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Plasma low-density lipoprotein receptor-related protein 1 concentration is not associated with human abdominal aortic aneurysm presence

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What this study adds: Polymorphisms within the LRP1 gene have been suggested to contribute to AAA risk. This study demonstrates that plasma concentrations of LRP1 are similar in men with and without AAA suggesting that this protein is an unsuitable marker to screen at-risk populations. This study confirms that that LRP1 expression is reduced in aortic biopsies collected from AAA patients compared to non-aneurysmal controls and demonstrates that LRP1 inhibition reduces the ability of vascular smooth muscle cells to internalise matrix metalloprotease 9 *in vitro*. These findings suggest that localised LRP1 dysregulation may be important in AAA pathogenesis, but is an unsuitable marker with which to screen at-risk populations.

Abstract

Objectives: Recent genetic data suggest that a polymorphism of the low density lipoprotein receptor-related protein 1 (LRP1) gene is an independent risk factor for abdominal aortic aneurysm. The aims of this study were to assess whether plasma and aortic concentrations of LRP1 were associated with abdominal aortic aneurysm, and to investigate the possible relevance of LRP1 to abdominal aortic aneurysm pathophysiology.

Design, Materials and Methods: Three analyses were conducted. Initially, plasma LRP1 concentrations were measured in community dwelling men with and without abdominal aortic aneurysm (n=189 and 309 respectively) using ELISA. Secondly, Western blotting analyses were employed to compare the expression of LRP1 protein in aortic biopsies collected from abdominal aortic aneurysm patients and non-aneurysmal post-mortem donors (n=6/group). Finally, the effect of *in vitro* LRP1 blockade on MMP9 clearance by vascular smooth muscle cells was assessed by zymography.

Results: Plasma LRP1 concentrations did not differ between groups of men with and without abdominal aortic aneurysm (median concentration 4.56 µg/mL (IQR 3.39-5.96) and 4.43 µg/mL (IQR 3.44-5.84); P=0.48), and were not associated with abdominal aortic aneurysm after adjusting for other risk factors (odds ratio: 1.10 (95% confidence interval: 0.91-1.32); P=0.35). In contrast, LRP1 expression was ~3.4 fold lower in aortic biopsies recovered from abdominal aortic aneurysm patients compared to controls (median (IQR) expression 1.72 (0.94-3.14) and 5.91 (4.63-6.94) relative density units; P=0.004). *In vitro* LRP1 blockade significantly reduced the ability of vascular smooth muscle cells to internalise extracellular MMP9.

Conclusions: These data suggest that aortic but not circulating LRP1 is down-regulated in abdominal aortic aneurysm patients, and indicates a possible role for this protein in clearing an aneurysm-relevant ligand.

Key words: Abdominal aortic aneurysm; Low density lipoprotein receptor related protein-1; biomarker; matrix metalloprotease 9.

Introduction

Abdominal aortic aneurysm (AAA) affects ~2% men and ~1% of women aged >65 years, and significantly increases the risk of mortality through aortic rupture and associated cardiovascular events.[1, 2] The aetiology of AAA remains unclear, however, inflammation, extracellular matrix degeneration and vascular smooth muscle cell (VSMC) loss appear to be important in the pathogenesis.[3, 4] AAA risk factors include advanced age, male sex and smoking.[1] A positive family history increases AAA risk ~2-fold, suggesting that genetic factors are important in AAA development.[5, 6] A genome-wide association study (GWAS) identified a positive association of the rs1466535 major (C) allele within the low-density lipoprotein receptor-related protein 1 (LRP1) gene with AAA presence (odds ratio ~1.2).[7] This association appeared AAA specific and was maintained after adjusting for cardiovascular risk factors leading to the suggestion that the *LRP1* rs1466535 C allele might significantly contribute to AAA risk.[8, 9] This is contradicted, however by recent data reporting a positive association between AAA and the LRP1 rs1466535 T (minor) allele in a geographically distinct patient population.[10]

LRP1 is structurally related to the low density lipoprotein receptor and comprises a 100 amino acid cytoplasmic domain, a membrane spanning region, and an extracellular loop with 4 ligand binding regions.[11, 12] LRP1 is predominantly expressed by VSMCs and facilitates the internalisation and clearance of bound ligands.[12-14] Previous studies suggest that LRP1 has functions beyond lipid metabolism and known LRP1 ligands include growth factors, matrix metalloproteases (MMPs), protease-inhibitor complexes and extracellular matrix components.[8, 11, 12] Conditional LRP1 knockout rodent models demonstrate that LRP1 deficiency results in abnormal VSMC proliferation and migration, increased extracellular matrix turnover and aneurysm formation within the mesenteric arteries. [14-16] Currently, only one study has directly investigated whether LRP1 expression is altered in human AAA.[17] In this study Chan *et al.* employed Western blotting and immunohistochemistry to demonstrate significant down-regulation of the LRP1 protein in aortic biopsies of Chinese AAA patients compared to non-aneurysmal controls. We hypothesised that reduced aortic LRP1 concentrations may result from release of this protein from the aneurysm wall, which may increase circulating LRP1 concentrations in AAA patients. Here, we assess plasma LRP1 as a potential AAA biomarker, confirm that aortic expression of LRP1 is significantly lower in AAA biopsies compared to controls, and suggest that this protein may play a role in clearing MMP9 from the aortic wall.

Materials and methods

Detailed materials and methods are provided in Supplementary File 1.

Participants: Samples collected from 3 Australian cohorts were used: 1) plasma samples collected from participants of the Health In Men Study (HIMS); 2) aortic biopsies from patients undergoing open surgery to repair large AAA; 3) aortic biopsies from non-aneurysmal heart beating brain dead organ donors.[18] The cohort characteristics and protocols of the HIMS have been previously reported.[18] For HIMS participants, an infra-renal aortic (IRA) diameter of 30-55 mm was defined as a small AAA; and IRA diameters >55 mm were considered large AAAs. Clinical information collected during the HIMS included age, medical history and smoking status. For patients undergoing AAA repair, risk factors were recorded as previously described (Supplementary File 1).[19, 20] Maximum IRA diameter was measured from axial computed tomography angiography (CTA) images as previously described.[21] No clinical information other than age and sex was available for organ donor participants. In all instances, written informed consent and institutional ethics approval was provided.

