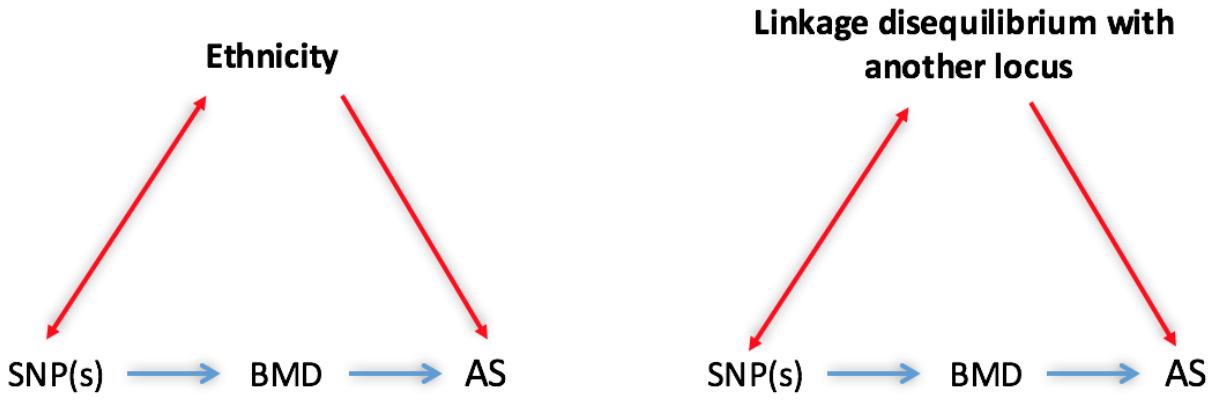
MR works based on the premise of Mendel's second law of independent assortment of alleles stating that all regions of the genome are passed on to the next generation independently of one another. However, this is not true for alleles that are near each other. Genetic loci that are in close proximity in the genome are said to be in linkage disequilibrium (LD). As a result, if one allele is passed on to the offspring, the other allele in LD with it is more likely than expected by chance to also pass on to the offspring[141]. Consequently, confounding by LD can occur if a locus

that is associated with the intermediate variable, is near another locus, which happens to also associate with the outcome of interest. The first locus instead of mediating its effect on the outcome through the intermediate variable of interest could be mediating the effect through another nearby gene in LD. For example, if a locus A decreases BMD and associates with CAVD, a causal association between BMD and CAVD is suggested. However, the association would be confounded if locus A is in LD with locus B which happens to cause CAVD through another mechanism (e.g. increasing Lp(a)). As a result, we would have overestimated the effect of BMD lowering on CAVD. Confounding by LD can be addressed by using multiple genetic variates that strongly associate with the intermediate variable and again looking for consistency across these variants. Consistent effects would suggest no confounding by LD because it would be unlikely that independent SNPs at different loci (or chromosomes) would lead to consistent effects via LD with other genes.

Population stratification: confounding by ethnicity

Different ethnic groups in the study population may have differences in the disease prevalence as well as differences in their genetic makeup. As a result, a variant might be associated with AS without any true association with the CAVD mechanism, but simply because the variant and the disease are both common in that race or ethnicity. Therefore, population stratification can be viewed as confounding by ethnicity. Population stratification can be circumvented by performing MR in an ethnically homogeneous population or using advanced genetic methods to adjust for population stratification. Our study population will be composed of individuals of European ancestry thereby limiting confounding by population stratification.

Figure 15: Left: Confounding by population stratification occurs when genetic ancestry is associated with both the risk allele and the outcome of interest. Right: Confounding by linkage disequilibrium occurs when another genetic marker near the risk allele associates with the outcome of interest.



Insufficient power: the need for a large sample size

If the associations between the SNP(s) and the intermediate variable and/or the association between the SNP(s) and the outcome are not strong, we may not observe an association between the intermediate variable and the outcome, despite the presence of a true causal association. This leads to false negative results. As a result, the larger the sample size and the higher the variation explained of the intermediate variable by the SNPs the better the chance of not missing true associations (i.e. false negatives).

Canalization: physiological compensation

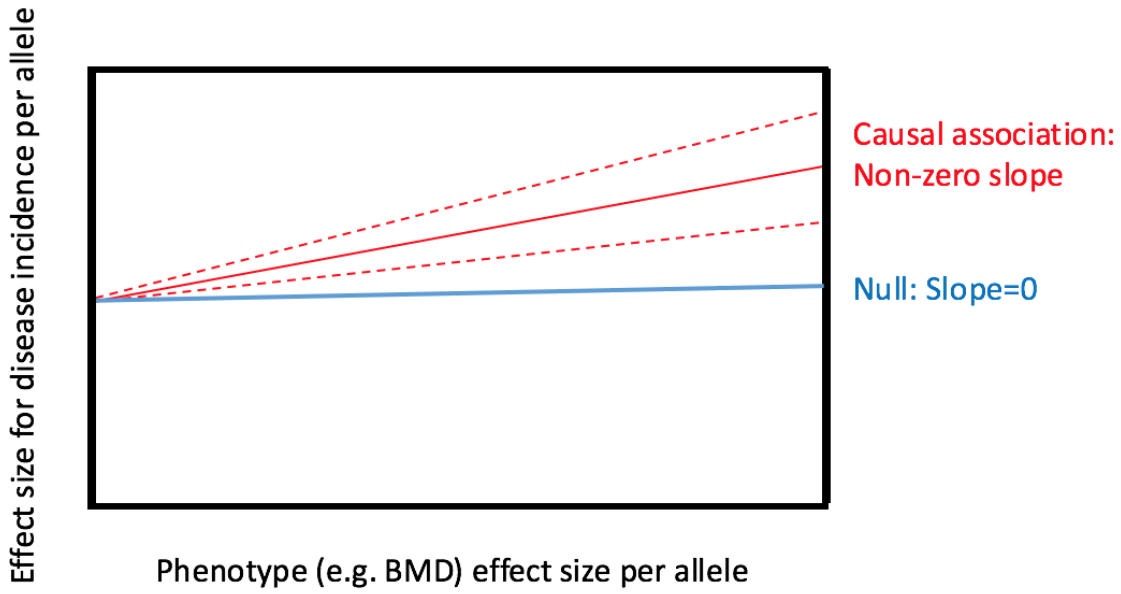
Canalization, also called biological robustness, refers to the ability of a population to reproduce the same phenotype regardless of its genotype by activation of mechanisms that bring a phenotype back to a set point[142]. In the context of our study and MR, if a BMD lowering SNP also

activates and promotes protective mechanisms against bone demineralization in utero and/or throughout lifetime, then the reduction of BMD may appear no longer as harmful and no longer lead to CAVD; consequently, it would be difficult to draw conclusions about a causal role of low BMD in AS. Canalization is difficult to control for in MR studies and its presence can never be entirely excluded.

Performing an MR analysis: e.g. Genetics ToolboX (GTX) package in R

The GTX package in R provides a single SNP meta-analysis association plot for Mendelian randomization using multiple variants[143]. Each point on the plot represents one SNP. Multiple SNPs that associate with our intermediate variable of interest are plotted on the X axis, the more to the right, the higher the effect size of that SNP on the phenotype. The effect sizes can be obtained from previous GWAS. The effect of the SNPs on the outcome variable is plotted on the Y axis. For example, in our study, the X axis is BMD effect size per variant, such that the SNPs plotted on the further right decrease the BMD the most. The effect size of the BMD-lowering SNPs on AS is plotted on the Y axis. If there is a causal relationship between a reduction in BMD and AS, we would observe a positive slope, if there is no association we would observe a zero slope (Figure 16). Using this approach, we sought to explore whether there was any evidence for a causal association between osteoporosis and AS using MR by examining whether common genetic variants that predispose individuals to BMD reduction are also associated with AS, individually or when combined in a genetic risk score (GRS).

Figure 16: Mendelian randomization done using GTX package in R. Red represents a hypothetical causal association between BMD and AS, the dotted red lines represent the confidence intervals. Blue represents a null effect or no association between BMD and AS.



Chapter IV: MANUSCRIPT

Genetic Predisposition to Lower Bone Mineral Density and Aortic Stenosis:

a Mendelian Randomization Study

Bandegi P, Dufresne L, Chen HY, Richards JB, Engert JC, Thanassoulis G

Introduction:

The most common form of heart valve disease in the developed world, Aortic stenosis (AS), remains a condition without any preventative treatment[3]. Currently, valve replacement is the only available option; thus, limiting patients to a deteriorating quality of life until diminished valve function demands intervention[7]. Transcatheter or surgical aortic valve replacement can have complications and carry a 30-day morality risk of about 3% to 7%[144-147]. Advances in understanding of molecular mechanisms in AS have determined promising pathways for medical intervention. Mounting studies report an inverse association between bone mineral density (BMD) and AS. Accordingly, an intriguing hypothesis is that common molecular pathways link these two diseases. It has been hypothesized that as individuals age and lose bone mass as a result of osteoporosis, the released calcium and phosphate in systemic circulation causes ectopic valvular calcification and AS. Understanding whether the relationship between BMD and AS is causal would guide future investigations and may suggest that treatments for BMD maintenance could be useful candidates to prevent or treat AS.

To date, no studies have evaluated whether a causal relationship exists between BMD and AS. Leveraging the random allocation of genetic polymorphisms at conception, Mendelian

randomization (MR) can evaluate existence of a causal influence of a modifiable risk factor on a disease outcome. Genetic risk scores (GRSs) for BMD explain about 5% of the genetic variance for this trait in children and adults and associate strongly with the risk of vertebral and non-vertebral fractures in both males and females, substantiating that a higher GRS leads to a life-time exposure to BMD reduction[148-152]. Using an MR study design, we sought to determine whether genetically mediated reduction of BMD associates with the presence of aortic valve disease in participants of the CHARGE cohorts as well as the large GERA cohort.

Methods:

In the three CHARGE cohorts, using summary level data, we evaluated associations of BMD GRS and aortic valve calcium with data from computed tomographic (CT) imaging. In the large GERA cohort, using both summary level data and individual level data, we evaluated the associations of BMD GRS and presence of aortic stenosis as reported by diagnostic and procedural codes in the electronic health records (EHR). The studies were approved by relevant regulatory bodies and McGill University Health Centre and participants provided informed consent.

Participants

CHARGE Consortium: The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium is a combination of five large longitudinal United States and Europe population cohorts with well-characterized individual genotype and clinical data; Psaty et al. have previously described each of these cohorts in detail[153]. We included three cohorts of individuals of White European descent with available genotype data and aortic valve calcium data measured by computed tomography (CT) scan. Namely, the Multi-Ethnic Study of Atherosclerosis (MESA, n

= 2527; cohort start to last follow-up: 2000-2012), the Age, Gene/Environment Susceptibility–Reykjavik Study (AGES-RS, n = 3120; from 2000-2012), and the Framingham Heart Study (FHS, n = 1295, from 1971-2013)[24].

