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## Major facilitator superfamily domain containing 5 inhibition reduces lipoprotein(a) uptake and calcification in valvular heart disease

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### Abstract

**BACKGROUND:** High circulating levels of lipoprotein(a) [Lp(a)], increase the risk of atherosclerosis and calcific aortic valve disease, affecting millions of patients worldwide. While

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#### SUPPLEMENTAL MATERIAL

Detailed methods

Figures S1–S7

Tables S1, S2, and Major Resources Table

Excel File S1

Uncropped Gel Blots

atherosclerosis is commonly treated with low-density lipoprotein-targeting therapies these do not reduce Lp(a) or calcific aortic valve disease risk, a disease with no available drug therapies. Targeting Lp(a) production and catabolism may provide therapeutic benefit, but little is known about Lp(a) cellular uptake.

**METHODS:** Here, unbiased ligand-receptor capture mass spectrometry was used to identify major facilitator superfamily domain containing 5 (MFSD5) as a novel receptor/co-factor involved in Lp(a) uptake.

**RESULTS:** Reducing *MFSD5* expression by a computationally-identified small molecule or siRNA suppressed Lp(a) uptake and calcification in primary human valvular endothelial and interstitial cells. *MFSD5* variants were associated with aortic stenosis ( $p=0.027$  after multiple hypothesis testing) with suggestive evidence of an interaction with plasma Lp(a) levels.

**CONCLUSIONS:** *MFSD5* knockdown suppressing human valvular cell Lp(a) uptake and calcification along with meta-analysis of *MFSD5* variants associating with aortic stenosis supports further preclinical assessment of *MFSD5* in cardiovascular diseases, the leading cause of death worldwide.

### Keywords

aortic valve stenosis; lipoprotein(a); MFSD5; lipoprotein receptors; cardiovascular calcification

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Calcific aortic valve disease (CAVD) is the most common valve disease, with aortic sclerosis affecting 25% and aortic stenosis affecting 2% of individuals over age 65.<sup>1</sup> Currently, the only treatment for symptomatic CAVD is invasive surgical or transcatheter valve replacements, which present some risk to patients and are not universally available. With no approved pharmaceutical therapies for CAVD, including statins that failed to provide benefit in clinical CAVD trials,<sup>2</sup> there is urgent need for therapeutics which are targeted for early intervention against both the initiation and progression of CAVD.

A major risk factor of CAVD and atherosclerosis is lipoprotein(a) [Lp(a)], a low-density lipoprotein (LDL)-like molecule. Current estimates predict over 1.4 billion people worldwide have high Lp(a);<sup>3</sup> however, there are no United States Food and Drug Administration-approved Lp(a)-lowering small-molecule therapies. Lp(a) abundance is controlled by genetics,<sup>4-6</sup> is mostly independent of diet, and is not consistently reduced by LDL lowering drugs, such as statins. Like LDL, Lp(a) contains apolipoprotein B (ApoB), but differs from LDL in that it also contains Apo(a). Lp(a) also contains other pro-atherogenic components, including oxidized phospholipids and apolipoprotein C III.<sup>7</sup> Apolipoprotein C III on Lp(a) associates with Lp(a)-mediated valve disease and may promote human valvular interstitial cell (VIC) calcification through mitochondrial dysfunction, oxidative stress, and inflammatory pathways.<sup>8,9</sup> While associated with increased cardiovascular disease risk,<sup>10,11</sup> the normal physiological functions of Lp(a) are unclear.<sup>12</sup> Complicating investigative efforts, Lp(a) is not endogenously produced in most experimental animal models, with its expression largely restricted to humans and other primates. Lp(a) can be produced in transgenic mice,<sup>13</sup> which have increased aorta lipid lesions, but there remains a paucity of animal models of Lp(a)-mediated CAVD pathology in

the absence of other genetic modifications, necessitating the use of human studies to better understand Lp(a) biology.

Overall, there are insufficient means to reduce circulating Lp(a) amounts to lower cardiovascular disease risk.<sup>3</sup> Strategies to limit Lp(a) effects include directly lowering Lp(a) levels and targeting Lp(a) uptake. Directly lowering Lp(a) levels can be achieved by lipoprotein apheresis, but this procedure is costly, semi-invasive, and severely limits patient mobility, which leads to high patient refusal and low adherence rates.<sup>14</sup> Proprotein convertase subtilisin/kexin type 9 inhibitors can reduce circulating Lp(a) abundance by 26.9%,<sup>15</sup> but whether further reduction of circulating Lp(a) provides improvements in cardiovascular disease outcomes is unknown. Clinical trials demonstrated that concentrations of circulating Lp(a) can be consistently and potently reduced by Apo(a) antisense therapy, reducing Lp(a) abundance below the critical 50 mg/dL threshold in patients with established cardiovascular disease.<sup>16</sup> Apo(a) antisense reduces circulating oxidized phospholipids in cardiovascular disease patients,<sup>16</sup> but if antisense also affects other cardiovascular disease-promoting molecules found on Lp(a) particles is unclear. Whether Apo(a) antisense and other *LPA* RNA-targeting therapies improve patient cardiovascular outcomes beyond the extent of existing cardiovascular drugs is currently being investigated in clinical trials.<sup>17</sup>

Inhibiting Lp(a) uptake specifically in cardiovascular tissue where it acts, may be a clinically beneficial approach. Several membrane proteins interact with Lp(a), and have been suggested to be putative Lp(a) receptors.<sup>18</sup> It is possible that several receptors mediate Lp(a) clearance in the liver, the major route of Lp(a) catabolism, in addition to other tissue-specific mechanisms of Lp(a) catabolism. There are conflicting reports on Lp(a) uptake by many of these putative Lp(a) receptors, including LDL receptor (LDLR);<sup>19</sup> as such, identification of definitive Lp(a) receptor(s) remains obscure in the liver and unknown in the aortic valve. In the aortic valve, Lp(a) has been observed in the extracellular matrix, and oxidized phospholipids on Lp(a), including lysophosphatidic acid, are metabolised and taken up by VICs, inducing calcification.<sup>7</sup> Whereas Apo(a)-antisense may work to reduce production of Lp(a) through limiting Apo(a), combinatory therapies inhibiting Lp(a) production and receptor-mediated uptake in cardiovascular cells may further improve cardiovascular outcomes. As an example, combination therapy with ezetimibe, an inhibitor of the cholesterol absorption protein, Niemann-Pick C1 Like 1 protein with simvastatin that inhibits cholesterol synthesis, improves cardiovascular outcomes compared to statin therapy alone.<sup>20</sup> Identifying receptors involved in Lp(a) catabolism would be advantageous, particularly in the context of CAVD, a disease with unmet therapeutic need. Therefore, we hypothesized that through an unbiased ligand-receptor capture mass spectrometry approach, Lp(a) receptor(s) controlling cellular Lp(a) uptake can be identified on VICs, a major cell type contributing to aortic valve calcification.<sup>7,21</sup>

## METHODS

Detailed methods and Major Resources Table provided in the Supplemental Material.