Measurement of plasma LRP1: Plasma LRP1 concentrations were measured using a commercially available ELISA (#E91010Hu, USCN Life Sciences, China). This ELISA employs antibodies against a soluble extra-cellular LRP1 immunogen suggesting suitability for plasma analysis. Reported inter and intra-assay variability are <12%. Due to limited plasma volumes, single measurements were taken for each patient as previously described.[19, 22]

In vitro experiments: Human aortic VSMC (CC-2571, Lonza) were maintained in growth medium in a humidified 5% CO_2 atmosphere at 37ºC and passaged when 70%–80% confluent. After 5 passages LRP1 blocking antibodies or isotype control antibodies (#MA1-27198 and MA5-14453, respectively, Thermo Fisher Scientific, Australia) were added to the culture media at concentrations of 30μg/mL. Cell culture supernatants were decanted after 24 hours and replaced with media containing 20ng/mL recombinant human matrix metalloprotease 9 (MMP9, #911-MP-010, R and D Systems, USA). Cell culture supernatants were harvested at 24 and 48 hours, and stored at -80ºC. Data presented are from 3 independent culture experiments.

Protein extraction and Western blotting: Isolation of aortic proteins and western blotting was performed as previously described.[23] Full-thickness human abdominal aortic samples were homogenized in the presence of protease inhibitors. Samples (30 μg protein/lane) were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and transferred onto a polyvinylidene difluoride membrane. The membrane was cut at ~60 kDa and each half separately blocked with 5% non-fat dry milk at 4ºC overnight. The 60-250 kDa proteins were incubated with anti-LRP1 antibody (R&D Systems MAB6360, USA), while the proteins <60 kDa were incubated with anti-β actin antibody, (Abcam, UK #AB75186) for 1 hour (room temperature). Membranes were washed and incubated with anti-mouse HRP- (LRP1 blot), or anti-rabbit (β actin)- conjugated IgG (DakoCytomation, Denmark #P0447 and P0448 respectively) for 1 hour (room temperature). Membranes were washed and proteins visualised with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, USA). The relative density of the LRP1 band was standardised by dividing by mean beta-actin density for the respective experimental group. Reported data refer to standardised LRP1 expression.

Gelatin zymography: Five µL of VSMC culture supernatant was loaded onto a 10% polyacrylamide gel containing 1 mg/mL gelatin. Samples were electrophoresed and washed twice in 2.5% (v/v) Triton X 100. Gels were incubated overnight in 50mM Tris-HCl (pH 8.0), 5mM $CaCl_2$ at 37°C and stained in 0.5% (w/v) Coomassie Blue R-250 for 30 mins. Gels were destained with 40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) water, to visualise protease activity and analysed via densitometry as above.

Statistical analyses: Data were analysed using SPSS v.21 (IBM) and Prism v.6 (GraphPad Software Inc, San Diego, USA). Continuous variables were analysed using the Mann-Whitney U test. Nominal variables were assessed using the chi-squared test. Correlations were assessed using Spearman Rho analyses. The correlation between aortic LRP1 expression, and AAA diameter was further assessed in leave-one-out sensitivity analyses. The association of plasma LRP1 with AAA presence was assessed via binary logistic regression corrected for confounders identified in univariate analyses. *In vitro* data comparing 3 groups were assessed using one-way ANOVA applying Tukey's *post-hoc* test for multiple comparisons. P values <0.05 were considered significant.

Results

Plasma LRP1 concentrations are not associated with AAA: LRP1 concentration was measured in plasma collected from 189 men with a small AAA, and 309 non-aneurysmal controls (total n=498). Participant characteristics are shown in Table 1. Participants had similar age and history of dyslipidemia, diabetes and stroke. The prevalence of smoking, hypertension and CHD were significantly higher in men with AAA. No differences in plasma LRP1 concentration were observed between the groups Median plasma LRP1 concentration was 4.56 µg/mL (interquartile range [IQR] (3.39-5.96) for men that had AAA and 4.43 µg/mL (IQR 3.44-5.84) for nonaneurysmal controls (p=0.48; Table 1, Fig 1A). Plasma LRP1 concentrations did not correlate with IRA diameter when assessing the whole population (Spearman r=0.06, P=0.22; Figure 1B), or the experimental groups (for men with AAA: Spearman r=0.056, P=0.441; for controls: Spearman r=0.054, P=0.342, data not shown).

Binary logistic regression was conducted to identify the major risk factors for AAA in the studied population. An increase in plasma LRP1 concentration of ~1 std dev (2.7 µg/mL) was not significantly associated with AAA presence in unadjusted analyses (odds ratio (OR): 1.07 (95% CI: 0.89-1.28; P=0.476), or in analyses adjusting for waist to hip ratio, smoking history, hypertension and CHD (OR: 1.10 (95% CI: 0.91-1.32); P=0.35; (Supplementary Table 1). A history of ever having smoked (OR: 3.44 (95% CI: 2.12-5.58), P<0.001), and CHD (OR: 1.94 (95% CI: 1.29-2.91), P<0.01) were independent risk factors for AAA in this population as previously described (Supplementary Table 1).[19, 22]

Western blot analysis of aortic LRP1 expression: As plasma LRP1 concentrations were similar in AAA patients and controls, Western blotting experiments were conducted to verify whether IRA LRP1 expression was lower in AAA patients compared to non-aneurysmal controls (n=6/group). Patients and controls were sex-matched (67% males/group, P=1.000). Younger AAA patients were specifically selected for this analysis to minimise age imbalances between the groups although AAA patients were older than organ donors (median (IQR) age 58.0 (54.8-66.6) and 45.0 (34.0-51.0) years respectively; P=0.026). Median IRA diameter for the AAA patients was 65.8 (IQR: 57.3-79.2) mm. IRA measurements were not available for controls. Western blotting identified a band at ~90k Da in all samples which was selected for analysis as advised by the antibody manufacturer (Fig 2A and Supplementary Figure 1). Standardised median aortic LRP1 expression was ~3.4-fold lower in AAA patients than non-aneurysmal controls (median (inter-quartile range [IQR]) expression 1.72 (0.94-3.14) and 5.91 (4.63-6.94) relative density units [RDU] respectively, P=0.004; Fig 2B). No significant correlation between standardised LRP1 expression and IRA diameter in the AAA patients was observed (Spearman r=0.600, P=0.242, Figure 2C). No significant correlation between tissue LRP1 expression and AAA diameter was observed following leave-one-out sensitivity analysis (Supplementary Table 2).