GERA cohort: The second population consists of the Genetic Epidemiology Research on Adult Health and Aging (GERA) cohort from Kaiser Permanente (KP), a health maintenance organization based in the United States[154]. KP Research Program on Genes in collaboration with the University of California—San Francisco have successfully genotyped 103,067 adults living in northern California using DNA from saliva samples via customized, ethnicity-specific Affymetrix Axiom™ 1.0 and 2.0 genotyping solution (beginning to end of DNA extraction: 2009-2011)[155,156]. The SNP genotyping success rate ranged from 98.1 to 99.4%. 670,176 SNPs were genotyped and over 15 million SNPs were imputed. Participants completed self-administered survey of behavioral and demographic variables. Responses from the KP Research Program on Genes, Environment, and Health (2007-2010) or the California Men's Health Study (2002-2003) questionnaires, genotypes, and EHR were linked using anonymized, unique patient identifiers (database of Genotypes and Phenotypes study accession phs000788.v1.p2). For this case-control study of AS (n = 3469), individuals aged 55 years or older with known AS status from January 1996 to December 2015, inclusive (n =44703) were included. Our analyses included participants who self-reported as only European descent, as there were not significant numbers of individuals available from other ethnicities.

Outcome Definitions

Aortic Valve Calcium in CHARGE Cohorts: Standard computed tomographic (CT) scans of aortic valve were performed on all participants and aortic valve calcium levels were measured. Presence of calcium was defined by standard Agatston methods as three or higher contiguous pixels with a minimum brightness level of 130 Hounsfield units[157]. Aortic valve calcium was noted when calcium was present in the valve leaflets or commissures[24].

Aortic Stenosis in GERA: Prevalence and incidence of AS diagnoses and aortic valve replacement (AVR) were obtained by extracting EHR data from January 1996 to December 2015, inclusive. AS cases were defined based on *The International Classification of Diseases, 9th Revision (ICD-9)* coding for aortic valve disorders (*ICD-9 424.1*) or history of undergoing AVR surgery[158]. AVR accounts for the patients who underwent surgical or transcatheter valve replacement. We have previously shown that the positive predictive value of this approach to accurately identify AS is greater than 90%[24]. Individuals with congenital heart disease (*ICD-9 746-747*) were excluded. Dyslipidemia was defined as 2 or more diagnoses of lipid metabolism disorders (*ICD-9272*) and 1 or more prescriptions for a statin, as noted in KP prescriptions database. Height, weight, smoking (ever/never), diabetes mellitus, hypertension, and osteoporosis were self-reported in the questionnaire. Ages older than 89 years (n = 389) were rounded down to 90 to protect the privacy participants[159]. Controls were GERA participants without an *ICD-9* code for AS or a procedure code for AVR (n= 41,234).

Genotyping, Imputation, and Construction of BMD Genetic Scores

CHARGE Cohorts: Genotyping details and HapMap imputation methods used in the FHS, AGES-RS, and MESA cohorts have been previously described[153].

GERA: Genotyping details, quality assurance, and quality control in the GERA Cohort has been previously described[154]. We used SHAPEIT2[160] and IMPUTE2[161,162] with the 1000 Genomes Project as the reference panel for the phasing and imputation steps[163]. The association of BMD variants with AS analysis was performed using SNPTEST version 2.5.2[164].

GRS construction: In the largest GWAS meta-analysis on BMD including more than 80,000 subjects of the Genetic Factors for Osteoporosis (GEFOS) consortium, Estrada et al. have reported 64 single-nucleotide polymorphisms (SNPs) associated with BMD at p value $< 5\times10^{-8}$ (GWAS significance level)[165]. We constructed a bone mineral density genetic risk score (BMD GRS) by using 60 of these 64 SNPs from this large scale GWAS meta-analysis. Two SNPs, rs7017914 and rs5934507, were not included in our GRS because they were only discovered in the meta-analysis of women only and men only respectively. Furthermore, to prune the SNPs for LD and find uncorrelated genetic loci, only the SNP with the lowest p value was selected per locus. As such the SNPs rs17482952 and rs7751941 were not included in our GRS, because their counterparts rs12407028 and rs4869742 map to the same locus/gene (1p31.3/WLS and 6q25.1/C6orf97 respectively). We created an unweighted GRS based on the number of BMD reducing alleles at each of these 60 loci, for each participant. A score of given 0 for homozygous wild type (protective for BMD), 1 for heterozygotes, and 2 for homozygotes risk allele. Therefore, the possible range of the unweighted BMD GRS was from 0 to 120. In addition, we constructed a weighted GRS in

which each SNP was weighted by its association with femoral neck or lumbar spine BMD. This was computed as the sum of the number of risk alleles at each SNP loci was multiplied by β coefficients of each individual SNP from the large-scale BMD GWAS study. The possible range of the weighted GRS was from 0 to -3.18.

Finally, we constructed five GRSs based on the involvement of each SNPs with the following bone physiology pathways: WNT (8 SNPs), RANK–RANKL–OPG (3 SNPs), endochondral ossification (5 SNPs), mesenchymal stem cell differentiation (3 SNPs), and 16 SNPs that have been previously shown to associate with fractures[165]. This was done by evaluating each SNP's proximity to genes that encode for proteins playing a role in bone biology. Subtyped SNPs were concordant with those reported by Mitchell et al. and Warrington et al[152,166]. The complete list of the 60 SNPs and the SNPs included each GRS subtype are available in the supplementary information (Appendix 1).

Statistical analyses

CHARGE Cohorts: To evaluate whether there is a causal relationship between a reduction in bone mineral density and aortic stenosis, we used GWAS summary-level data (β coefficients) for BMD and aortic valve calcium levels in 3 CHARGE participating cohorts with aortic valve calcium data. The GWAS for CHARGE aortic valve calcium has been previously conducted using an age- and sex-adjusted additive model (previously described)[24]. To estimate the association for BMD GRS with presence of aortic valve calcium, we extracted summary level SNP data from the CHARGE aortic valve calcium GWAS for all the BMD SNPs identified. We then used the Genetics Toolbox (gtx) package in R to generate GRS effect sizes (β coefficients) expresses as

odd radios such that OR = e^{β} and 95% confidence intervals for presence of aortic calcium. Using summarized data in MR have been shown to be as effective as using individual level data given variants are uncorrelated[167]. Finally, we plot the effect size of each SNP on BMD against odds ratio of aortic valve calcium for that allele.

GERA: Similar to the analysis in CHARGE, using the Genetics Toolbox R package, we plotted β coefficients of BMD SNPs against SNPs associated with AS in GERA. Therefore, we generated association estimates for each BMD SNP with presence of AS, which were expressed as log of odds ratios (Log Odds AS). In GERA, in addition to the summary-level data, we used direct individual genotype and phenotype data (participant-level data). A multi-locus BMD genetic risk score was calculated for each individual in GERA (n = 44,703) by summing the number of risk alleles (dosage) for each SNP. The association of the BMD GRS with incident clinical aortic stenosis was evaluated using univariate regression analysis. Adjustments were made for common covariates, age, sex, BMI, hypertension, diabetes, osteoporosis, and smoking status, in multivariate model. A p value below 0.05 was deemed to be statistically significant. We also investigated whether a GRS comprising variants that belong to a certain genetic pathway associate with AS. Finally, we used MR-Egger regression analysis to ensure that our results were robust to bias from potentially invalid instrumental variables (SNPs) due to unknown pleiotropy[168].

Results:

Baseline characteristics of CHARGE and GERA cohorts are shown in Table 1 and Table 2. Details of CHARGE cohort characteristics have been previously described. In brief, they consist of individuals in the following cohorts: FHS (n = 1298), AGES-RS, MESA (n = 2527), and GERA (n = 44,703). The prevalence of aortic valve calcium across the three CHARGE cohorts was 32% (n = 2245).

Table 1. Characteristics of the CHARGE cohort. The discovery cohorts include individuals from White European ancestry in FHS (Framingham Heart Study), AGES-RS (Age, Gene, Environment, Susceptibility Reykjavik Study), and MESA (Multi-Ethnic Study of Atherosclerosis).

Characteristics	FHS	AGES-RS	MESA
Genotyping platform	Affymetrix, version 5.0	Illumina Hu370CNV	Affymetrix, version 6.0
Imputation software	MACH	MACH, version 1.0.16	IMPUTE, version 2.1.1
Country of origin	United States	Iceland	United States
Population	White European	White European	White European
No. of participants	1298	3120	2527
Age—years ±SD	60±9	76±5	63±10
Female No. (%)	616 (47)	1811 (58)	1321 (52)
Presence of aortic valve calcium No. (%)	510 (39)	1338 (43)	397 (16)

There were 44,703 individuals in GERA available for analysis. The sample had 41,234 controls with an average age of 69.3 years and 3,469 (8.4%) AS cases with an average age of 74.6 years old. 51.3% of the control individuals were female and 44% of the cases were females. 11.9% of the AS patients had osteoporosis compared with 9.8% of the controls. Age, BMI, presence of hypertension, diabetes, and smoking status were higher in cases than controls (table 3.)

Table 2*. Characteristics of the GERA study population.

Characteristics	**GERA**
Genotyping platform	Affymetrix Axiom 1.0 and 2.0
Country of origin	United States
Population	White European
No. of participants	44703
Age median [IQR]	69.0 [63.0, 76.0]
Female No. (%)	22684 (50.7)
BMI median [IQR]	25.9 [23.6, 29.2]
Hypertension (%)	18906 (42.3)
Diabetes (%)	5097 (11.4)
Osteoporosis (%)	4438 (9.9)
Smoking now Status (%)	1861 (4.16)
Coronary artery disease (%)	12136 (27.1)

*Non-parametric data were presented as median with inter-quartile ranges (IQRs). Categorical data were presented as n (%).

Table 3*. Characteristics of cases and controls in the GERA study population (n = 44703).

Covariates	Control (N=41234)	Case (N=3469)	p value
Age median [IQR]	68.0 [63.0, 75.0]	75.0 [68.0, 81.0]	$p < 2.20 \times 10^{-16}$
Female (%)	21158 (51.3)	1526 (44.0)	$p < 2.20 \times 10^{-16}$
BMI median [IQR][a&]	25.9 [23.5, 29.2]	26.5 [24.0, 29.9]	$p = 5.93 \times 10^{-10}$
Hypertension (%)	16961 (41.1)	1945 (56.1)	$p < 2.20 \times 10^{-16}$
Diabetes (%)	4491 (10.9)	606 (17.5)	$p < 2.20 \times 10^{-16}$
Osteoporosis (%)	4025 (9.8)	413 (11.9)	$p = 7.37 \times 10^{-05}$
Smoking ever Status[b] (%)	20247 (49.1)	1834 (52.9)	$p = 2.41 \times 10^{-07}$

*Non-parametric data were presented as median with inter-quartile ranges (IQRs). Categorical data were presented as n (%) and tested using Fisher's exact test.
a Data on body mass index were available for 42,962 participants.
& Calculated as weight in kilograms divided by height in meters squared.
b Data on smoking were available for 42,535 participants.

Associations between osteoporosis and AS

In unadjusted analyses, osteoporosis was associated with 25% higher odds of AS (95% CI: 1.13-1.39, $p = 5.97 \times 10^{-05}$). When adjusting for possible confounders, osteoporosis status remains significantly associated with AS (OR =1.17, 95% CI: 1.04-1.32, p = 0.012).