## Cell Culture

Human aortic valve tissue was obtained with informed consent in accordance with the deceleration of Helsinki. Human aortic valve samples were handled under institutional review board protocol 2011P001703, approved by the Beth Israel Deaconess Medical Center and Partners Human Research Committee institutional review boards, and included written consent from all donors. Researchers were blinded to all clinical characteristics and patient data of the valve tissues used. Primary human VICs were obtained from human aortic valve tissues by collagenase digestion. Following removal of valvular endothelial cells (VECs) by scraping with a razor blade, valves were divided into small cubes (1–2mm) and digested in 1 mg/mL collagenase (Sigma Aldrich, St. Louis, MO) in Dulbecco's Minimum Essential Medium (DMEM, Thermo Fisher Scientific) for 1 hour with agitation. Following removal of the 1-hour digest, valve pieces were redigested with 1 mg/mL collagenase for 3 hours. Resultant supernatant was then centrifuged (400g, 5 minutes, room temperature) and the VIC pellet resuspended in DMEM supplemented with 10% fetal bovine serum (unless otherwise stated, 1% penicillin/streptomycin (P/S), cultured (37°C 5% CO<sub>2</sub>, 90% humidity) and used between passages 1 and 5. VIC purity was validated by immunofluorescence staining for vimentin<sup>+</sup> (abcam #ab92547) and negative for CD31<sup>-</sup> (abcam #ab9498), which was further verified by proteomics analysis. VECs (Factor VIII<sup>+</sup>, alpha-SMA<sup>-</sup>) were obtained from Lonza and cultured in the same manner as VICs. HepG2 cells were obtained from ATCC (Manassas, VA) and cultured in Eagle's Minimum Essential Medium (Thermo Fisher Scientific) with 10% FBS (unless otherwise noted) and 1% P/S.

## Lipoprotein(a) Uptake Assay

Uptake assays were performed as follows: briefly, VICs, VECs, and HepG2 cells were cultured in lipoprotein-deficient serum for 48 hours before incubation with Dil-labeled LDL (Thermo Fisher Scientific) or Lp(a) for 6 hours. To confirm removal of Lp(a) not taken up by cells, cells were stringently washed at room temperature (RT) as follows: three times in phosphate buffered saline (PBS) with 0.8% bovine serum albumin (BSA), twice in PBS with 0.8% BSA and 0.2 M ε-ACA for five minutes each, twice with 0.2 M acetic acid (pH 2.5) with 0.2 M NaCl for ten minutes each, and finally twice in PBS. Cells were stained with DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific), 488-conjugated wheat germ agglutinin (WGA) (Thermo Fisher Scientific) for the nuclei and membrane respectively, and imaged on a Nikon Confocal A1 microscope (Nikon, Tokyo, Japan). Fluorescence was quantified with an ImageXpress Pico digital fluorescence system (Molecular Devices, San Jose, CA), and assessed for percentage positive (Dil-Lp[a]/DAPI). Alternatively fluorescence intensity was quantified using NIH ImageJ software from the Nikon Confocal A1 microscope images.

## Calcification Assay

To induce calcification, 100% confluent VICs were cultured in DMEM for up to 21 days, in either normal medium (NM; DMEM supplemented with 10% FBS and 1% PS) or in pro-calcifying medium (PM; NM with 10 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 50 μg/mL L-ascorbic acid). 10 μg/mL Lp(a) was included in PM when noted. VICs were stained with 2% Alizarin red (Lifeline Cell Technology, Frederick,

MD) at day 14 and 21 of culture and quantified by extracting the stain with 100 mmol/L cetylpyridinium chloride (Thermo Fisher Scientific) at room temperature for 1 hour, and the absorbance was measured at 540 nm.

### TriCEPS Mass Spectrometry

TriCEPS reagents were commercially obtained as Flow-TriCEPS and ligand-receptor capture-TriCEPS kits (Dual Systems, Zurich, Switzerland). Dot blots to validate TriCEPS reagent coupling to Lp(a) (20 µg) were performed according to the manufacturer's instructions and blotted with three serial dilutions (1:10, 1:100, 1:1000). Transferrin was used as an active comparator and glycine as a negative control that quenches the amine-reactive moieties of TriCEPS. Fluorescence-activated cell sorting (FACS) of VICs incubated without Lp(a), with fluorescently labeled TriCEPS coupled to Lp(a), or fluorescently labeled TriCEPS coupled to Lp(a) that was quenched with glycine was assessed using an FACS Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ; Harvard Immunology FACS core facility) and commercially obtained Flow-TriCEPS reagents according to manufacturer's instructions. For TriCEPS experiments, six equally pooled VIC donors were used with a commercially obtained TriCEPS kit. VICs were plated at 100% confluency and  $2 \times 10^7$  cells per sample were used. For ligand-receptor capture TriCEPS, the TriCEPS reagent was coupled to Lp(a) (300 µg ligand coupled to 150 µg TriCEPs) according to manufacturer's instructions and incubated with VICs (4°C, 1 hour) to enable Lp(a) and receptor binding but not Lp(a) internalization. VICs were oxidized according to the TriCEPS kit instructions, allowing for crosslinking of TriCEPS-coupled Lp(a) to membrane proteins that were affinity purified and analyzed by LC-MS/MS.

### MFSD5 siRNA

Knockdown studies in VICs and HepG2 cells were performed with siRNA targeting *MFSD5* (20 nM, Assay IDs s39790, s224982 Thermo Fisher and Dharmacon Smartpool *MFSD5* siRNA), *LDLR* (20 nM, Assay ID S4, Thermo Fisher) and non-targeting control siRNA (20 nM, Assay ID#4390843, Thermo Fisher) with Lipofectamine RNAi MAX transfection reagent (Thermo Fisher Scientific) according to manufacturer's protocol. For VECs, 40 nM siRNAs were used. Three validated *MFSD5* siRNAs were tested, and similar results were observed for all three with knockdown efficiency.