In vitro LRP1 blockade inhibits VSMC MMP9 clearance: The relevance of LRP1 under-expression to AAA pathology remains unclear. LRP1 dysfunction has been suggested to promote AAA through inefficient clearance of extracellular ligands including proteases.[9] The effect of LRP1 blockade on extracellular clearance of MMP9 by VSMCs was assessed *in vitro*. VSMCs were exposed to LRP1 blocking antibodies, isotype control-IgG or vehicle for 24 hours, prior to adding recombinant MMP9. Conditioned media MMP9 activity was assessed 24 and 48 hours after MMP9 addition using gelatin zymography (Supplementary Figure 2).

After 24 hours, the MMP9 activity of culture supernatants was comparable between cells exposed to MMP9 with anti-LRP1 antibodies, isotype control IgG, or vehicle (mean (±

standard error of the mean [SEM]) MMP9 activity: 3.69 (±0.50), 3.85 (±0.26) and 4.58 (±0.31 RDU respectively; P=0.270; Figure 3A). The MMP9 activity of media from all 3 groups was significantly greater than negative control cells which did not receive recombinant protease (all P values <0.05; data not shown). After 48 hours extracellular MMP9 activity differed significantly between VSMCs receiving anti-LRP1 antibodies, isotype control IgG, or vehicle (mean (± SEM): 11.27 (±0.44), 8.29 (±0.45) and 9.80 (±0.75) RDU respectively; P=0.027; Figure 3B). Between-group comparisons demonstrated that conditioned media from VSMCs receiving LRP1-blocking antibodies exhibited significantly higher MMP9 activity than the control IgG1-exposed cells (mean difference between groups: 2.97; 95% CI: 0.53-5.42; p<0.05). MMP9 activity from all 3 groups remained higher than the negative controls at this time point (all P-values <0.05; data not shown).

Discussion

Recent data suggest that the LRP1 rs1466535 polymorphism may be a specific AAA risk factor.[7, 9, 10] A previous study has reported that LRP1 expression within the IRA is significantly lower in AAA patients compared to organ donor controls.[17] We and others have demonstrated that proteins liberated from the aneurysm wall may enter the bloodstream and act as potential biomarkers for AAA.[24-26] Consequently, we hypothesised that weak aortic LRP1 expression in AAA patients may increase circulating concentrations of this protein, although plasma LRP1 concentrations were similar in men with (n=189) and without AAA (n=309). Given this finding we attempted to verify the previous finding that LRP1 was downregulated within the aorta of AAA patients. We assessed LRP1 expression in IRA biopsies collected from AAA patients and organ donor controls. Currently, only one study has reported significant reductions in LRP1 expression in aortic biopsies collected from AAA patients compared to non-aneurysmal organ donor controls.[17] Using antibodies against a different LRP1 immunogen and an alternative house-keeping protein, we confirm that aortic LRP1 protein expression was down-regulated in Australian patients with large AAAs compared to non-aneurysmal controls. Collectively, these findings suggest that LRP1 dysregulation is localised to the aortic wall in AAA patients. This was assessed *in vitro* and our data suggest that LRP1 blockade significantly impairs the ability of VSMCs to remove the AAA relevant protease MMP9 from the surrounding media.

Bown *et al.* suggested that the LRP1 rs1466535 CC genotype is associated with increased LRP1 expression (~1.2 fold) compared to the TT genotype.[7] It should be noted that Bown and colleagues analysed ascending aortic biopsies, compared to the IRA samples analysed in the current study and the previous investigation by Chan *et al.*[17] Gene expression patterns differ between aortic regions and work is needed to assess whether the rs1466535 CC genotype is associated with differential IRA LRP1 expression.[27] Thus, the mechanisms underpinning reduced IRA LRP1 expression in AAA patients remain unclear. Chan *et al.* hyothesised that LRP1 may be regulated by miR205.[17] Kim and colleagues demonstrated that aortic expression of miR205 is significantly increased in apolipoprotein E deficient mice which developed AAA in response to angiotensin-II infusion.[28] Moreover administration of anti-miR205 oligonucleotides to aortic endothelial cells greatly attenuated angiotensin-II induced AAA development. They also showed that LRP1 expression markedly decreased in response to angiotensin-II infusion, but was not rescued by *in vivo* anti-miR205 administration, suggesting that miR205 may exert AAA-protective effects through other pathways.[28] Recent data also demonstrate that normocholesterolaemic mice deficient in smooth muscle LRP1 (smLRP1$^{-/-}$) are no more susceptible to angiotensin-II induced AAA than wild type controls, although the formation of large aneurysms within the ascending aorta and mesenteric artery was reported.[29] It should be noted that normocholesterolaemic mice appear more resistant to angiotensin-II induced AAA than dyslipidaemic strains and future studies employing other AAA induction approaches in these mice are needed to further assess the role of LRP1 in AAA formation.[23, 30, 31]

Although there is no direct evidence of a role for LRP1 in AAA formation, studies of the smLRP$^{-/-}$ mouse have demonstrated an important role for this protein in maintaining vascular integrity.[14, 16, 32] smLRP$^{-/-}$ mice aortas show Marfan Syndrome-like phenotypes with disruption of the elastic laminae and VSMC hyperplasia, increased vessel tortuosity, thickness and dilatation.[14-16, 32] Comparable circulating cholesterol concentrations in smLRP1$^{-/-}$ and control mice suggests that the observed pathologies are independent of lipoprotein metabolism.[14] Intriguingly, mice deficient in both smLRP1 and LDLR appear more susceptible to atherosclerosis than smLRP1^{+}LDLR$^{-/-}$ controls demonstrated by widespread lesion formation following high cholesterol feeding.[14, 16, 32] However, the aetiologies of AAA and atherothrombosis appear distinct.[20, 33] AAA is associated with VSMC apoptosis and extracellular matrix degeneration, rather than cellular proliferation. MMP9, an LRP1 ligand, is over-expressed in human and mouse AAA biopsies, and is implicated in AAA pathogenesis.[1, 31] The current study suggests a role for LRP1 in regulating aortic MMP9 concentrations evidenced by heightened proteolytic activity in supernatants from LRP1 compromised VSMCs. This is supported by prior data demonstrating increased MMP9 activity in conditioned media harvested from mouse embryonic fibroblasts treated with an LRP1 antagonist, and an accumulation of MMP9 within the aortas of smLRP1$^{-/-}$ mice. [14, 34] These findings suggest that aortic LRP1 dysregulation may exacerbate extracellular matrix proteolysis, although LRP1 has potential to contribute towards multiple AAA pathogenic mechanisms due to the broad spectrum of ligands for this receptor.[9]

Extracellular forms of LRP1 are thought to result from shedding of the LRP1 ectodomain.[35-37] No association of circulating LRP1 concentration with AAA was observed suggesting that low IRA LRP1 expression in AAA patients does not result from heightened extracellular shedding. Immunohistochemistry data by Chan and colleagues demonstrate that LRP1 is predominantly expressed within the aortic media and adventitia, and confirm that this is reduced in human AAA although the cells which express LRP1 in the normal aorta were not investigated in this study. LRP1 is known to be expressed by VSMCs. Thus, it is possible that the loss of VSMCs typically demonstrated within the aortas of AAA patients may have contributed to the down-regulation of LRP1 observed in the current study. [17] Further studies are required to directly assess this.