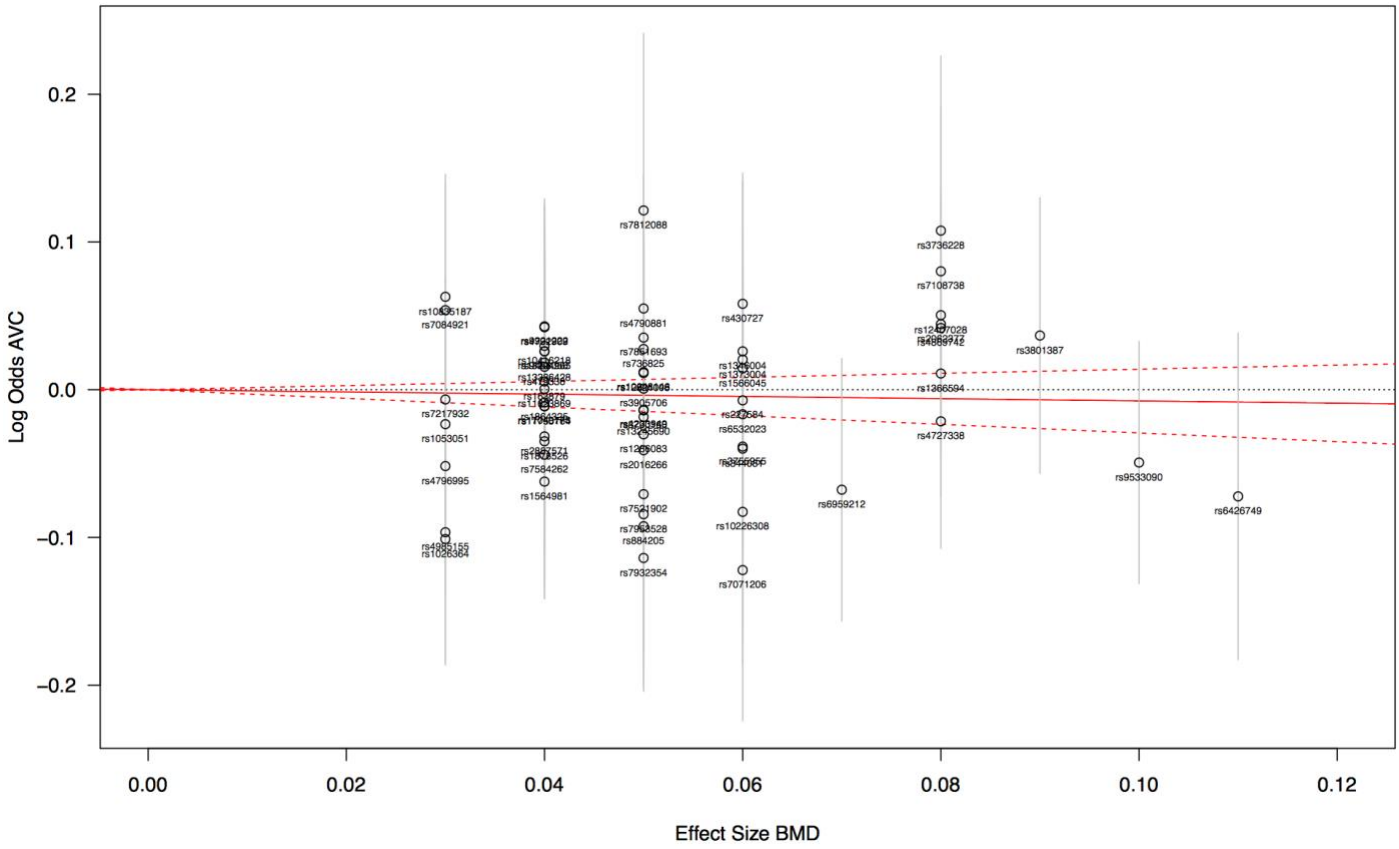
Associations of BMD GRS with AVC and AS using summary level data

In the CHARGE cohorts, the GRS did not associate with aortic valve calcium (OR, 0.93 [95% CI, 0.75-1.15]; p= 0.48). Similarly, in GERA, BMD SNPs did not associate with AS (OR, 0.99 [95% CI, 0.87-1.13]; p= 0.93). The MR-Egger regression yielded similar results: AVC OR, 1.29 [95% CI, 0.62-2.68]; p= 0.49 and AS OR, 0.85 [95% CI, 0.58-1.25]; p= 0.40 (Table 4.). The

regression slope from a β_{AS} vs β_{BMD} plot was not significantly different from zero indicating no evidence of a causal association between BMD and AS. Similar results were observed for β_{AVC} (Figure 1).

As a positive control and to verify BMD SNPs, presence of osteoporosis in GERA was plotted against the magnitude of genetic decrease in BMD across all BMD SNPs. Across all BMD–associated SNPs, a given genetic increase in BMD significantly decreases the odds of osteoporosis (Figure 2).

Figure 1 (next page): Magnitude of genetic decrease in BMD and aortic valve calcium (panel A) and incident AS (panel B) across all BMD SNPs in CHARGE (top) and GERA (bottom). Each point represents a single bone mineral density (BMD) single-nucleotide polymorphism (SNP). Across all BMD–associated SNPs, a given genetic increase in BMD does not have any correlation odds of aortic valve calcium (AVC) or odds of aortic stenosis (AS). The solid red line represents the best line of fit, and the dashed red lines represent the 95% CI for this relationship.


Log Odds AVC
Effect Size BMD
0.2
0.1
0.0
−0.1
−0.2
0.00
0.02
0.04
0.06
0.08
0.10
0.12
rs10835187
rs7084921
rs7812088
rs3736228
rs7108738
rs4790881
rs430727
rs3801387
rs7217932
rs1053051
rs4796995
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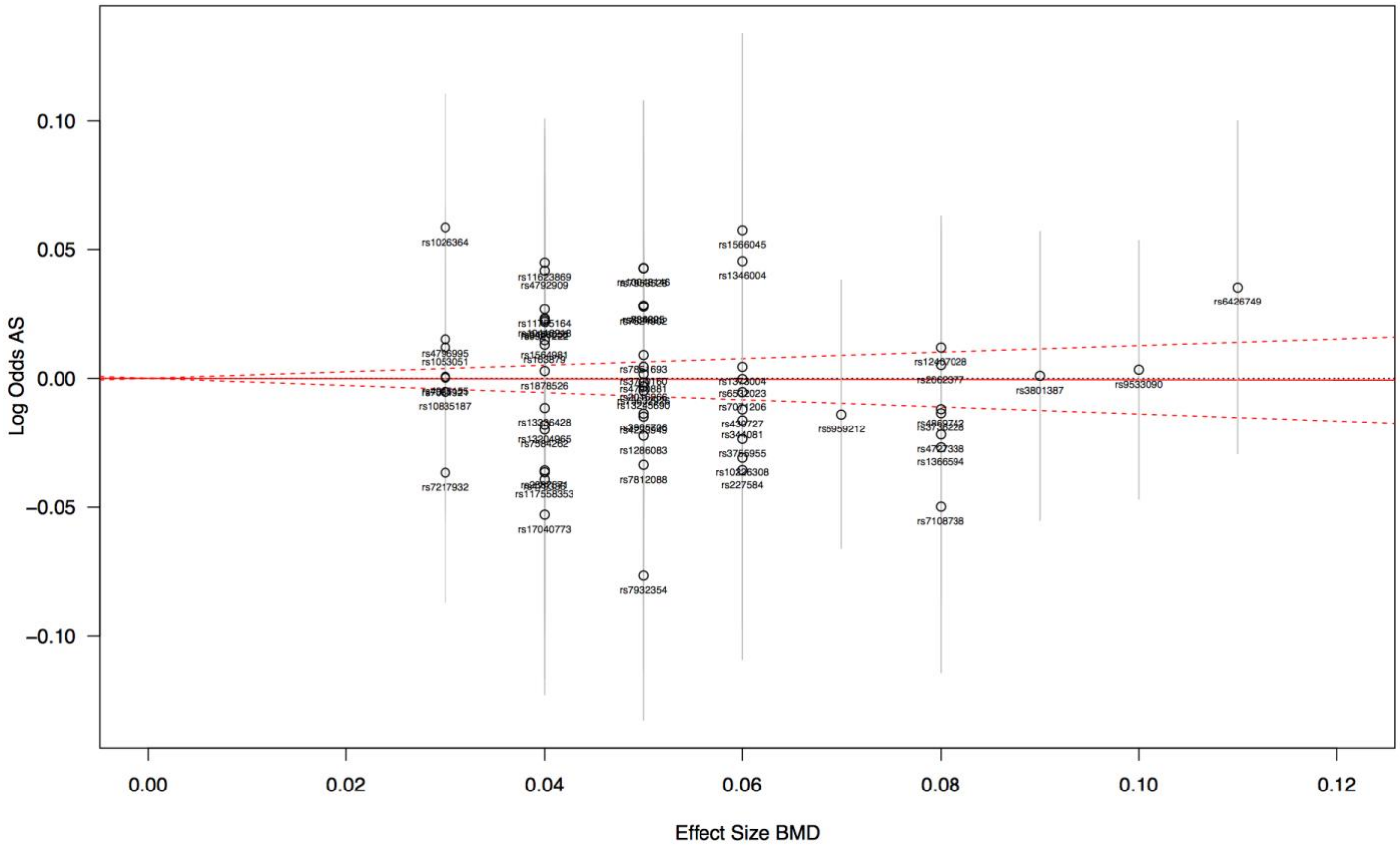
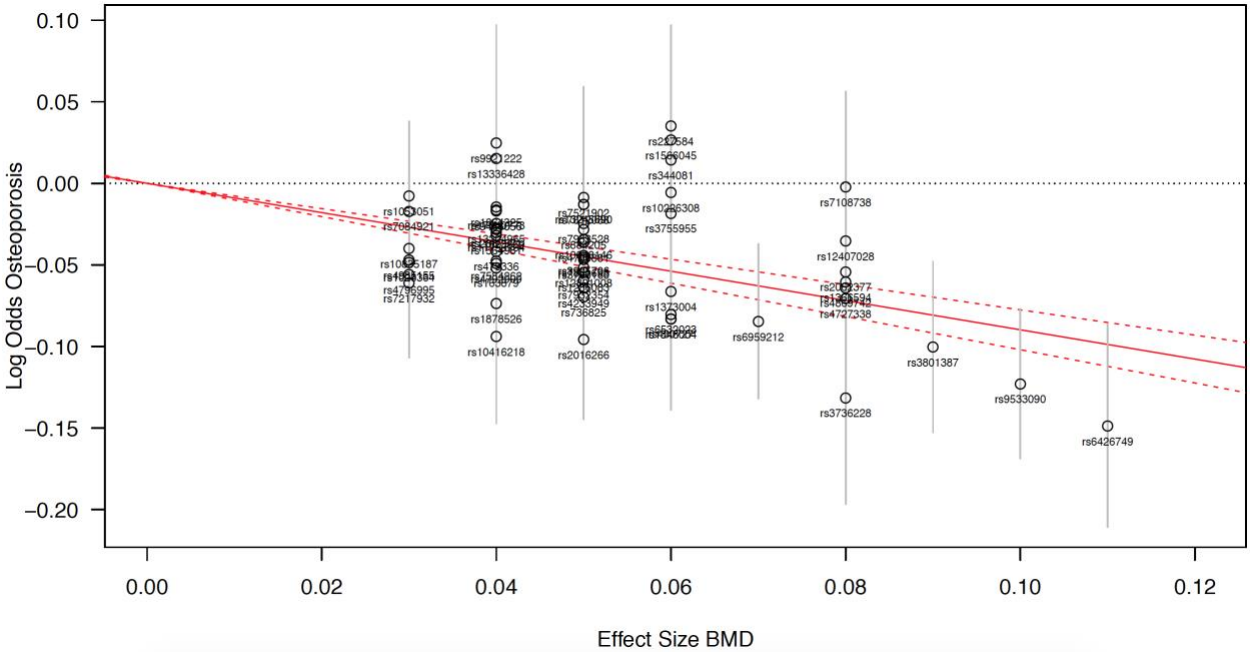