### Lp(a) Interaction Analysis

The UK Biobank is a prospective cohort comprised of > 500,000 participants in the United Kingdom with broad phenotyping electronic health records, death registries, blood-based analyses, genotyping, and other methodologies.<sup>22</sup> Genotyping had been performed using the Applied Biosystems UK Biobank Axiom Array and Applied Biosystems UK BiLEVE Axiom Array, followed by imputation using the 1000 Genomes phase 3, Haplotype Reference Consortium, and UK10K reference panels.<sup>23</sup> Serum Lp(a) had been measured using an immuno-turbidimetric assay manufactured by Randox Bioscience, UK. Measures were set below or above the reportable range defined by the manufacturer (3.8 to 189 nmol/L) to 3.8 nmol/L or 189 nmol/L, respectively. The analysis included unrelated participants aged 55 years or older at baseline with genetically-confirmed White British ancestry. Individuals with congenital valvular heart disease in their hospital inpatient records

(*International Classification of Diseases, Ninth Revision [ICD-9]* 746–747 or *International Classification of Diseases, Tenth Revision [ICD-10]* Q20-Q23) were excluded. Among the remaining 247,417 participants, aortic stenosis cases were identified through the presence of *ICD-9* 424.1, *ICD-10* I35.0, or *OPCS Classification of Surgical Operations and Procedures, Fourth Revision* codes K26.1, K26.2, K26.3, K26.4, K31.2, K32.2, or K35.2 in the hospital inpatient records, with all other participants deemed to be controls. Associations with aortic stenosis were estimated in logistic regression models using PLINK version 2,<sup>24</sup> adjusted for age, age squared, sex, genotype batch, and 20 principal components.

The Genetic Epidemiology Research on Adult Health and Aging cohort consists of > 100,000 individuals in northern California who are in the Kaiser Permanente healthcare delivery system.<sup>25</sup> Genotyping had been performed using customized Affymetrix Axiom arrays,<sup>26</sup> followed by imputation using the Michigan Imputation Server<sup>27</sup> with the Haplotype Reference Consortium version r1.1 as the reference panel.<sup>28</sup> Analysis included unrelated European ancestry participants aged 55 years or older. Using electronic health record data from January 1996 to December 2015, inclusively, individuals with congenital valvular heart disease were excluded (*ICD-9* 746–747) and identified aortic stenosis cases in the remaining 55,180 participants through the presence of *ICD-9* 424.1 or a procedure code for aortic valve replacement. All other participants were defined as controls. Associations with aortic stenosis were estimated in logistic regression models using PLINK version,<sup>24</sup> adjusted for age, age squared, sex, and 10 principal components.

Firstly, analysis with a fixed effect model, inverse-variance weighted meta-analysis for aortic stenosis of the 291 variants within 50 kb of *MFSD5* which had a minor allele frequency 0.001 and an imputation quality 0.3 in both cohorts was performed (consisted of 87 independent variants). For the independent ( $r^2 < 0.1$ ) variants with  $p < 0.01$  in the meta-analysis (5 variants), the (i) direct effect on the natural logarithm of Lp(a) and (ii) the interaction with the natural logarithm of Lp(a) for the outcome of aortic stenosis were examined. For a variant which a significant interaction ( $p < 0.05$ ), the prevalence of aortic stenosis stratified by copies of the minor allele and tertiles of Lp(a) was examined and performed logistic regression modelling the association of combinations of minor allele copies and Lp(a) tertiles with aortic stenosis. For all analyses, we adjusted p-values based on the number of independent variants tested by Bonferroni correction.

### Data Availability

Data generated or analysed during the current study are included in the article and its Supplemental Data. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium by the PRIDE,<sup>29</sup> partner repository with the dataset identifier PXD026861. Username: reviewer\_pxd026861@ebi.ac.uk; password: uadLa8rZ.

### Statistical Analysis

Analysis of variance (ANOVA) with Tukey's multiple comparison testing and Welch's t tests were performed with Prism 9 (GraphPad, La Jolla, CA). Data were analyzed for normality and equal variance to determine whether applied parametric tests were appropriate.

## RESULTS

### Ligand-receptor Capture Mass Spectrometry Identified a Previously Uncharacterized Lp(a) Receptor/Co-Factor on Human VICs

TriCEPS ligand-receptor capture mass spectrometry<sup>30</sup> was used to identify membrane proteins that bind Lp(a) and mediate its uptake in human valves. For this experiment we used VICs isolated from human valves, validated by cells staining positive for an interstitial cell marker, vimentin<sup>+</sup> and negative for an endothelial cell marker, CD31<sup>-</sup> (Figure S1A and S1B). We confirmed the presence and absence of these markers and further characterized these VICs using cell-fractionated proteomics for membrane, cytosolic, and nuclear proteins (Figure S2 and Excel File S1). TriCEPS reagent specifically couples to the N-terminus and lysines of ligands, with the whole complex then binding to plasma membrane receptors. Oxidization of cells allows for the TriCEPS reagent to crosslink and then be affinity purified, with the bound membrane proteins identified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (Figure 1A). Dot blot analysis confirmed that Lp(a) could be coupled to the TriCEPS reagent using TriCEPS-transferrin plasma membrane receptor coupling as an active comparator and glycine that quenches the amine-reactive moieties of the TriCEPS reagent as a negative control (Figure S3A). Lp(a) was readily coupled to TriCEPS in a similar manner to that of transferrin supporting that the TriCEPS reagent is compatible with Lp(a) ligand (Figure S3A). This experimental process did not alter VIC viability, assessed by a live/dead cell assay (Figure S3B). Supporting that TriCEPS-coupled Lp(a) binds to VICs, TriCEPS-labeled Lp(a) increased VIC fluorescence that was partially suppressed by glycine quenching, as assessed by FACS (Figure S3C).

To identify which protein(s) TriCEPS-coupled Lp(a) bound to on VICs, ligand-receptor capture mass spectrometry was performed with Lp(a)-coupled TriCEPS and transferrin-coupled TriCEPS as a positive control. VICs took up Lp(a) in a time-dependent manner (Figure S3D), and incubation at 4°C inhibited internalization of Lp(a), enabling surface Lp(a) receptor identification using LC-MS/MS. Validating the experimental protocol, transferrin incubation lead to enrichment of the transferrin receptor (Figure 1B). Additional membrane proteins enriched in transferrin-incubated VICs included LDLR and mannose receptor C type 2 (Figure 1B). Rapid endocytosis in Lp(a) incubated cells could result in an apparent enrichment of Lp(a) receptors in the transferrin-incubated VIC group compared to the Lp(a)-incubated group, due to receptor internalization upon Lp(a) binding. However, since the TriCEPS incubation was carried out at 4°C, a condition that inhibits endocytosis therefore making this scenario less likely, we focused on receptors enriched with Lp(a) incubation in VICs. Most of the proteins enriched in the Lp(a)-incubated VICs were as expected, known components of Lp(a) particles,<sup>8</sup> including Apo(a) and ApoB (Figure 1C). Given the expectation of an Lp(a) receptor being plasma membrane-localized enabling it to bind circulating Lp(a), we looked for enrichment of membrane-bound proteins. There was only one plasma membrane protein that was enriched in TriCEPS-coupled Lp(a)-incubated VICs, a ubiquitously expressed protein, major facilitator superfamily domain containing 5 (MFSD5) (Figure 1C). Additional Lp(a) receptors not identified by the TriCEPS capture method cannot be excluded from participating in Lp(a) uptake; however, identification of MFSD5 as the only markedly enriched receptor by TriCEPS-coupled Lp(a)

immunopurification in VICs led us to focus on assessment of MFSD5 as a putative Lp(a) receptor.