There are several limitations to the current study. Firstly, the sample sizes employed in the Western blotting analyses were relatively small. As most AAAs are now managed by endovascular repair aortic wall biopsies can only be obtained from the occasional patient undergoing open surgery for a large AAA.[1] Similarly, ethical and practical considerations prevent the collection of aortic biopsies from large numbers of control donors, however, sample sizes here are similar to other published studies.[17, 22, 38-40] Secondly the organ donor controls were younger than the AAA patients and the possibility that the age imbalance may have contributed to differences in aortic LRP1 expression cannot be excluded. Limited clinical details were available for the organ donor controls and variation in cardiovascular risk factors between groups could not be adjusted for. However, organ donors are often the only viable source of healthy aortic biopsies.[22, 41] Some previous studies have utilised macroscopically healthy tissues proximal to the AAA sac as matched control tissues, however these were not available for the current study.[24, 38] A recent genome-wide expression study comparing biopsies from the proximal aneurysm neck with IRA biopsies from organ donors demonstrated significant reductions in LRP1 expression in the AAA patients.[42] This suggests that LRP1 dysregulation precedes macroscopic AAA dilatation. Thirdly, no plasma samples were available from the AAA patients or organ donors who provided aortic biopsies. Thus, IRA and plasma LRP1 concentrations could not be directly compared in these individuals. Instead, plasma samples were obtained from men with screen-detected AAAs, not hospital referred patients. Consequently most aneurysmal blood donors had small (<50 mm) AAAs. A difference in plasma LRP1 concentration may be seen when comparing patients with large AAAs to healthy controls, although this was not possible to assess in this study. Furthermore, the ELISA we used recognises an extracellular LRP1 immunogen whereas the Western blotting antibody employed is raised against an intracellular epitope. This complicates direct comparison of the two tests. Limited sample availability prevented us from assessing aortic tissue extracts by ELISA to cross-validate the assay. Moreover it is possible that minor differences in plasma LRP1 concentration between the groups may have been masked by inter-assay variability (reported as <12%). Finally LRP1 genotype data were not available for any participants in this study. The rationale for assigning participants to experimental groups was therefore based on AAA.

In summary, plasma LRP1 concentrations were similar in relatively large groups of community-dwelling men with and without AAA (n=189 and 309 respectively), minimising the biomarker potential for this molecule. Our data support an inverse association between IRA LRP1 expression and AAA presence, and suggest that LRP1 inhibition in VSMCs may potentially contribute to MMP9 accumulation within the aortic wall. Studies employing larger biopsy numbers and alternative methodologies such as RNA interference are required to validate these findings, and to further assess the impact of reduced LRP1 expression on AAA pathology.

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Conflict of interests: None

References

1. Moxon JV, Parr A, Emeto TI, Walker P, Norman PE, Golledge J. Diagnosis and monitoring of abdominal aortic aneurysm: Current status and future prospects. Curr Probl Cardiol. 2010;35(10):512-48.

2. Svensjo S, Mani K, Bjorck M, Lundkvist J, Wanhainen A. Screening for abdominal aortic aneurysm in 65-year-old men remains cost-effective with contemporary epidemiology and management. Eur J Vasc Endovasc Surg. 2014;47(4):357-65.

3. Hellenthal FA, Buurman WA, Wodzig WK, Schurink GW. Biomarkers of AAA progression. Part 1: extracellular matrix degeneration. Nat Rev Cardiol. 2009;6(7):464-74.

4. Hellenthal FA, Buurman WA, Wodzig WK, Schurink GW. Biomarkers of abdominal aortic aneurysm progression. Part 2: inflammation. Nat Rev Cardiol. 2009;6(8):543-52.

5. Golledge J, Kuivaniemi H. Genetics of abdominal aortic aneurysm. Curr Opin Cardiol. 2013;28(3):290-6.

6. Larsson E, Granath F, Swedenborg J, Hultgren R. A population-based case-control study of the familial risk of abdominal aortic aneurysm. J Vasc Surg. 2009;49(1):47-50; discussion 1.

7. Bown MJ, Jones GT, Harrison SC, Wright BJ, Bumpstead S, Baas AF, et al. Abdominal aortic aneurysm is associated with a variant in low-density lipoprotein receptor-related protein 1. Am J Hum Genet. 2011;89(5):619-27.

8. Giusti B, Galora S, Saracini C, Pratesi G, Gensini GF, Pulli R, et al. Role of rs1466535 low density lipoprotein receptor-related protein 1 (LRP1) gene polymorphism in carotid artery disease. Atherosclerosis. 2014;237(1):135-7.

9. Wild JB, Stather PW, Sylvius N, Choke E, Sayers RD, Bown MJ. Low density lipoprotein receptor related protein 1 and abdominal aortic aneurysms. Eur J Vasc Endovasc Surg. 2012;44(2):127-32.

10. Galora S, Saracini C, Pratesi G, Sticchi E, Pulli R, Pratesi C, et al. Association of rs1466535 LRP1 but not rs3019885 SLC30A8 and rs6674171 TDRD10 gene polymorphisms with abdominal aortic aneurysm in Italian patients. J Vasc Surg. 2015;61(3):787-92.

11. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 2001;108(6):779-84.

12. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. Physiol Rev. 2008;88(3):887-918.

13. Basford JE, Moore ZW, Zhou L, Herz J, Hui DY. Smooth muscle LDL receptor-related protein-1 inactivation reduces vascular reactivity and promotes injury-induced neointima formation. Arterioscler Thromb Vasc Biol. 2009;29(11):1772-8.

14. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science. 2003;300(5617):329-32.