Log Odds AS
Effect Size BMD
0.10
0.05
0.00
−0.05
−0.10
0.00
0.02
0.04
0.06
0.08
0.10
0.12
rs1026364
rs1566045
rs1346004
rs6426749
rs4796995
rs1053051
rs10835187
rs7217932
rs1878526
rs1286083
rs7812088
rs7932354
rs17040773
rs6959212
rs3801387
rs9533090
rs1366594
rs7108738

Figure 2. Magnitude of genetic decrease in BMD and presence of osteoporosis across all BMD SNPs in GERA. Each point represents a single bone mineral density (BMD) single-nucleotide polymorphism (SNP). Across all BMD–associated SNPs, a given genetic increase in BMD does significantly decreases the odds of osteoporosis. The solid red line represents the best line of fit, and the dashed red lines represent the 95% CI for this association.



Associations of BMD GRS and AS in the GERA cohort using individual level data

The BMD GRS constructed for GERA participants followed a normal distribution with a mean of 59.89, a SD of ± 4.73, a minimum GRS of 41.08, and a maximum GRS of 80.81. In GERA participants, the BMD GRS was not significantly associated with aortic stenosis (OR per GRS increment, 1.00 [95%CI, 0.99-1.01]; p = 0.85). When adjusting for common covariates the BMD GRS remained not significant (OR, 1.00 [95%CI, 0.99-1.01]; p = 0.78). The weighted BMD GRS also followed a normal distribution (N = 44,703, Mean = -2.98, Median = -2.98, SD = ± 0.26, Min = -4.05, Max = -1.76) and similar to the unweighted analysis, it did not associate with AS both in unadjusted and adjusted analysis (unadjusted OR,1.01 [95%CI, 0.87-1.14]; p= 0.94 and adjusted OR,1.07 [95%CI, 0.92-1.21]; p= 0.38) (Table 5.)

We sought to evaluate whether any BMD-lowering SNPs alone had any significant effect on AS. Forest plot (figure 4.) demonstrates the meta-analysis for the association between BMD-lowering SNPs and self-reported osteoporosis, and BMD-lowering SNPs and aortic stenosis. BMD GRS is a strong predictor of self-reported osteoporosis OR, 1.05 [95% CI, 1.04-1.05]. However, it does not associate with AS OR, 1.00 [95% CI, 0.99-1.01]. Individual SNPs that significantly increase the odds of osteoporosis do not increase the odds of aortic stenosis (Figure 4.).

Secondary analyses

We constructed five GRS based on the involvement of each SNP in the following bone physiology pathways: WNT (8 SNPs), RANK–RANKL–OPG (3 SNPs), endochondral ossification (5 SNPs), mesenchymal stem cell differentiation (3 SNPs), and fractures (16 SNPs);

while all of the pathway-based GRSs were associated significantly with osteoporosis, none of the them were significantly associated with AS (Table 6 and 7).

Table 4: Mendelian randomization results (GTX and MR-Egger) using summary level data in CHARGE (outcome of aortic valve calcification) and GERA (outcome of AS presence).

	MR-Egger		GTX		M
	OR* (95% CI)	p Value	OR* (95% CI)	p Value	
Aortic valve calcification	1.29 (0.62, 2.68)	0.49	0.93 (0.75, 1.15)	0.48	60
AS presence	0.85 (0.58, 1.25)	0.40	0.99 (0.87, 1.13)	0.93	60

* OR = Odds ratio of aortic valve calcification or AS

Table 5: Logistic regression analysis of osteoporosis and aortic stenosis versus unweighted and weighted BMD GRS.

	Osteoporosis		Aortic Stenosis		N
	OR (95% CI)	p Value	OR (95% CI)	p Value	
Unweighted GRS					
Unadjusted BMD GRS[1]	1.04 (1.04-1.05)	2.12×10^{-35}	1.00 (0.99-1.01)	0.85	44703
Adjusted* BMD GRS	1.05 (1.04-1.05)	2.52×10^{-39}	1.00 (0.99-1.01)	0.78	44703
Adjusted** BMD GRS	1.05 (1.04-1.06)	1.34×10^{-36}	1.00 (0.99-1.01)	0.78	40911
Weighted GRS					
Unadjusted w-BMD GRS[2]	1.86 (1.74-1.98)	5.28×10^{-25}	1.01 (0.87-1.14)	0.94	44703
Adjusted* w-BMD GRS	2.00 (1.88-2.12)	1.60×10^{-28}	1.01 (0.87-1.14)	0.94	44703
Adjusted** w-BMD GRS	2.03 (1.90-2.15)	6.79×10^{-27}	1.07 (0.92, 1.21)	0.38	40911

1. BMD GRS = Unweighted BMD genetic risk score (n = 60 SNPs)
2. w-BMD GRS = Weighted BMD genetic risk score (n = 60 SNPs)
*adjusted for age, age^2 and sex
**adjusted for age, sex, BMI, hypertension, diabetes, dyslipidemia, and smoking

Table 6. Association of pathway-based unweighted BMD genetic risk scores with osteoporosis and aortic stenosis.

Biologic Pathway	Osteoporosis		Aortic Stenosis		N
	OR (95% CI)	p Value	OR (95% CI)	p Value	
WNT					
Unadjusted WNT GRS[1]	1.07 (1.05-1.09)	6.25×10^{-12}	1.01 (0.99-1.03)	0.33	44703
Adjusted* WNT GRS	1.08 (1.06-1.10)	1.54×10^{-13}	1.01 (0.99-1.03)	0.47	44703
Adjusted** WNT GRS	1.09 (1.06-1.11)	2.05×10^{-14}	1.00 (0.98-1.03)	0.72	40911
RANK/ RANKL/ OPG					
Unadjusted R/RL/O GRS[2]	1.07 (1.04-1.10)	3.46×10^{-07}	0.99 (0.96-1.02)	0.47	44703
Adjusted* R/RL/O GRS	1.08 (1.05-1.11)	3.15×10^{-07}	0.99 (0.96-1.02)	0.50	44703
Adjusted** R/RL/O GRS	1.08 (1.04-1.11)	1.41×10^{-06}	0.99 (0.96-1.03)	0.70	40911
Endochondral Ossification					
Unadjusted ENDOS GRS[3]	1.06 (1.03-1.08)	6.15×10^{-07}	1.02 (0.99-1.04)	0.14	44703
Adjusted* ENDOS GRS	1.06 (1.04-1.09)	1.29×10^{-07}	1.02 (1.00-1.05)	0.11	44703
Adjusted** ENDOS GRS	1.06 (1.04-1.09)	1.51×10^{-06}	1.02 (0.99-1.05)	0.14	40911
Mesenchymal Stem Cell					
Unadjusted MesSC GRS[4]	1.06 (1.03-1.09)	1.55×10^{-05}	1.01 (0.98-1.04)	0.74	44703
Adjusted* MesSC GRS	1.06 (1.03-1.09)	1.03×10^{-05}	1.00 (0.98-1.04)	0.76	44703
Adjusted** MesSC GRS	1.06 (1.03-1.09)	4.90×10^{-05}	1.01 (0.98-1.04)	0.69	40911
Fracture					
Unadjusted Fracture GRS[5]	1.06 (1.05-1.08)	5.03×10^{-21}	1.00 (0.99-1.02)	0.88	44703
Adjusted* Fracture GRS	1.07 (1.06-1.08)	2.19×10^{-23}	1.00 (0.99-1.01)	0.93	44703
Adjusted** Fracture GRS	1.07 (1.06-1.09)	7.94×10^{-22}	1.00 (0.98-1.02)	0.98	40911

1. WNT GRS = Genetic risk score with BMD SNPs that are involved in the WNT pathway (n = 8)
2. R/RL/O GRS = Genetic risk score with BMD SNPs that are involved in the RANK/RANKL/OPG pathway (n = 3)
3. ENDOS GRS = Genetic risk score with BMD SNPs that are involved in endochondral ossification pathway (n = 5)
4. MesSC GRS = Genetic risk score with BMD SNPs that are involved in mesenchymal stem cell differentiation (n = 3)
5. Fracture GRS = Genetic risk score with BMD SNPs that are significantly associated with fracture (n= 16)

*adjusted for age, age^2 and sex

**adjusted for age, age^2, sex, BMI, hypertension, diabetes, dyslipidemia, and smoking

Table 7. Association of pathway-based weighted BMD genetic risk scores with osteoporosis and aortic stenosis.

Biologic Pathway	Osteoporosis		Aortic Stenosis		N
	OR (95% CI)	p Value	OR (95% CI)	p Value	
WNT					
Unadjusted w-WNT GRS[1]	2.96 (2.69-3.23)	3.14×10^{-15}	1.01 (0.87-1.14)	0.94	44703
Adjusted* w-WNT GRS	3.32 (3.04-3.60)	6.40×10^{-17}	1.04 (0.74-1.35)	0.80	44703
Adjusted** w-WNT GRS	3.65 (3.36-3.95)	9.68×10^{-18}	0.99 (0.67-1.32)	0.97	40911
RANK/ RANKL/ OPG					
Unadjusted w-R/RL/O GRS[2]	2.51 (2.18-2.84)	4.25×10^{-08}	1.01 (0.87-1.14)	0.94	44703
Adjusted* w-R/RL/O GRS	2.66 (2.31-3.00)	2.32×10^{-08}	0.91 (0.53-1.28)	0.61	44703
Adjusted** w-R/RL/O GRS	2.62 (2.26-2.98)	1.66×10^{-07}	0.92 (0.52-1.32)	0.68	40911
Endochondral Ossification					
Unadjusted w-ENDOS GRS[3]	2.56 (2.14-2.97)	9.59×10^{-06}	1.01 (0.87-1.14)	0.93	44703
Adjusted* w-ENDOS GRS	2.82 (2.39-3.25)	2.57×10^{-06}	1.44 (0.97-1.91)	0.13	44703
Adjusted** w-ENDOS GRS	2.59 (2.14-3.05)	4.08×10^{-05}	1.45 (0.95-1.95)	0.15	40911
Mesenchymal Stem Cell					
Unadjusted w-MesSC GRS[4]	4.28 (3.63-4.93)	1.22×10^{-05}	1.01 (0.87-1.14)	0.94	44703
Adjusted* w-MesSC GRS	4.76 (4.08-5.44)	6.57×10^{-06}	1.04 (0.30,1.77)	0.92	44703
Adjusted** w-MesSC GRS	4.26 (3.54-4.97)	6.85×10^{-05}	1.07 (0.29-1.85)	0.87	40911
Fracture					
Unadjusted w-Fracture GRS[5]	2.80 (2.60-3.00)	1.68×10^{-23}	1.01 (0.87-1.14)	0.94	44703
Adjusted* w-Fracture GRS	3.10 (2.89-3.31)	9.96×10^{-26}	1.01 (0.78-1.24)	0.93	44703
Adjusted** w-Fracture GRS	3.22 (3.00-3.45)	6.21×10^{-25}	1.01 (0.77-1.26)	0.92	40911