### Reducing *MFSD5* Expression Inhibited Lp(a) Uptake

To determine whether MFSD5 participated in Lp(a) uptake in human cells, the effects of validated *MFSD5* specific siRNA (confirmed by three separate siRNAs) on Lp(a) uptake in VICs was assessed. Purified Lp(a) was used throughout these experiments that was validated by apolipoprotein content and gel electrophoresis (Figure S4A and S4B). Lp(a) purity was additionally validated by Apo(a) immunogold electron microscopy, which showed that all lipoprotein particles were positively labeled with Apo(a), as well as demonstrating Lp(a) uptake by VICs (Figure S4C). Significant cellular uptake of Lp(a) was achieved at 10 ug/mL in human liver HepG2 cells (Figure S4D), a concentration previously associated with VIC calcification,<sup>21</sup> and was therefore used in our experiments. We also conducted a competition assay of Dil-labeled LDL and unlabeled Lp(a) (in excess; 10ug/mL) in HepG2 cells. With Lp(a) in excess, LDL was not taken up as readily in HepG2 cells as when incubated with Dil-LDL alone (Figure S4E). *MFSD5* siRNA incubation with VICs was validated by quantitative PCR (Figure 2A) and reduced Lp(a) uptake assessed by immunofluorescence and Western blotting (Figure 2B; Figure S5). Given the similarity of Lp(a) to LDL, the ability of *MFSD5* siRNA to alter Dil-LDL uptake by VICs was also assessed. Reduction of *LDLR* by siRNA did not reduce VIC *MFSD5* mRNA (Figure 2A) or Lp(a) uptake in VICs (Figure 2B; Figure S5). As a control for *LDLR* knockdown, *LDLR* siRNA but not *MFSD5* siRNA reduced *LDLR* mRNA (Figure 2C) and Dil-LDL uptake in VICs (Figure 2D). Similarly, *MFSD5* siRNA reduced *MFSD5* mRNA (Figure 3A) and Lp(a) uptake (Figure 3B) in primary human VECs. *LDLR* siRNA but not *MFSD5* siRNA reduced VEC *LDLR* mRNA (Figure 3C) and Dil-LDL uptake (Figure 3D). These data support MFSD5 as a previously uncharacterized Lp(a) receptor or co-factor mediating Lp(a) uptake in human valvular cells.

As Lp(a) catabolism occurs primarily in the liver and *MFSD5* has ubiquitous expression, the functional role of MFSD5 in human liver HepG2 cells was also assessed. Reduced *MFSD5* with *MFSD5-specific* siRNA incubation was validated by quantitative PCR analysis in HepG2 cells (Figure S6A). *MFSD5* siRNA reduced Lp(a) uptake in HepG2 cells (Figure S6B). No change in *MFSD5* levels were observed with *LDLR* specific siRNA in HepG2 cells (Figure S6A). Suppression of HepG2 cells *LDLR* mRNA (Figure S6C) by *LDLR* siRNA did not reduce Lp(a) uptake (Figure S6B), but did reduce Dil-LDL uptake (Figure S6D) in our experimental conditions. Together these data demonstrate that MFSD5 is unlikely to interact with ApoB protein present on both Lp(a) and LDL.

Next, small molecule inhibition of MFSD5 Lp(a) uptake was conducted through the L1000 platform,<sup>31,32</sup> in which genes and drugs are connected by common gene-expression signatures enabling prediction of compounds that modulate genes of interest. L1000 analysis supported aminopurvalanol A, a cyclin-dependent kinase inhibitor, as a top, commercially available compound that could modulate *MFSD5* expression. Quantitative PCR demonstrated that aminopurvalanol A could suppress *MFSD5* mRNA levels in VICs (Figure S7A). VICs incubated with aminopurvalanol A were then incubated with Lp(a), to



confirm a reduction in uptake. In agreement with the *MFSD5* siRNA data, Lp(a) uptake was reduced by aminopurvalanol A incubation (Figure S7B). These data provide an initial in vitro proof-of-concept that *MFSD5* and associated Lp(a) uptake can be regulated by small molecules, in addition to providing an independent validation of our *MFSD5* siRNA data.

### Reducing *MFSD5* Expression Suppressed VIC Calcification

To assess the potential of reducing VIC Lp(a) uptake in the etiology of CAVD through reduced *MFSD5*, we first used siRNA to suppress *MFSD5* expression. The presence of *MFSD5* in human aortic valve tissues from which primary VICs were obtained was shown with RNAscope (Figure 4A). After isolation, purified VICs were pre-incubated with *MFSD5* or control siRNA and then incubated in pro-calcifying media (PM) containing inorganic phosphate and ascorbic acid, with or without Lp(a). Lp(a) incubation led to earlier calcification compared to that induced by PM alone (Figure 4B). In agreement with a role of *MFSD5* in mediating Lp(a) uptake that promotes calcification, *MFSD5* siRNA suppressed calcification in VICs, assessed by Alizarin red staining (Figure 4C). Similarly, aminopurvalanol A incubation inhibited primary human VIC calcification by Lp(a) (Figure S7C).

### *MFSD5* Variants Associated with Aortic Stenosis and may Modify Lp(a) Mediated Risk in Human Aortic Stenosis

To further evaluate the contribution of *MFSD5* to Lp(a)-related phenotypes in humans, the role of *MFSD5*-region variants on Lp(a) driven CAVD was assessed. Five independent ( $r^2 < 0.1$ ) variants (Table S1) were selected within 50 kb of *MFSD5* associated with aortic stenosis at  $p < 0.01$  in an inverse-variance weighted, fixed effects meta-analysis of 247,417 UK Biobank White British participants (3,443 cases) and 55,180 Genetic Epidemiology Research on Adult Health and Aging European-ancestry participants (3,469 cases) for 291 variants. One variant, rs61760168, was found to be significantly associated with AS in meta-analysis after adjustment for multiple hypothesis testing (OR 2.03, 95% CI 1.38,2.97;  $p = 3 \times 10^{-4}$ ,  $p_{\text{adj}} = 0.027$  for multiple comparisons). No significant direct effect of *MFSD5* variants on Lp(a) levels in the UK Biobank was found (Table S1). We observed a differential effect of Lp(a) on aortic stenosis by the *MFSD5* rs73101935 genotype (interaction  $p = 0.031$ ;  $p_{\text{adj}} = 0.155$  for multiple comparisons, Figure 5). Among individuals in the highest tertile of Lp(a), the prevalence of aortic stenosis was 1.8% in individuals with no copies of rs73101935-T, compared to 4.7% in individuals with one copy (Figure 5). In regression analysis, compared to individuals in the lowest tertile of Lp(a) and with zero copies of rs73101935-T, the odds of aortic stenosis were 54% higher for individuals in the highest tertile of Lp(a) but with zero copies of rs73101935-T (odds ratio, 1.54; 95% CI, 1.41 to 1.67;  $p = 2.0 \times 10^{-23}$ ), and 341% higher for individuals in the highest Lp(a) tertile with one copy of rs73101935-T (odds ratio, 4.41; 95% CI, 2.64 to 7.35;  $p = 1.3 \times 10^{-8}$ ) (Table S2).