15. Muratoglu SC, Belgrave S, Hampton B, Migliorini M, Coksaygan T, Chen L, et al. LRP1 protects the vasculature by regulating levels of connective tissue growth factor and HtrA1. Arterioscler Thromb Vasc Biol. 2013;33(9):2137-46.

16. Zhou L, Takayama Y, Boucher P, Tallquist MD, Herz J. LRP1 regulates architecture of the vascular wall by controlling PDGFRbeta-dependent phosphatidylinositol 3-kinase activation. PLoS One. 2009;4(9):e6922.

17. Chan CY, Chan YC, Cheuk BL, Cheng SW. A Pilot Study on Low-density Lipoprotein Receptor-related Protein-1 in Chinese Patients with Abdominal Aortic Aneurysm. Eur J Vasc Endovasc Surg. 2013;46(5):549-56.

18. Norman PE, Flicker L, Almeida OP, Hankey GJ, Hyde Z, Jamrozik K. Cohort profile: The Health In Men Study (HIMS). Int J Epidemiol. 2009;38(1):48-52.

19. Golledge J, Clancy P, Jamrozik K, Norman PE. Obesity, adipokines, and abdominal aortic aneurysm: Health in Men study. Circulation. 2007;116(20):2275-9.

20. Moxon JV, Liu D, Wong G, Weir JM, Behl-Gilhotra R, Bradshaw B, et al. Comparison of the serum lipidome in patients with abdominal aortic aneurysm and peripheral artery disease. Circ Cardiovasc Genet. 2014;7:71-9.

21. Parr A, Jayaratne C, Buttner P, Golledge J. Comparison of volume and diameter measurement in assessing small abdominal aortic aneurysm expansion examined using computed tomographic angiography. Eur J Radiol. 2011;79(1):42-7.

22. Moxon JV, Padula MP, Clancy P, Emeto TI, Herbert BR, Norman PE, et al. Proteomic analysis of intra-arterial thrombus secretions reveals a negative association of clusterin and thrombospondin-1 with abdominal aortic aneurysm. Atherosclerosis. 2011;219(2):432-9.

23. Krishna SM, Seto SW, Moxon JV, Rush C, Walker PJ, Norman PE, et al. Fenofibrate increases high-density lipoprotein and sphingosine 1 phosphate concentrations limiting abdominal aortic aneurysm progression in a mouse model. Am J Pathol. 2012;181(2):706-18.

24. Emeto TI, Moxon JV, Biros E, Rush CM, Clancy P, Woodward L, et al. Urocortin 2 is associated with abdominal aortic aneurysm and mediates anti-proliferative effects on vascular smooth muscle cells via corticotrophin releasing factor receptor 2. Clin Sci. 2014;126(7):517-27.

25. Dejouvencel T, Feron D, Rossignol P, Sapoval M, Kauffmann C, Piot JM, et al. Hemorphin 7 Reflects Hemoglobin Proteolysis in Abdominal Aortic Aneurysm. Arterioscler Thromb Vasc Biol. 2010;30(2):269-75.

26. Golledge J, Clancy P, Moran C, Biros E, Rush C, Walker P, et al. The novel association of the chemokine CCL22 with abdominal aortic aneurysm. Am J Pathol. 2010;176(5):2098-106.

27. Lillvis JH, Erdman R, Schworer CM, Golden A, Derr K, Gatalica Z, et al. Regional expression of HOXA4 along the aorta and its potential role in human abdominal aortic aneurysms. BMC Physiol. 2011;11:9.

28. Kim CW, Kumar S, Son DJ, Jang IH, Griendling KK, Jo H. Prevention of Abdominal Aortic Aneurysm by Anti-MicroRNA-712 or Anti-MicroRNA-205 in Angiotensin II-Infused Mice. Arterioscler Thromb Vasc Biol. 2014;34(7):1412-21.

29. Davis FM, Rateri DL, Balakrishnan A, Howatt DA, Strickland DK, Muratoglu SC, et al. Smooth muscle cell deletion of low-density lipoprotein receptor-related protein 1 augments angiotensin II-induced superior mesenteric arterial and ascending aortic aneurysms. Arterioscler Thromb Vasc Biol. 2015;35(1):155-62.

30. Moran CS, Jose RJ, Biros E, Golledge J. Osteoprotegerin deficiency limits angiotensin II-induced aortic dilatation and rupture in the apolipoprotein E-knockout mouse. Arterioscler Thromb Vasc Biol. 2014;34(12):2609-16.

31. Moxon JV, Liu D, Moran CS, Crossman DJ, Krishna SM, Yonglitthipagon P, et al. Proteomic and genomic analyses suggest the association of apolipoprotein C1 with abdominal aortic aneurysm. Proteomics Clin Appl. 2014;8(9-10):762-72.

32. Boucher P, Li WP, Matz RL, Takayama Y, Auwerx J, Anderson RG, et al. LRP1 functions as an atheroprotective integrator of TGFbeta and PDFG signals in the vascular wall: implications for Marfan syndrome. PLoS One. 2007;2(5):e448.

33. Golledge J, Norman PE. Atherosclerosis and abdominal aortic aneurysm: Cause, response, or common risk factors? Arterioscler Thromb Vasc Biol. 2010;30(6):1075-7.

34. Hahn-Dantona E, Ruiz JF, Bornstein P, Strickland DK. The low density lipoprotein receptor-related protein modulates levels of matrix metalloproteinase 9 (MMP-9) by mediating its cellular catabolism. J Biol Chem. 2001;276(18):15498-503.

35. Grimsley PG, Quinn KA, Chesterman CN, Owensby DA. Evolutionary conservation of circulating soluble low density lipoprotein receptor-related protein-like ("LRP-like") molecules. Thromb Res. 1999;94(3):153-64.

36. Liu Q, Zhang J, Tran H, Verbeek MM, Reiss K, Estus S, et al. LRP1 shedding in human brain: roles of ADAM10 and ADAM17. Mol Neurodegener. 2009;4:17.

37. Selvais C, D'Auria L, Tyteca D, Perrot G, Lemoine P, Troeberg L, et al. Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1 (LRP-1) and clearance function. FASEB J. 2011;25(8):2770-81.

38. Biros E, Moran CS, Wang Y, Walker PJ, Cardinal J, Golledge J. microRNA profiling in patients with abdominal aortic aneurysms: the significance of miR-155. Clin Sci. 2014;126(11):795-803.