1. w-WNT GRS = Weighted Genetic risk score (GRS) with BMD SNPs that are involved in the WNT pathway (n = 8)
2. w-R/RL/O GRS = Weighted GRS with BMD SNPs that are involved in the RANK/RANKL/OPG pathway (n = 3)
3. w-ENDOS GRS = Weighted GRS with BMD SNPs that are involved in endochondral ossification pathway (n = 5)
4. w-MesSC GRS = Weighted GRS with BMD SNPs that are involved in mesenchymal stem cell differentiation (n = 3)
5. w-Fracture GRS = Weighted GRS with BMD SNPs that are significantly associated with fracture (n= 16)

*adjusted for age, age^2 and sex

**adjusted for age, age^2, sex, BMI, hypertension, diabetes, dyslipidemia, and smoking

Figure 3. Mendelian Randomization of BMD and Risk of Aortic Stenosis in GERA. The aim of Mendelian randomization is to provide a robust test of the association between BMD and aortic stenosis (association 3). It is possible to test association 3 by using standard epidemiologic methods, but these methods may be biased (e.g., confounding, reverse causality, etc.) In order to overcome this bias, Mendelian randomization indirectly tests association 3 by first establishing via linear regression that BMD–related SNPs decrease BMD (association 1)[169,152]. These BMD SNPs are then tested for an association with aortic stenosis (association 2). Under the assumption that the entire effect of the BMD SNPs on aortic stenosis (association 2) is mediated by their effect on decreasing BMD (association 1), an un-confounded, and unidirectional assessment of association 3 can be obtained (i.e., instrumental variable estimate). [DEXA= dual energy X-ray scans, FN-BMD= bone mineral density at femoral neck, LS-BMD= bone mineral density at lumbar spine].

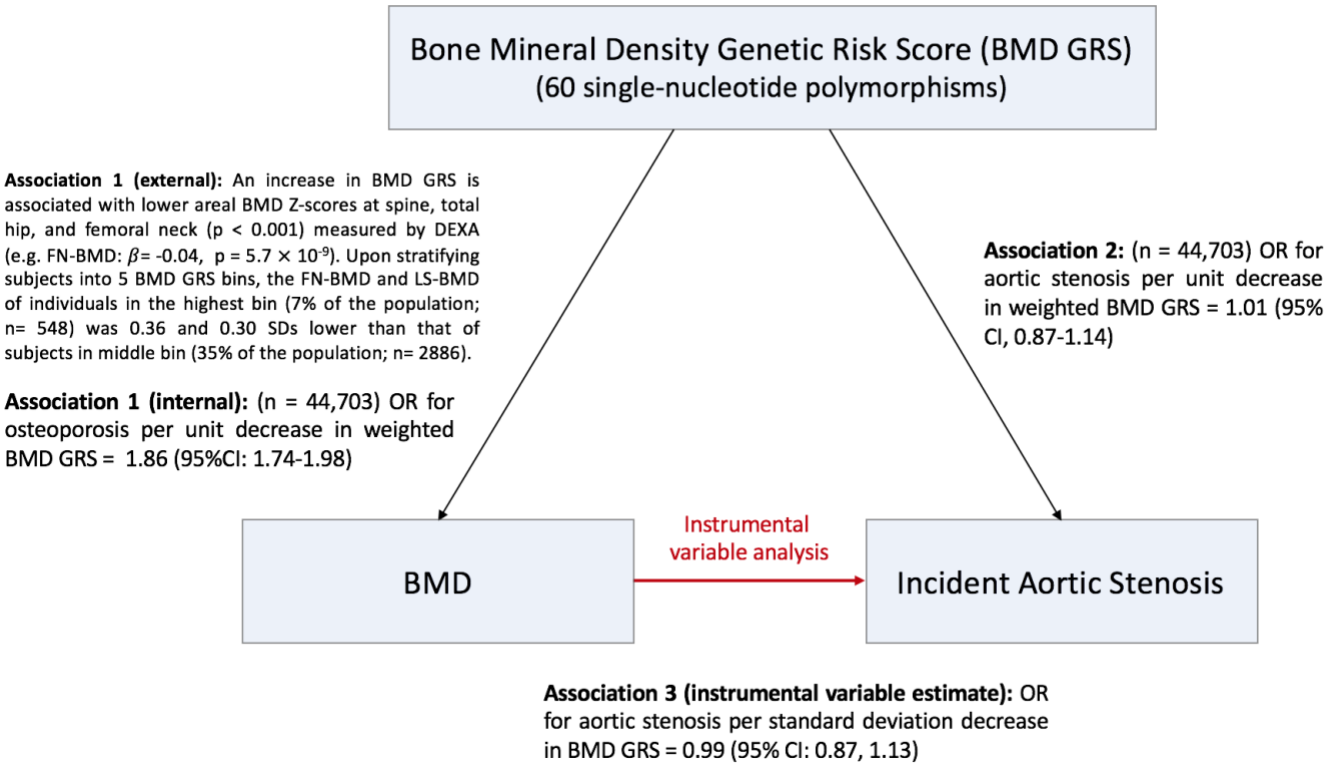
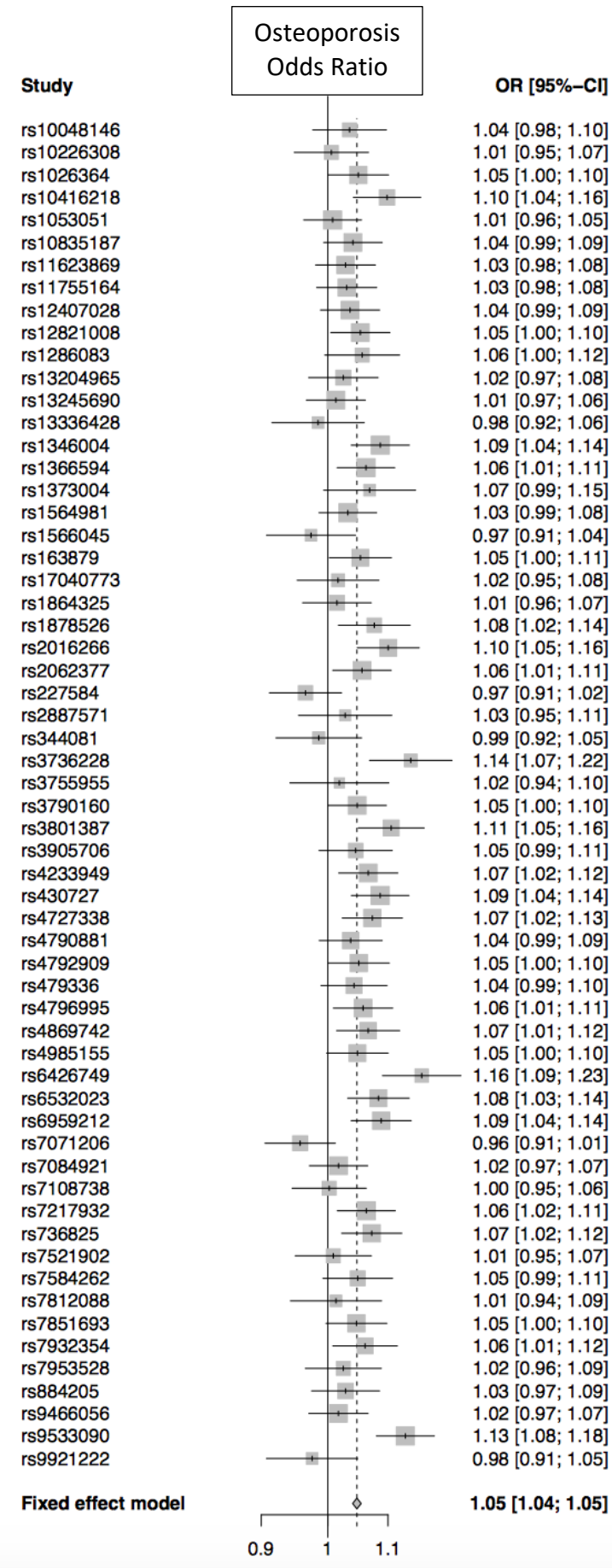
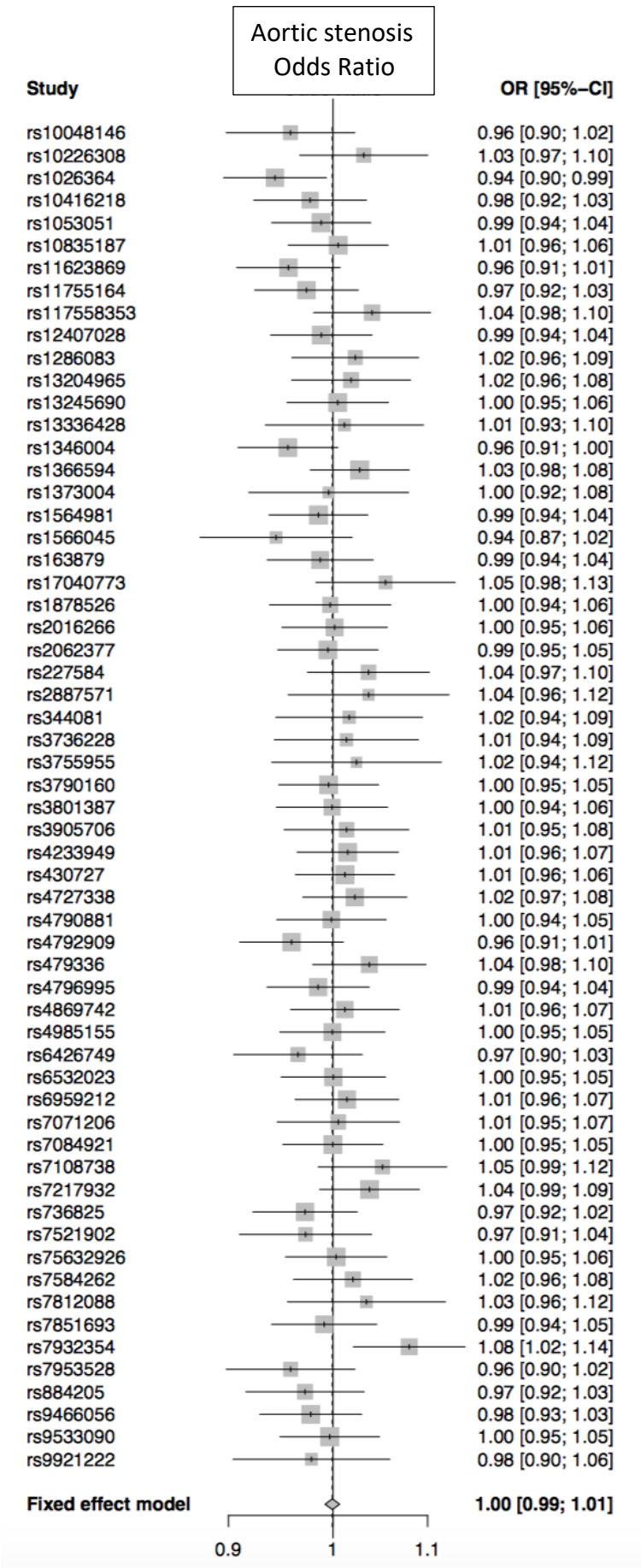