## DISCUSSION

The current study demonstrates a role of *MFSD5* as a new receptor/co-factor involved in Lp(a) uptake and aortic stenosis. Moreover, the data reveals *MFSD5* binding of Lp(a) through a crosslinking affinity purification assay. *MFSD5* contribution to Lp(a) uptake and

VIC calcification was further demonstrated by reducing *MFSD5* expression. An *MFSD5* variant, which enhanced the effect of Lp(a) on aortic stenosis greater than 4-fold was identified in the UK Biobank genetic dataset that further supports a role for *MFSD5* in Lp(a)-mediated cardiovascular disease. While we observed a reduction of Lp(a) uptake in HepG2 cells with *MFSD5* siRNA, albeit less than detected in valvular cells, we did not find a direct effect on Lp(a) plasma concentrations in the UK Biobank dataset. Lp(a) production is largely impacted by the *LPA* locus and a lack of direct effect on Lp(a) levels by variants in *MFSD5* does not preclude a proposed role of *MFSD5* as an Lp(a) receptor or co-factor.

Despite the clinical importance of Lp(a), the cellular and biochemical pathways involved in Lp(a) catabolism remain unclear. Reducing Lp(a) mediated pathology might be achieved by altering its uptake, like statins in reducing the effects of LDL in part via increasing LDL receptor abundance. However, unlike LDL, the receptors involved in Lp(a) catabolism remain unclear. Several types of receptors have been suggested to be involved in Lp(a) uptake including lipoprotein receptors, scavenger receptors, toll-like receptors, lectins, and plasminogen receptors, and Lp(a) uptake may involve multiple receptors.<sup>18</sup> In our experimental conditions, *LDLR* specific siRNA did not reduce Lp(a) uptake in human valvular or HepG2 cells; however, we cannot exclude a role of *LDLR* in Lp(a) catabolism in other conditions. Lp(a) contains Apo(a) that may impair the ability of *LDLR* to bind ApoB on Lp(a) particles, resulting in slower Lp(a) uptake than LDL, which was reduced by *LDLR* specific siRNA in human valvular and HepG2 cells. Whether *MFSD5* interacts with oxidized lipids is unknown. *MFSD5* and lysophosphatidic acid receptor, a receptor associated with aortic stenosis mediated by oxidative transformation of low-density lipoprotein,<sup>33</sup> was suggested as one of 56,000 candidate interactions identified by an affinity purification-mass spectrometry approach.<sup>34</sup> As such, if *MFSD5* acts as a co-factor with other receptors associated with Lp(a) and oxidized lipids could be further explored, but is beyond the scope of the present study.

*MFSD5* is a largely unstudied protein and the physiologic function of *MFSD5*, a plasma membrane protein, is unknown, but it may act in transport and metabolic functions. To our knowledge, this is the first report of *MFSD5* function using human cells and tissues. *MFSD5* overexpression experiments in yeast cells suggest that it is a solute transporter that transfers molybdate anions.<sup>35</sup> *MFSD5* interacts with overexpressed glucagon-like peptide-1 receptor in Chinese hamster ovary cells,<sup>36</sup> a receptor involved in controlling blood glucose levels through increasing insulin secretion. *MFSD5* is a member of the mammalian solute carrier family, a group with at least 395 members in humans and which translocate a broad range of substrates across membranes.<sup>37</sup> Given that *MFSD5* is evolutionarily conserved, *MFSD5* likely has additional functions unrelated to Lp(a). Whereas rodents do not endogenously produce Lp(a), *Mfsd5* mRNA is reduced by either starvation or high-fat diet in mice, suggesting a possible additional function in energy regulation.<sup>37</sup> *MFSD5* involvement in metabolism may be related to cardiovascular disease pathology. We demonstrated inhibition of *MFSD5* expression suppressed Lp(a) uptake in VICs and VECs. Lp(a) enhances glycolysis that induces endothelial cell inflammation associated with transendothelial migration of leukocytes in the arterial wall.<sup>38</sup> In addition to regulating recruitment of immune cells and expression of pro-calcific proteins, VECs can undergo

an endothelial-to-mesenchymal transformation into osteogenic cells,<sup>39</sup> and high glucose stimulates endothelial-to-mesenchymal transformation of VECs.<sup>40</sup>

Our current TriCEPS ligand-receptor capture affinity purification results support MFSD5 binding to Lp(a) and implicate a functional role in a lipid metabolism. It remains unclear what portion of Lp(a) that MFSD5 interacts with, but it is unlikely to be ApoB given that *MFSD5* siRNA did not reduce LDL uptake in vitro. How increased Lp(a) uptake promotes VIC calcification is an active area of research. We and others have shown the oxidized phospholipid component of Lp(a) increases inflammatory and osteogenic gene transcription in VICs.<sup>7,21</sup> Similar VIC phenotype changes were also observed with a proinflammatory component of Lp(a), apolipoprotein C III.<sup>9</sup> Additionally, Lp(a) promotes the release of calcifying extracellular vesicles in human VICs.<sup>41</sup> While these inflammatory and osteogenic changes would be expected to be suppressed by reduced *MFSD5* expression, as this condition reduced Lp(a) uptake and calcification, the key molecular pathways through which MFSD5 regulates calcification with high Lp(a) requires further study. Given the association we observed with the *MFSD5* variant rs73101935 and increased odds of aortic stenosis in patients with high Lp(a), understanding the molecular changes associated with this variant may help elucidate mechanisms through which high Lp(a) increases aortic stenosis risk.

A limit of our study is a need for further demonstration of direct MFSD5 and Lp(a) binding. Our TriCEPS ligand-receptor capture experimental approach suggests an interaction of MFSD5 with Lp(a) but does not exclude other nearby proteins directly binding to Lp(a). A more direct biochemical means could be explored in future studies to show specific binding of Lp(a) and MFSD5 that could mechanistically explain why Lp(a) uptake was suppressed in vitro when we inhibited MFSD5 expression. In human VICs, a computationally identified *MFSD5* suppressing small molecule, aminopurvalanol A, inhibited calcification, supporting our siRNA data and the potential druggability of MFSD5. However, aminopurvalanol A is not a specific MFSD5 inhibitor, and we cannot exclude other mechanisms contributing to its anti-calcification and Lp(a) uptake suppression effects. Another limitation of the present study is the lack of an in vivo animal model supporting our in vitro and human genetics data. While not trivial, development of in vivo MFSD5-deficient models with high Lp(a)-driven CAVD pathology could provide a means to gain further mechanistic insight into the role of MFSD5 in this disease.