39. Biros E, Walker PJ, Nataatmadja M, West M, Golledge J. Downregulation of transforming growth factor, beta receptor 2 and Notch signaling pathway in human abdominal aortic aneurysm. Atherosclerosis. 2012;221(2):383-6.

40. Lenk GM, Tromp G, Weinsheimer S, Gatalica Z, Berguer R, Kuivaniemi H. Whole genome profiling reveals a significant role for immune function in human abdominal aortic aneurysms. BMC Genomics. 2007;8(237).

41. Moxon JV, Padula MP, Herbert BR, Golledge J. Challenges, current status and future perspectives of proteomics in improving understanding, diagnosis and treatment of vascular disease. Eur J Vasc Endovasc Surg. 2009;38(3):346-55.

42. Biros E, Moran CS, Rush CM, Gabel G, Schreurs C, Lindeman JH, et al. Differential gene expression in the proximal neck of human abdominal aortic aneurysm. Atherosclerosis. 2014;233(1):211-8.

Table 1: Clinical characteristics of 498 population screened men included in the plasma LRP1 analyses.

Characteristic	**Men without AAA (AOD <30 mm; n=309)**	**Men with AAA (AOD >30 mm n=189)**	**P-value**
Infra-renal aortic diameter (mm)	19.5 (19.2-20.2)	33.7 (31.7-40.3)	<0.001
Age (years)	72 (68-75)	72 (68-75)	0.275
Waist:hip ratio	0.95 (0.91-1.00)	0.96 (0.93-1.00)	0.069
Plasma LRP1 (µg/mL)	4.43 (3.44-5.84)	4.56 (3.39-5.96)	0.476
Past history of:			
Ever smoking	196 (63.4%)	163 (86.2%)	<0.001
Hypertension	126 (40.8%)	97 (51.3%)	0.022
Dyslipidaemia	133 (43.0%)	90 (47.6%)	0.319
Diabetes	34 (11.0%)	18 (9.5%)	0.600
Stroke	19 (6.2%)	17 (9.0%)	0.234
CHD	83 (26.9%)	83 (43.9%)	<0.001

CHD: Coronary heart disease. Nominal variables are presented as numbers and valid % and compared using the Chi-squared test. Continuous variables are presented as median and interquartile range and compared using the Mann-Whitney U test.

Figure legends

Figure 1: Plasma LRP1 is not associated with AAA presence. A) ELISA analysis revealed no difference in circulating LRP1 concentrations in men with AAA (n=189) and non-aneurysmal controls (n=309, Mann-Whitney U test). B) No correlation was seen between plasma LRP1 concentration and infrarenal aortic diameter. Non-aneurysmal controls are boxed.

Figure 2: Western blot analysis of LRP1 expression in infra-renal aortic biopsies recovered from AAA patients and non-aneurysmal organ donors. A) Immunoblot detailing relative infra-renal aortic LRP1 expression in AAA patients (AAA) and controls (CTRL). B) Comparisons between groups revealed standardised aortic LRP1 expression to be significantly lower in AAA patients than controls. Symbols represent individual samples, horizontal bars denote median and inter-quartile ranges. C) Standardised LRP1 expression did not significantly correlate with infra-renal aortic diameter in the AAA patients.

Figure 3: The effect of *in vitro* LRP1 blockade on extracellular MMP9 activity. VSMCs were cultured in the presence of recombinant MMP9 after exposure to LRP1 blocking antibodies, control IgG1, or vehicle. MMP9 activity in cell culture supernatants was assessed 24 (A) and 48 (B) hours after MMP9 addition via gelatin zymography. Cells which did not receive MMP9 are included as negative controls, but are not included in further analyses. Mean ± SEM MMP9 activity are shown for each group (n=3 for all). MMP9 activity was assessed between experimental groups via one-way ANOVA, tables show mean differences

and 95% confidence intervals (CI) for comparisons between groups. NS: Non-significant, *: $p<0.05$ after correction via Tukey’s post-hoc test for multiple comparisons.

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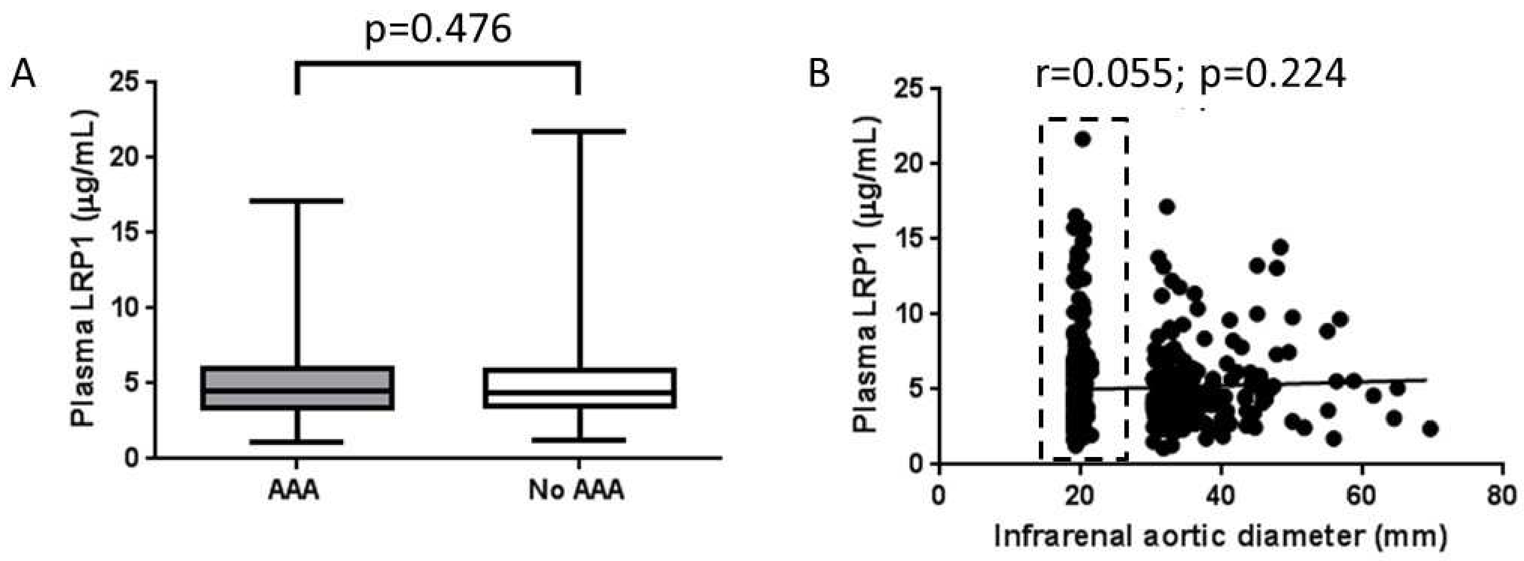