Figure 4 (next page). Forest plot demonstrating the meta-analysis for the association between BMD-lowering SNPs and self-reported osteoporosis (left), and BMD-lowering SNPs and aortic stenosis as reported by physician *ICD-9* coding or prior AVR procedure (right). BMD GRS is a strong predictor of self-reported osteoporosis OR = 1.05 and 95% CI = (1.04-1.05). However, BMD GRS does not associate with AS OR = 1.00 and 95% CI = (0.99-1.01). For example, we can visualize that two SNPs that significantly increase the odds of osteoporosis do not increase the odds of aortic stenosis. SNP rs6426749 that significantly associates with osteoporosis OR= 1.16 and 95% CI = (1.09-1.23), does not associate with AS OR= 0.97 and 95% CI = (0.90-1.03). Similarly, SNP rs9533090 associates significantly with osteoporosis OR=1.13 and 95% CI = (1.08-1.18) but not with AS OR= 1.00 95% CI = (0.95-1.05).


Osteoporosis
Odds Ratio
Study
OR [95%–CI]
rs10048146 1.04 [0.98; 1.10]
rs10226308 1.01 [0.95; 1.07]
rs1026364 1.05 [1.00; 1.10]
rs10416218 1.10 [1.04; 1.16]
rs1053051 1.01 [0.96; 1.05]
rs10835187 1.04 [0.99; 1.09]
rs11623869 1.03 [0.98; 1.08]
rs11755164 1.03 [0.98; 1.08]
rs12407028 1.04 [0.99; 1.09]
rs12821008 1.05 [1.00; 1.10]
rs1286083 1.06 [1.00; 1.12]
rs13204965 1.02 [0.97; 1.08]
rs13245690 1.01 [0.97; 1.06]
rs13336428 0.98 [0.92; 1.06]
rs1346004 1.09 [1.04; 1.14]
rs1366594 1.06 [1.01; 1.11]
rs1373004 1.07 [0.99; 1.15]
rs1564981 1.03 [0.99; 1.08]
rs1566045 0.97 [0.91; 1.04]
rs163879 1.05 [1.00; 1.11]
rs17040773 1.02 [0.95; 1.08]
rs1864325 1.01 [0.96; 1.07]
rs1878526 1.08 [1.02; 1.14]
rs2016266 1.10 [1.05; 1.16]
rs2062377 1.06 [1.01; 1.11]
rs227584 0.97 [0.91; 1.02]
rs2887571 1.03 [0.95; 1.11]
rs344081 0.99 [0.92; 1.05]
rs3736228 1.14 [1.07; 1.22]
rs3755955 1.02 [0.94; 1.10]
rs3790160 1.05 [1.00; 1.10]
rs3801387 1.11 [1.05; 1.16]
rs3905706 1.05 [0.99; 1.11]
rs4233949 1.07 [1.02; 1.12]
rs430727 1.09 [1.04; 1.14]
rs4727338 1.07 [1.02; 1.13]
rs4790881 1.04 [0.99; 1.09]
rs4792909 1.05 [1.00; 1.10]
rs479336 1.04 [0.99; 1.10]
rs4796995 1.06 [1.01; 1.11]
rs4869742 1.07 [1.01; 1.12]
rs4985155 1.05 [1.00; 1.10]
rs6426749 1.16 [1.09; 1.23]
rs6532023 1.08 [1.03; 1.14]
rs6959212 1.09 [1.04; 1.14]
rs7071206 0.96 [0.91; 1.01]
rs7084921 1.02 [0.97; 1.07]
rs7108738 1.00 [0.95; 1.06]
rs7217932 1.06 [1.02; 1.11]
rs736825 1.07 [1.02; 1.12]
rs7521902 1.01 [0.95; 1.07]
rs7584262 1.05 [0.99; 1.11]
rs7812088 1.01 [0.94; 1.09]
rs7851693 1.05 [1.00; 1.10]
rs7932354 1.06 [1.01; 1.12]
rs7953528 1.02 [0.96; 1.09]
rs884205 1.03 [0.97; 1.09]
rs9466056 1.02 [0.97; 1.07]
rs9533090 1.13 [1.08; 1.18]
rs9921222 0.98 [0.91; 1.05]
Fixed effect model 1.05 [1.04; 1.05]
0.9
1
1.1


Aortic stenosis
Odds Ratio
Study
OR [95%–CI]
rs10048146 0.96 [0.90; 1.02]
rs10226308 1.03 [0.97; 1.10]
rs1026364 0.94 [0.90; 0.99]
rs10416218 0.98 [0.92; 1.03]
rs1053051 0.99 [0.94; 1.04]
rs10835187 1.01 [0.96; 1.06]
rs11623869 0.96 [0.91; 1.01]
rs11755164 0.97 [0.92; 1.03]
rs117558353 1.04 [0.98; 1.10]
rs12407028 0.99 [0.94; 1.04]
rs1286083 1.02 [0.96; 1.09]
rs13204965 1.02 [0.96; 1.08]
rs13245690 1.00 [0.95; 1.06]
rs13336428 1.01 [0.93; 1.10]
rs1346004 0.96 [0.91; 1.00]
rs1366594 1.03 [0.98; 1.08]
rs1373004 1.00 [0.92; 1.08]
rs1564981 0.99 [0.94; 1.04]
rs1566045 0.94 [0.87; 1.02]
rs163879 0.99 [0.94; 1.04]
rs17040773 1.05 [0.98; 1.13]
rs1878526 1.00 [0.94; 1.06]
rs2016266 1.00 [0.95; 1.06]
rs2062377 0.99 [0.95; 1.05]
rs227584 1.04 [0.97; 1.10]
rs2887571 1.04 [0.96; 1.12]
rs344081 1.02 [0.94; 1.09]
rs3736228 1.01 [0.94; 1.09]
rs3755955 1.02 [0.94; 1.12]
rs3790160 1.00 [0.95; 1.05]
rs3801387 1.00 [0.94; 1.06]
rs3905706 1.01 [0.95; 1.08]
rs4233949 1.01 [0.96; 1.07]
rs430727 1.01 [0.96; 1.06]
rs4727338 1.02 [0.97; 1.08]
rs4790881 1.00 [0.94; 1.05]
rs4792909 0.96 [0.91; 1.01]
rs479336 1.04 [0.98; 1.10]
rs4796995 0.99 [0.94; 1.04]
rs4869742 1.01 [0.96; 1.07]
rs4985155 1.00 [0.95; 1.05]
rs6426749 0.97 [0.90; 1.03]
rs6532023 1.00 [0.95; 1.05]
rs6959212 1.01 [0.96; 1.07]
rs7071206 1.01 [0.95; 1.07]
rs7084921 1.00 [0.95; 1.05]
rs7108738 1.05 [0.99; 1.12]
rs7217932 1.04 [0.99; 1.09]
rs736825 0.97 [0.92; 1.02]
rs7521902 0.97 [0.91; 1.04]
rs75632926 1.00 [0.95; 1.06]
rs7584262 1.02 [0.96; 1.08]
rs7812088 1.03 [0.96; 1.12]
rs7851693 0.99 [0.94; 1.05]
rs7932354 1.08 [1.02; 1.14]
rs7953528 0.96 [0.90; 1.02]
rs884205 0.97 [0.92; 1.03]
rs9466056 0.98 [0.93; 1.03]
rs9533090 1.00 [0.95; 1.05]
rs9921222 0.98 [0.90; 1.06]
Fixed effect model 1.00 [0.99; 1.01]
0.9
1
1.1

Discussion:

In this study of 6,945 participants with subclinical data on aortic valve calcium and 44,703 participants with longitudinal *ICD-9* coding for aortic stenosis, we confirm the association between osteoporosis and aortic stenosis but demonstrate that genetic predisposition to a reduction in bone mineral density (i.e. BMD GRS) is not associated with presence of aortic valve calcium or incident aortic stenosis. We find no evidence of an association between WNT, RANK-RANKL-OPG, endochondral ossification, and mesenchymal stem cell differentiation mediated BMD GRS and aortic stenosis, suggesting that reduction of BMD via these pathways does not play a causal role in the development of aortic stenosis. Our mendelian randomization results suggest that confounding or other biases may explain the association between reduced BMD or osteoporosis and AS.

The results of this study corroborate findings from structural and functional imaging studies that have demonstrated 1) disparities in hydroxyapatite structures of valvular calcific lesions versus bone mineral and 2) a lack of correlation between the calcification processes present in the aortic valve versus bone. Firstly, Bertazzo et al. use nano-analytical electron microscopy techniques and show that calcific lesions in aortic valves are crystallographically and architecturally different from bone mineral[170]. While skeletal bone is composed of poorly crystalline hydroxyapatite forming plate-like structures,[171] aortic valve calcific lesions are composed of highly crystalline hydroxyapatite forming spherical particles. Additionally, while bone mineralization happens within and along collagen fibres by nucleation and growth of calcium phosphate crystals[172], calcific aortic valve lesions are believed to be generated by the laying down

of apatite in concentric layers without association with collagen fibrils. Interestingly, these particles were also present in 46% of aortic valves that did not present any macroscopically observable calcific lesions on the valve or in surrounding tissue and 80% of putatively normal aortic valves with macroscopically observable calcific lesions on surrounding tissue. These mineralized spherical particles are present at all stages of CAVD and their diameter was found to have a trend with increasing AS severity. Furthermore, while osteoblastic proteins (Runx2, Sp7, and osteocalcin) were present in AV tissue adjacent to compact calcification, they were not present in AV tissue with only mineralized spherical particles. Therefore, spherical particles may precede osteoblastic cells and dense mineralized matrix and may contribute to CAVD initiation as well as propagation. Indeed, stiffer matrices are known to drive mesenchymal stem cells towards the osteoblast fate[173] and degree of calcification at baseline is the strongest predictor of disease progression in aortic stenosis[174]. Taken together, we conclude calcification mechanisms genetically and structurally distinct from that in bone begin in the valve and likely proceed independent of bone deposition. Secondly, Dweck et al. conducted positron emission tomography (PET) for quantifying current activity of calcific processes using 18F-sodium fluoride (18F-NaF) tracer and computed tomography (CT) for quantifying established macroscopic calcification and providing a BMD estimate (measured as mean Hounsfield Unit density at thoracic vertebrae)[109]. 18F-NaF uptake is known to increase in cases of accelerated bone turnover such as in Paget's diseases[175]; 18F-NaF PET has also been used in cardiovascular calcification where it is believed to show increased uptake in areas of increased calcification activity in the aortic valve[176]. This PET/CT study showed that while 18F-NaF activity in the aortic valve correlated strongly with the severity of aortic stenosis as measured by the peak trans-valvular velocity on echocardiography (r^2 =0.419, P <0.001), it did not correlate with BMD (r^2 =0.000, P =0.766)[109]. Furthermore, thoracic

vertebra 18F-NaF uptake did not inversely correlate with 18F-NaF uptake in the aortic valve (r^2 = 0.001, P= 0.806). Taken together, it can be concluded that an active calcification process independent of that in skeletal bone is at work in the pathophysiology of CAVD. Our findings concur with this lack of causal association between BMD and AS, and extend it to specifically demonstrate there is no association between the pathways important in bone homeostasis (WNT, RANK-RANKL-OPG, endochondral ossification, and mesenchymal stem cell differentiation) and development of AS.