In conclusion, this study identifies a previously unknown biological function of MFSD5 in lipid metabolism and provides initial proof-of-concept and support for further preclinical assessment of MFSD5 in cardiovascular disease, including through perturbation of Lp(a) metabolism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Disclosures**

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**Non-standard Abbreviations and Acronyms:**

<b>DAPI</b>	4',6-diamidino-2-phenylindole
<b>Apo(a)</b>	apolipoprotein(a)
<b>ApoB</b>	apolipoprotein B
<b>CAVD</b>	calcific aortic valve disease
<b>DMEM</b>	Dulbecco's Minimum Essential Medium
<b>FACS</b>	fluorescence-activated cell sorting
<b>Lp(a)</b>	lipoprotein(a)
<b>LC-MS/MS</b>	liquid chromatography-mass spectrometry/mass spectrometry
<b>LDL</b>	low-density lipoprotein
<b>LDLR</b>	low-density lipoprotein receptor
<b>MFSD5</b>	major facilitator superfamily domain containing 5
<b>NM</b>	normal media
<b>P/S</b>	penicillin/streptomycin
<b>PheWAS</b>	phenome-wide association studies
<b>PBS</b>	phosphate buffered saline
<b>PM</b>	pro-calcifying media
<b>VIC</b>	valvular intersitial cell
<b>VEC</b>	valvular endothelial cell
<b>WGA</b>	wheat germ agglutinin

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### Clinical Perspective

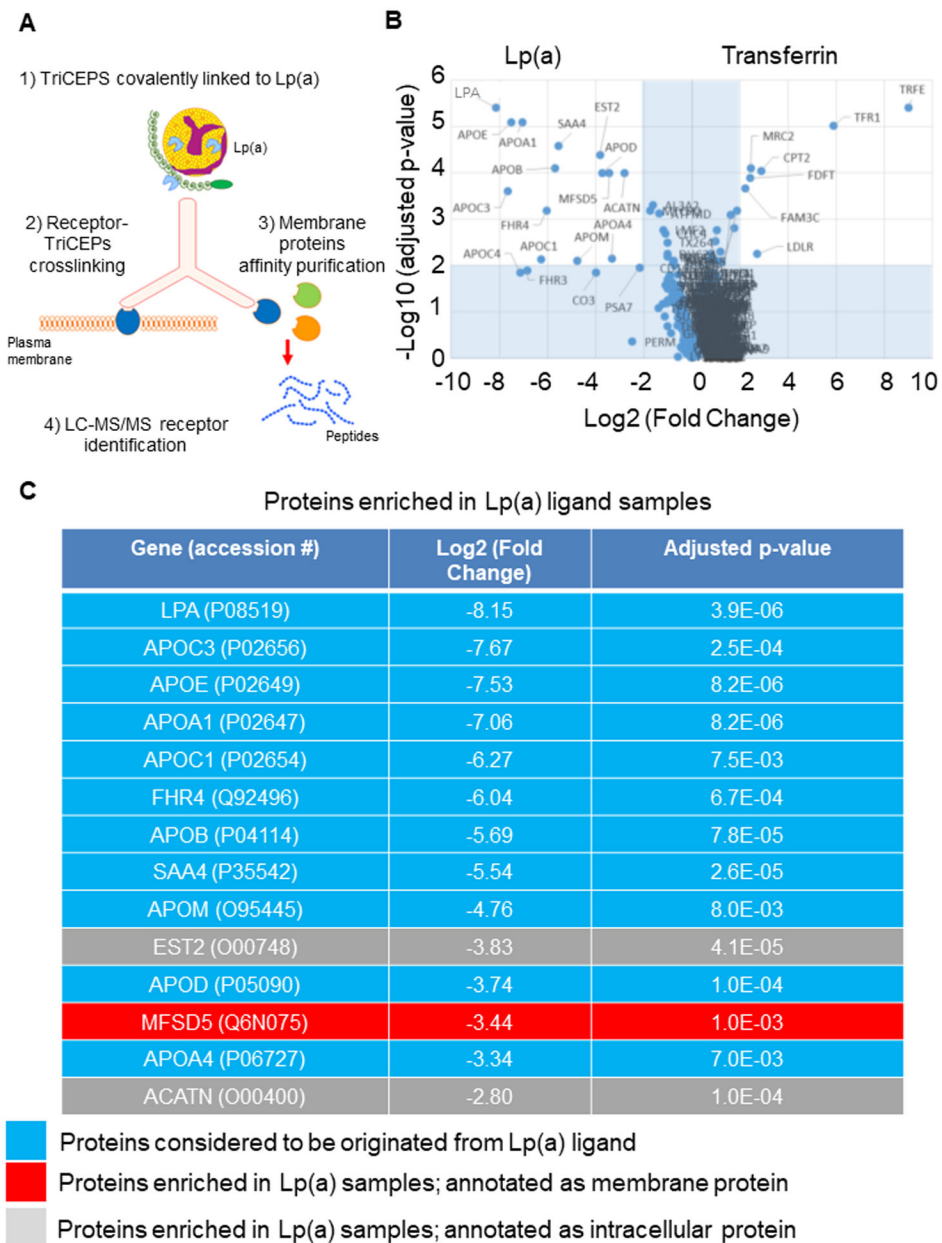
#### What is new?

- First identification of MFSD5 as a receptor/co-factor involved in Lp(a) uptake and calcific aortic valve disease.
- *MFSD5* variant rs73101935 magnified the effect of high Lp(a) on aortic stenosis.