Figure 1

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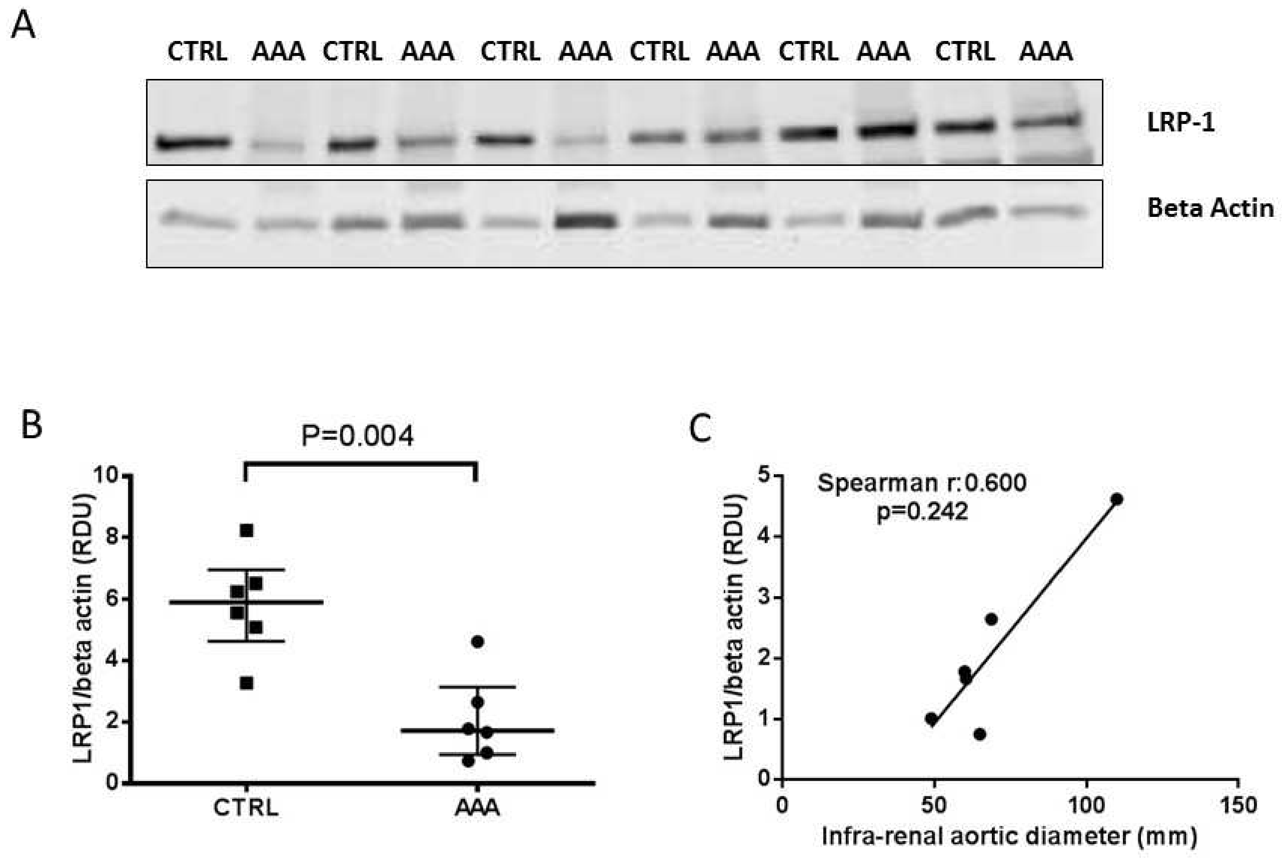
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LRP1/beta actin (RDU)
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150
Infra-renal aortic diameter (mm)

Figure 2

	Mean Diff.	95% CI	Significance
MMP9 vs. MMP9 + Anti-LRP1	0.8863	-0.7122 - 2.485	NS
MMP9 vs. MMP9 + Control IgG1	0.7264	-0.8721 - 2.325	NS
MMP9 + Anti-LRP1 vs. MMP9 + Control IgG1	-0.1599	-1.758 - 1.439	NS

	Mean Diff.	95% CI	Significance
MMP9 vs. MMP9 + Anti-LRP1	-1.463	-3.908 - 0.9819	NS
MMP9 vs. MMP9 + Control IgG1	1.509	-0.9358 - 3.954	NS
MMP9 + Anti-LRP1 vs. MMP9 + Control IgG1	2.972	0.5271 - 5.416	*

Figure 3

Moxon *et al.* Aortic wall but not plasma low-density lipoprotein receptor-related protein 1 is down-regulated in human abdominal aortic aneurysm. Supplementary material.

Supplementary File 1 – Detailed materials and methods.

Supplemetary Table 1 – Independent risk factors for AAA in 498 community dwelling men screened for AAA.

Supplementary Table 2 – Leave one out sensitivity analysis assessing the correlation between aortic LRP1 expression and AAA diameter.

Supplementary Figure 1 – Raw Western blot images detailing IRA LRP1 and beta-actin expression in AAA patients and organ donor controls.

Supplementary Figure 2 – Raw zymography gels showing MMP9 activity from *in vitro* VSMC culture experiments.

SUPPLEMENTARY FILE 1 – DETAILED MATERIALS AND METHODS

Participants: Samples collected from 3 Australian cohorts were used in different stages of the study as follows: 1) aortic biopsies from patients undergoing open surgery to repair AAA, 2) aortic biopsies from non-aneurysmal heart beating brain dead organ donors, and 3) plasma samples collected from participants of the Health In Men Study (HIMS).[18] For patients undergoing AAA repair risk factors were recorded as previously described.[19, 20] Briefly, characteristics collected for each patient included sex, age, history of hypertension, diabetes, coronary heart disease (CHD) and prescribed medications. Diabetes and hypertension were defined by a prior history of diagnosis or treatment of these conditions. Smoking status was defined as having ever or never smoked. CHD diagnosis was based on a history of angina, myocardial infarction or coronary artery revascularisation. Maximum infra-renal aortic diameter was measured from axial computed tomography angiography (CTA) images using the viewer function on a Philips workstation (MxView Visualization Workstation Software, Philips Electronics) and recorded in millimeters as previously reported.[21] No clinical information other than age and sex was available for organ donor participants. The cohort characteristics and protocols of the HIMS have been previously described.[18] HIMS was a community based screening program assessing AAA which used ultrasound to measure infra-renal aortic diameter in Western Australian men aged >65 years. Maximum infra-renal aortic diameter of 30-55 mm was defined as a small AAA; and infrarenal aortic diameters >55 mm were considered large AAAs. Relevant to the current study, clinical information collected for each HIMS participant included age, medical history and smoking status. Anthropomorphic measurements including height, weight and hip and waist circumference were also recorded. Participants in the current study were selected based on blood sample availability, and completeness of clinical data. In all instances, appropriate written informed consent and institutional ethics approval to use biological samples and clinical data for research purposes was provided.