So how do we reconcile the lack of a causal association between BMD and AS with the substantial evidence for an association between BMD[106,108], Paget's disease[177] or disorders of calcium metabolism[178] with AS? Indeed, our observational results confirm an association between osteoporosis and incident aortic stenosis. Our Mendelian randomization results suggest that confounding and/or other biases likely explain these observational results. For example, minimally oxidized low-density lipoprotein (MM-LDL) and oxidative stress have been shown to enhance vascular cell and inhibit bone cell differentiation[51,52]. Additionally, while in bone, a pro-inflammatory microenvironment activates osteoclasts and reduces bone mineral, in diseased valvular cells, it promotes an osteogenic phenotype causing increased mineral deposition[40,105,179-181]. As such, oxidative stress or inflammatory mechanisms may be the confounders that drive both bone demineralization and ectopic valvular calcification. We conclude that genetic predisposition to lower BMD and systemic calcium and phosphate is unlikely to cause AS, and demineralizing mechanisms present in skeletal bone are likely independent from ectopic calcification that occurs in CAVD.

It is noteworthy to elaborate on the scope and implications of our findings. Our Mendelian randomization study show that SNPs that lead to reduced BMD do not significantly associate with presence of aortic valve calcium or presence of aortic stenosis. This does not disprove or discredit the similarities in the pathophysiology of AS and skeletal bone turnover. Importantly, the receptor activator of nuclear factor kappa-B interaction with its ligand, RANK-RANKL, has been shown to have opposite effects in bone and aortic valve. In bone the interaction induces osteoclasts and leads to bone resorption, while the same interaction in the aortic valve, induces osteogenic differentiation and calcification[102]. This is thought to be due to the progenitor cell population present at each site, osteoclast progenitors are more populous in skeletal bone[12]. Therefore, this contrast in progenitor cell population may account for the association between BMD reduction and AS without any significant role for systemic calcium and phosphate release from demineralizing skeletal bone. As a result, RCTs for the effectiveness of osteoporosis medications, bisphosphonates and denosumab, in aortic stenosis may be successful by acting locally at the aortic valve. However, our study implicates that maintenance of bone mass, per se, is likely not an effective strategy in preventing CAVD or slowing its progression.

While RCTs are the gold standard to change clinical practice, Mendelian randomization studies like ours have important implications especially in guiding future research. From a research perspective, whether or not bone loss and systemic mineral release plays a role in CAVD is an intriguing hypothesis that cannot be tested in humans via RCTs due to ethical reasons. With our Mendelian randomization study design, leveraging genetic variation among individuals at conception, we were able to rule out a causal association between bone loss and aortic stenosis in a naturally randomized experiment. While many studies show an association between BMD and

AS, our work is the first to evaluate whether this association is causal. With the possibility of a direct causal link now ruled out, targeting BMD modification specifically is unlikely to be an effective treatment for AS. As a result, other therapeutic avenues to AS prevention and treatment should be prioritized. Furthermore, although our MR analysis rules out the specific hypothesis that loss of BMD does not cause AS, osteoporosis medications could still be a promising tool in AS prevention through direct action on valve interstitial cells. For example, Denosumab is a monoclonal antibody against RANKL which similarly to OPG binds and sequesters to RANKL and inhibits its activity, which is to induce to osteogenic differentiation and calcification in stenotic aortic valves and an RCT is underway[111]. If this RCT is successful in reducing valve calcification, our MR analysis may aid in the interpretation of such results by attributing the beneficial effects to a local specific valve mechanism, as opposed to a generalized BMD reduction effect.

This study has multiple strengths including analysis in the large GERA cohort with AS (n=44, 703) as well as in the CHARGE consortium (n=6,945) with AVC measures. The Mendelian randomization study design prevents common confounders provides evidence in favour of causal associations. Our study has several limitations that deserve mention. First, the summary-level data from the CHARGE consortium precluded adjustment for additional covariates. However, due to inherent randomization, MR studies at this scale are likely not confounded by common covariates. Indeed, adjusting for potential AS covariates in the GERA individual GRS analyses did not change our findings. Second, while our study included a large cohort, the power to identify an association with AS may have been low. We had 80% power to detect a true odds of ratio aortic stenosis greater or equal to 1.225 per standard deviation of BMD. Therefore, if the effect size of a BMD GRS on AS is more modest, we would be underpowered and unable to detect such an association.

Finally, our study was performed only in participants of European descent; whether these results apply to other ethnicities will require further investigation.

Conclusion

In summary, in a large-scale case-control study, we confirm that osteoporosis is strongly associated with aortic stenosis. However, our genetic analyses show that exposure to a greater genetic predisposition for BMD does not associate with the presence of AS. Our study provides new evidence that the association of BMD and osteoporosis with AS is likely not causal and that confounding and/or other biases may explain the observational associations. Direct maintenance of bone mineral density and avoidance of osteoporosis is unlikely to prevent aortic stenosis; however, whether local modulation of specific pro-calcific pathways at the valve may be beneficial cannot be excluded.

Chapter V: Summary and Future Work

Aortic stenosis is the most common form of valve disease in the developed world and remains a condition without preventative medical treatment. With the aging of the population, the prevalence of AS is projected to increase by 2-fold by the year 2040, and by 3-fold by 2060[182]. The pathophysiology of calcific aortic valve disease is complex and unique, yet similarities with bone turnover mechanism are apparent. The fact that disorders of bone metabolism associate with AS motivated us to explore whether this association is causal. Our objective was to better understand the nature of this association and provide preliminary evidence for the possibility of effectiveness of BMD maintenance to prevent AS initiation and/or progression. Our work demonstrated while BMD lowering SNPs strongly associate with osteoporosis, they do not associate with AS. As such, our mendelian randomization results suggest that there is no causal association between reduced BMD and AS, and the associations shown in observational studies are likely confounded. Therefore, the clinical implication of our work is the conclusion that BMD maintenance is unlikely to be a successful strategy for AS treatment.

Given the complexity of the genetics of the BMD trait and our inability to appreciate fully the systemic changes that BMD reducing SNPs confer, it is difficult to draw any conclusion about the causal effects of serum calcium or phosphate levels on AS. A recent MR study showed that SNPs that raise serum calcium levels are associated with increased risk of coronary artery disease[183]. Interestingly, the SNPs that are GWAS significant for serum calcium levels are mutually exclusive from those that are GWAS significant for BMD (our 60 BMD SNPs). Therefore, it is important to keep in mind that the scope of our findings is delimited by the biological consequences of BMD SNPs. For example, bone disorders might result in a rise of

serum calcium levels independent of BMD and as such associate with cardiovascular calcification. Therefore, while we rule out the causal role of BMD in the pathophysiology of AS, no conclusions about other modifiable risk factors affected by bone disorders can be made. In other words, bone disorders may causally associate with AS through inflammation, serum biomarker level changes, etc., but not through BMD. In the future, we aim to conduct MR studies for serum calcium SNPs and AS to further our conclusions about the intriguing associations between bone disorders and aortic stenosis.

Chapter VI: Appendix

(continued on next page)

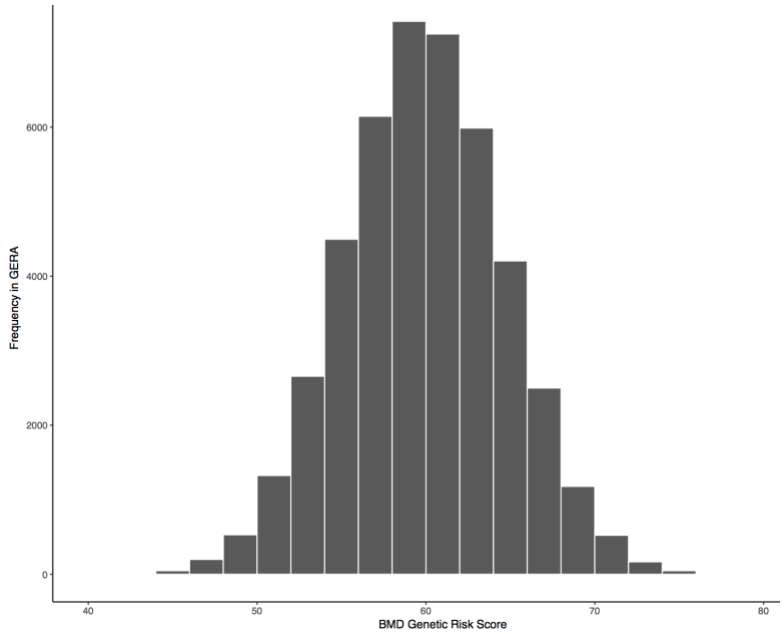
Appendix 1. SNPs associated with BMD at GWAS significance level and their biologic pathway assignment.