#### What are the clinical implications?

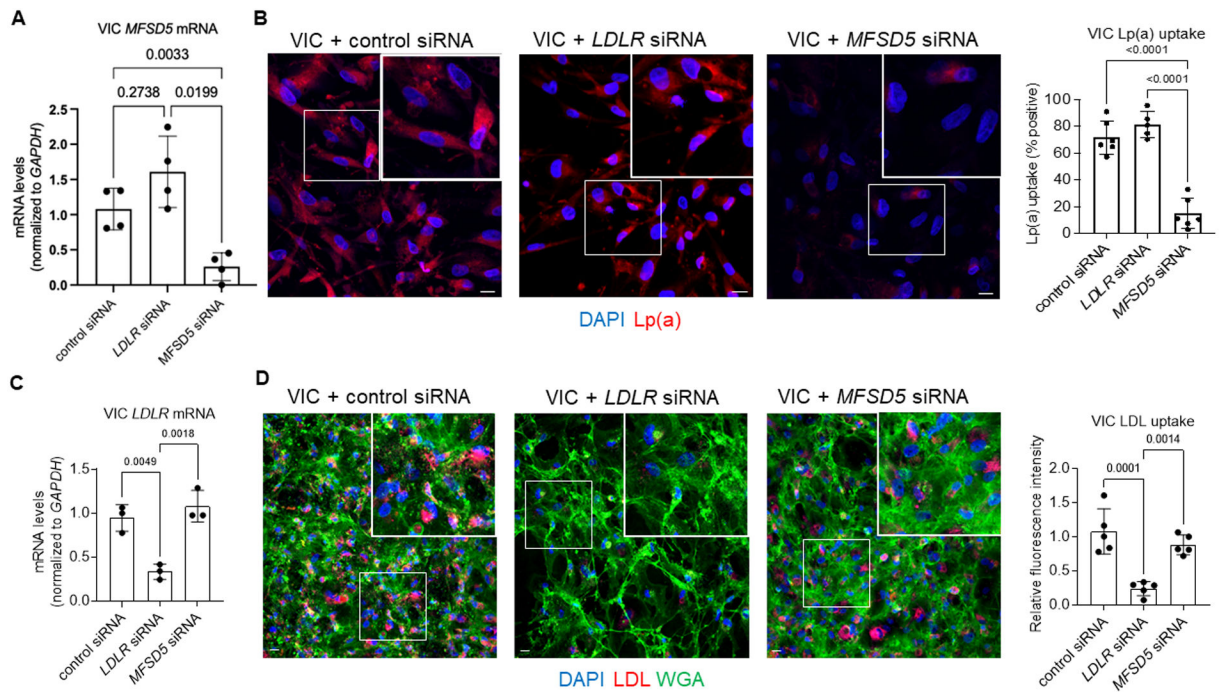
- MFSD5 is a novel and potentially druggable target with a computationally identified small molecule and *MFSD5* siRNA suppressing *MFSD5* expression, Lp(a) uptake and calcification in primary human valvular cells.





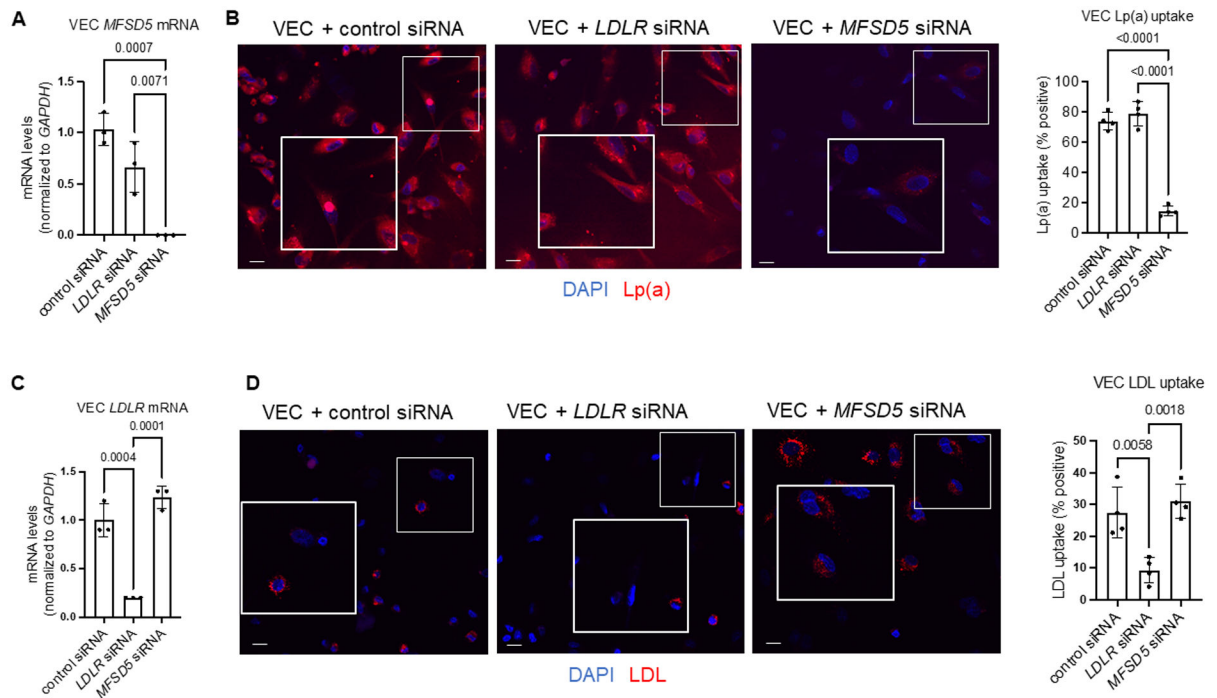
**Figure 1. MFSD5 in human aortic VICs binds to Lp(a).**

**A**, Cartoon showing the TriCEPS mass spectrometry experimental setup used to identify Lp(a) receptors on human aortic VICs. **B**, Volcano plot showing enrichment of mass spectrometry-identified proteins with TriCEPS-labeled Lp(a) incubation or TriCEPS-labeled transferrin used as an incubation control. **C**, Proteins purified and significantly enriched by TriCEPS-labeled Lp(a) capture in VICs shown. MFSD5 indicated in red; (n=3 replicate experiments with 6 pooled donors/experiment).



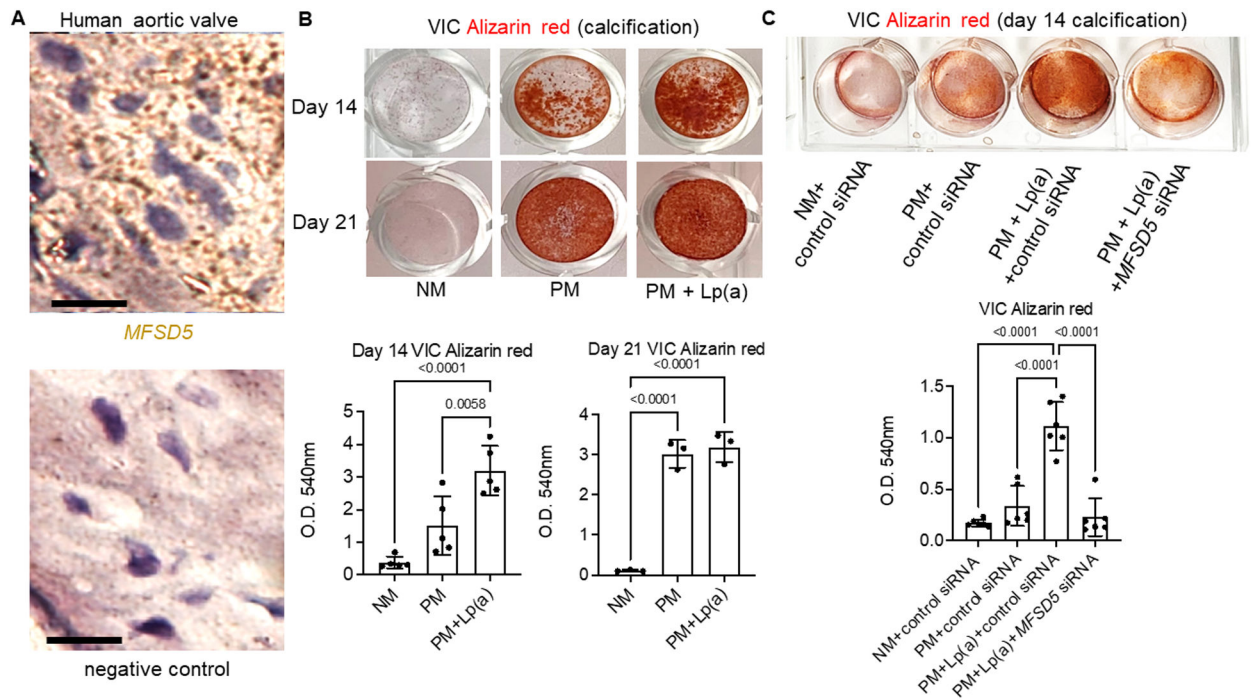
**Figure 2. *MFSD5* specific siRNA reduced Lp(a) uptake by human VICs.**

**A**, Analysis of *MFSD5* mRNA abundance in VICs incubated with the indicated siRNAs (n=4 donors). **B**, Left: Confocal microscopy analysis of Dil-Lp(a) uptake by VICs incubated with the indicated siRNAs. Quantification of Lp(a) uptake by the indicated cells (n=5–6 donors). **C**, Analysis of *LDLR* mRNA abundance in VICs incubated with the indicated siRNAs (n=3 donors). **D**, Left: Confocal microscopy analysis of Dil-LDL uptake by VICs incubated with the indicated siRNAs. DAPI was used to stain nuclei and wheat germ agglutinin (WGA) was used to stain membranes. Right: Quantification of Dil-LDL uptake by VICs incubated with the indicated siRNAs (n=5 donors). Data are mean  $\pm$  SD and were analyzed by ANOVA. Scale bars = 20  $\mu$ m, higher magnification inset areas are indicated by white boxes.



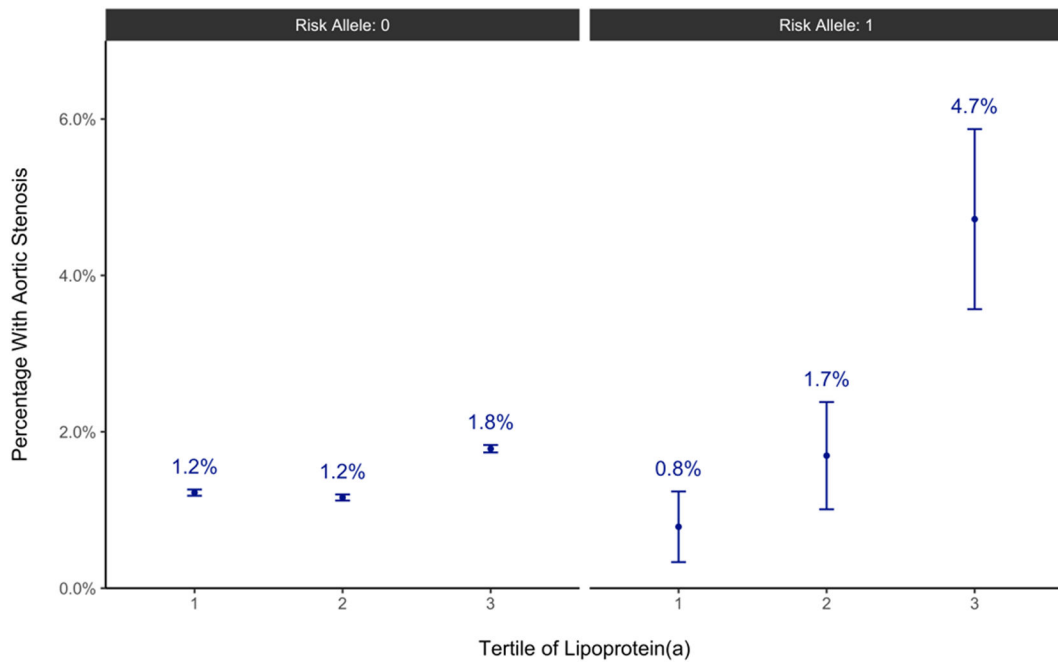
**Figure 3. *MFSD5* specific siRNA reduced Lp(a) uptake by human VECs.**

**A**, Analysis of *MFSD5* mRNA abundance in VECs incubated with the indicated siRNAs (n=3 donors). **B**, Left: Confocal microscopy analysis of Dil-Lp(a) uptake by VECs incubated with the indicated siRNAs. Quantification of Lp(a) uptake by the indicated cells (n=4 donors). **C**, Analysis of *LDLR* mRNA abundance in VECs incubated with the indicated siRNAs (n=3 donors). **D**, Left: Confocal microscopy analysis of Dil-LDL uptake by VECs incubated with the indicated siRNAs. DAPI was used to stain nuclei. Right: Quantification of Dil-LDL uptake by VECs incubated with the indicated siRNAs (n=4 donors). Data are mean  $\pm$  SD and were analyzed by ANOVA. Scale bars = 20  $\mu$ m, higher magnification inset areas are indicated by white boxes.



**Figure 4. *MFSD5* specific siRNA reduced Lp(a)-mediated calcification of human VICs.**

**A**, *MFSD5* RNAscope in human aortic valves (n=5 donors with an example donor image shown stained with an *MFSD5* probe (brown stain) and a negative control probe in the same region of a serial section). Scale bars = 20  $\mu$ m. **B**, VIC calcification (Alizarin red) images (top) and quantification (bottom) for VICs incubated in normal control media (NM) or pro-calcifying media (PM) with or without Lp(a) (n=5 donors for day 14, and 3 donors for day 21; analyzed by ANOVA). **C**, VICs incubated with the indicated siRNAs (n=6 donors; analyzed by ANOVA). Data are mean  $\pm$  SD.



**Figure 5. Prevalence of aortic stenosis in the UK Biobank, stratified by the number of *MFSD5* rs73101935-T alleles and tertiles of Lp(a).**

Prevalence is expressed as mean  $\pm$  SE (n=75,774, 75,792, and 75,808 participants for the 1, 2, and 3 respective Lp(a) tertiles with Risk allele: 0, and 382, 354, and 339 participants for the 1, 2, and 3 respective Lp(a) tertiles with Risk allele: 1). A table format of the data is included in the Supplemental Material, Tables S1 and S2.