						FN-BMD		LS-BMD		
SNP	**Locus**	**Closest Gene**	**A1***	**A2**	**Freq***	**Beta**	**p Value**	**Beta**	**p Value**	**Genetic Risk Score Subtype****
rs10048146	16q24.1	*FOXL1*	a	g	0.8	0.05	1.00×10^{-14}	0.05	3.09×10^{-11}	
rs10226308	7p14.1	*TXNDC3*	a	g	0.84	-0.02	0.02	-0.06	6.40×10^{-13}	
rs1026364	3q13.2	*KIAA2018*	t	g	0.37	0.03	4.08×10^{-10}	0.02	7.57×10^{-04}	
rs10416218	19q13.11	*GPATCH1*	t	c	0.73	-0.03	5.52×10^{-08}	-0.04	6.64×10^{-11}	
rs1053051	12q23.3	*C12orf23*	t	c	0.52	-0.03	9.60×10^{-10}	-0.03	7.90×10^{-08}	
rs10835187	11p14.1_1	*LIN7C*	t	c	0.55	-0.01	0.03	-0.03	4.90×10^{-08}	
rs11623869	14q32.32	*MARK3*	t	g	0.35	-0.04	5.20×10^{-16}	-0.04	5.12×10^{-11}	
rs11755164	6p21.1	*SUPT3H*	t	c	0.4	-0.01	0.05	-0.04	5.60×10^{-11}	Mesenchymal Stem Cell
rs12407028	1p31.3	*WLS*	t	c	0.6	0.05	3.44×10^{-23}	0.08	3.11×10^{-45}	
rs12821008	12q13.12	*DHH*	t	c	0.39	0.03	3.34×10^{-07}	0.05	1.17×10^{-15}	
rs1286083	14q32.12	*RPS6KA5*	t	c	0.81	-0.05	2.02×10^{-15}	-0.05	1.75×10^{-14}	Fracture
rs13204965	6q22.32	*RSPO3*	a	c	0.76	0.04	8.12×10^{-12}	0.04	3.61×10^{-10}	
rs13245690	7q31.31	*C7orf58*	a	g	0.65	0.02	8.20×10^{-04}	0.05	1.65×10^{-11}	
rs13336428	16p13.3_2	*C16orf38*	a	g	0.43	-0.04	1.49×10^{-16}	-0.04	1.66×10^{-13}	
rs1346004	2q24.3	*GALNT3*	a	g	0.5	-0.05	1.08×10^{-25}	-0.06	3.87×10^{-30}	
rs1366594	5q14.3	*MEF2C*	a	c	0.54	0.08	4.47×10^{-61}	0.01	0.01	
rs1373004	10q21.1	*MBL2*	t	g	0.13	-0.04	1.45×10^{-08}	-0.06	1.56×10^{-12}	Fracture
rs1564981	16q12.1	*CYLD*	a	g	0.5	-0.02	4.38×10^{-05}	-0.04	1.95×10^{-10}	
rs1566045	16q12.1	*SALL1*	t	c	0.8	-0.06	1.94×10^{-22}	-0.01	0.04	
rs163879	11p14.1_2	*DCDC5*	t	c	0.68	-0.03	2.06×10^{-08}	-0.04	2.19×10^{-11}	Fracture
rs17040773	2q13	*ANAPC1*	a	c	0.76	0.04	1.51×10^{-09}	0.01	0.19	
rs1864325	17q21.31_2	*MAPT*	t	c	0.22	-0.03	7.47×10^{-05}	-0.04	4.89×10^{-11}	WNT
rs1878526	2q14.2	*INSIG2*	a	g	0.22	0	0.79	0.04	1.22×10^{-10}	
rs2016266	12q13.13	*SP7*	a	g	0.68	-0.03	3.67×10^{-10}	-0.05	2.95×10^{-20}	Mesenchymal Stem Cell & Endochondral Ossification
rs2062377	8q24.12	*TNFRSF11B*	a	t	0.57	-0.06	9.06×10^{-25}	-0.08	3.16×10^{-39}	RANK-RANKL-OPG
rs227584	17q21.31_1	*C17orf53*	a	c	0.7	-0.06	2.56×10^{-24}	-0.04	9.92×10^{-10}	Fracture
rs2887571	12p13.33	*ERC1*	a	g	0.76	-0.03	6.49×10^{-09}	-0.04	5.59×10^{-12}	WNT
rs344081	3q25.31	*LEKR1*	t	c	0.87	0.04	2.22×10^{-06}	0.06	4.46×10^{-12}	
rs3736228	11q13.2	*LRP5*	t	c	0.16	-0.05	4.83×10^{-11}	-0.08	2.08×10^{-26}	Fracture & WNT
rs3755955	4p16.3	*IDUA*	a	g	0.16	-0.06	1.46×10^{-14}	-0.06	5.24×10^{-15}	
rs3790160	20p12.2	*JAG1*	t	c	0.5	0.04	3.61×10^{-12}	0.05	3.07×10^{-19}	
rs3801387	7q31.31	*WNT16*	a	g	0.74	-0.08	5.02×10^{-40}	-0.09	3.17×10^{-51}	Fracture & WNT
rs3905706	10p11.23	*MPP7*	t	c	0.22	-0.01	0.03	0.05	2.41×10^{-16}	
rs4233949	2p16.2	*SPTBN1*	c	g	0.38	0.02	5.91×10^{-06}	0.05	2.25×10^{-18}	Fracture

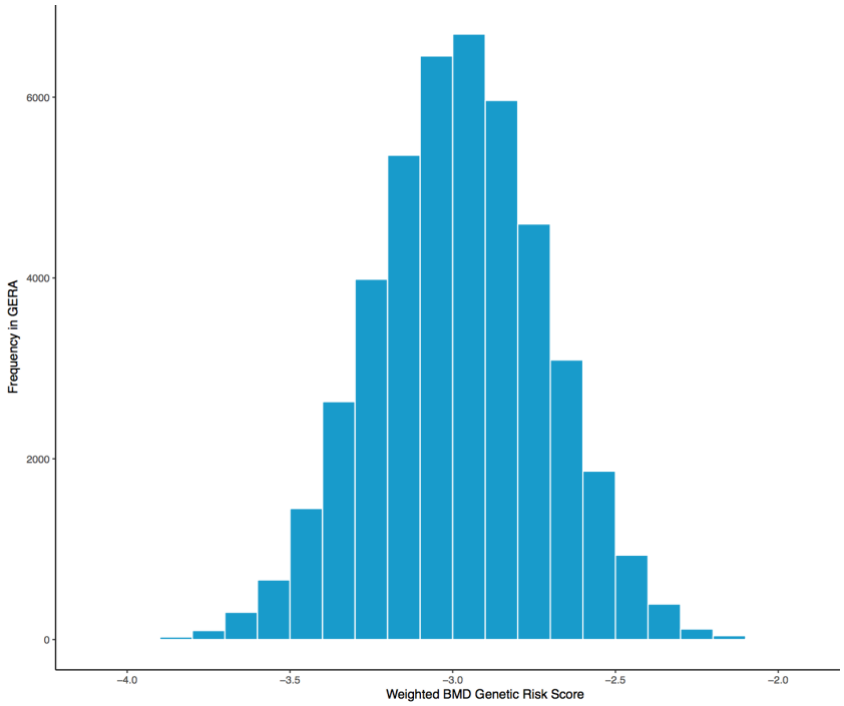
SNP	Locus	Closest Gene	A1*	A2	Freq*	Beta	p Value	Beta	p Value	Genetic Risk Score Subtype**
rs430727	3p22.1	*CTNNB1*	t	c	0.48	-0.06	4.41×10^{-25}	-0.05	1.54×10^{-18}	Fracture & WNT
rs4727338	7q21.3	*SLC25A13*	c	g	0.67	0.08	8.10×10^{-48}	0.07	2.13×10^{-35}	Fracture
rs4790881	17p13.3	*SMG6*	a	c	0.69	0.05	9.75×10^{-19}	0.03	3.38×10^{-09}	
rs4792909	17q21.31_1	*SOST*	t	g	0.37	0.04	1.95×10^{-11}	0.04	9.43×10^{-10}	Fracture
rs479336	1q24.3	*DNM3*	t	g	0.74	-0.04	8.51×10^{-15}	-0.03	2.14×10^{-05}	
rs4796995	18p11.21	*C18orf19*	a	g	0.63	0.03	4.85×10^{-08}	0.02	6.65×10^{-04}	Fracture
rs4869742	6q25.1	*C6orf97*	t	c	0.31	-0.05	4.15×10^{-18}	-0.08	3.95×10^{-35}	
rs4985155	16p13.11	*NTAN1*	a	g	0.67	-0.03	1.74×10^{-10}	-0.03	2.15×10^{-09}	
rs6426749	1p36.12	*ZBTB40*	c	g	0.17	0.11	7.39×10^{-57}	0.1	1.86×10^{-44}	Fracture & WNT
rs6532023	4q22.1	*MEPE*	t	g	0.34	0.06	4.95×10^{-26}	0.06	1.23×10^{-27}	Fracture & Endochondral Ossification
rs6959212	7p14.1	*STARD3NL*	t	c	0.32	-0.04	1.18×10^{-13}	-0.07	3.76×10^{-38}	Fracture & WNT
rs7071206	10q22.3_1	*KCNMA1*	t	c	0.78	0	0.81	-0.06	5.02×10^{-19}	
rs7084921	10q24.2	*CPN1*	t	c	0.39	0.03	9.03×10^{-10}	0.03	9.15×10^{-07}	
rs7108738	11p15.2	*SOX6*	t	g	0.83	-0.08	1.08×10^{-32}	-0.03	2.14 x 10-06	Endochondral Ossification
rs7217932	17q24.3	*SOX9*	a	g	0.46	0.03	1.92×10^{-11}	0.01	0.08	Mesenchymal Stem Cell & Endochondral Ossification
rs736825	12q13.13	*HOXC6*	c	g	0.56	0.04	1.06×10^{-09}	0.05	7.68 x 10-16	
rs7521902	1p36.12	*WNT4*	a	c	0.31	-0.04	2.85×10^{-09}	-0.05	9.66 x 10-11	Fracture
rs7584262	2p21	*PKDCC*	t	c	0.23	0.04	1.27×10^{-09}	0.01	0.07	Endochondral Ossification
rs7812088	7q36.1	*ABCF2*	a	g	0.13	0.05	7.28×10^{-09}	0.04	2.24 x 10-07	
rs7851693	9q34.11	*FUBP3*	c	g	0.64	0.05	3.37×10^{-22}	0.03	6.08 x 10-08	Fracture
rs7932354	11p11.2	*ARHGAP1*	t	c	0.31	0.05	5.12×10^{-18}	0.04	5.45 x 10-12	
rs7953528	12p11.22	*KLHDC5*	a	t	0.18	0.05	1.87×10^{-12}	-0.01	0.13	
rs884205	18q21.33	*TNFRSF11A*	a	c	0.27	-0.04	3.18×10^{-10}	-0.05	1.58 x 10-17	RANK-RANKL-OPG
rs9466056	6p22.3	*CDKAL1*	a	g	0.38	-0.04	2.73×10^{-13}	-0.03	3.56 x 10-08	
rs9533090	13q14.11	*AKAP11*	t	c	0.49	-0.05	4.94×10^{-23}	-0.1	4.82 x 10-68	RANK-RANKL-OPG
rs9921222	16p13.3_1	*AXIN1*	t	c	0.48	-0.04	5.18×10^{-12}	-0.04	1.00 x 10-16	WNT

*Beta estimates (effect of each copy of the SNP allele on standardized BMD) and frequencies are reported for the A1 allele.
**All 60 SNPs were included in the overall BMD genetic risk score. The SNPs contributing to pathway specific genetic risk scores are indicated.
FN-BMD is BMD at the femoral neck and LS-BMD is BMD at lumbar spine.
Beta and p Values are from the meta-analysis results for loci associated with BMD at GWS level (FN-BMD n = up to 83,894 and LS-BMD n = up to 77,508)[165].
In summary level data analysis, the beta for the BMD with the lower p value was used.

Appendix 2. Distribution of BMD GRS in GERA (N = 44,703, Mean = 59.89, Median = 59.87 SD = ± 4.73, Min = 41.08, Max = 80.81)



Appendix 3. Distribution of weighted BMD GRS in GERA (N = 44,703, Mean = -2.98, Median = -2.98, SD = ± 0.26, Min = -4.05, Max = -1.76)



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