In vitro experiments: Commercial human aortic VSMC (CC-2571, Lonza) were maintained in growth medium (DMEM (DMEM/F12, Gibco) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 1% L-Glutamine and 1% penicillin/streptomycin, Gibco) in a humidified 5% CO_2 atmosphere at 37°C. Cells were passaged when 70%–80% confluent. After 5 passages VSMCs were seeded into 12 well plates at a density of $1x10^6$ cells/mL and allowed to adhere overnight. LRP1 blocking antibodies or isotype control antibodies (#MA1-27198 and MA5-14453, respectively, Thermo Fisher Scientific, Australia) were added to the culture media to final concentrations of 30μg/mL based on a previous publication using these antibodies in VSMCs.[43] Cell culture supernatants were decanted after 24 hours and replaced with media containing recombinant human matrix metalloprotease 9 (MMP9, #911-MP-010, R and D Systems, USA), to a final concentration of 20ng/mL. This concentration was chosen as prior data have shown this is the approximate median plasma MMP9 concentration in patients with large stable AAAs,[44] and was considered physiologically relevant. Cell culture supernatants were harvested at 24 and 48 hours, and stored at -80°C for later analysis. Data presented are from 3 independent culture experiments.

Protein extraction and Western blotting: Isolation of aortic proteins and western blotting was performed as previously described.[23] Full-thickness human abdominal aortic samples, or cultured VSMCs were homogenized in the presence of protease inhibitors to obtain protein extracts. Protein concentrations were determined using a Bradford protein assay kit (BioRad, USA). Samples (30 μg of protein per lane) were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed (100 V, 90 min), and transferred (15 mA, 60 min) onto a polyvinylidene difluoride membrane (BioRad, USA). The membrane <30 kDa was removed as mid-low molecular weight proteins were not targeted in this investigation. The remaining membrane was halved (cut at ~60 kDa) and each half separately blocked with 5% non-fat dry milk at 4ºC overnight. The membrane containing the high molecular weight proteins (~60-250 kDa) was incubated with anti-LRP1 antibody (1:1000 dilution in PBSt [1 x PBS pH 7.4 + 1% (v/v) Tween 20], R&D Systems MAB6360, USA), proteins <60 kDa were incubated with anti-β actin antibody, 1:10,000 (Abcam, UK #AB75186). After 1 hour at room temperature, membranes were washed in TBSt (1 x TBS pH 7.4 + 1% (v/v) Tween 20, 3 x 10 mins) and incubated with anti-mouse HRP- (LRP1 blot), or anti-rabbit (β actin)- conjugated IgG (DakoCytomation, Denmark #P0447 and P0448 respectively) diluted 1:1,000 in PBSt for 1 hour at room temperature. Membranes were washed (2 washes in TBSt, 1 wash in TBS for 10 mins each). Protein expression was visualised with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, USA) and quantified using the Quantity One v4.6.7 software package (BioRad, USA). The relative density of the LRP1 band was standardised by dividing by mean beta-actin density for the respective experimental group. All presented Western blot data refer to standardised LRP1 expression.

Gelatin zymography: Five µL of VSMC culture supernatant was loaded into each well of a 10% polyacrylamide gel containing 1 mg/mL gelatin. Samples were electrophoresed as above and then washed in 2.5% (v/v) Triton X 100 for 15 minutes. After 2 washes, gels were incubated overnight in developing buffer (50mM Tris-HCl (pH 8.0), 5mM $CaCl_2$) at 37ºC prior to staining in 0.5% (w/v) Coomassie Blue R-250 for 30 mins. Gels were placed in destain solution (40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) water), to visualise areas of protease activity. Gels were analysed via densitometry as described above.

Measurement of plasma LRP1: Plasma LRP1 concentrations were measured using a commercially available ELISA according to the manufacturer's directions (#E91010Hu, USCN Life Sciences, China). This ELISA employs antibodies raised against a soluble extra-cellular LRP1 immunogen (ser4373-glu4409), suggesting suitability for plasma analysis. The manufacturer's guidelines state that the minimum detectable concentration of human LRP1 with this assay is 2.91 pg/mL and that no significant cross-reactivity with human LRP1 analogues is reported. Reported inter and intra-assay variability are both <12%. Due to limited sample availability, single measurements were taken for each patient as previously described.[19, 22]

Statistical analyses: Data were analysed using the SPSS v.21 statistical package (IBM). Plots were generated using Prism v6 software (GraphPad Software Inc, San Diego, USA). Continuous variables were compared between groups using the Mann-Whitney U test. Nominal variables were assessed using the chi-squared test. Correlation between variables was assessed using Spearman Rho analyses. The correlation between aortic LRP1 expression, and AAA diameter was further assessed in leave-one-out sensitivity analyses in which individual patients were systematically excluded. The association of plasma LRP1 with AAA presence was assessed via binary logistic regression corrected for waist:hip ratio and a history of ever smoking, hypertension or CHD based on significant, or near-significant differences between groups identified via univariate analysis. *In vitro* data comparing 3 groups were assessed using one-way ANOVA applying Tukey's *post-hoc* test for multiple comparisons. For all tests, P values <0.05 were considered significant.

References

1. Norman PE, Flicker L, Almeida OP, Hankey GJ, Hyde Z, Jamrozik K. Cohort profile: The Health In Men Study (HIMS). Int J Epidemiol. 2009;38(1):48-52.
2. Golledge J, Clancy P, Jamrozik K, Norman PE. Obesity, adipokines, and abdominal aortic aneurysm: Health in Men study. Circulation. 2007;116(20):2275-9.
3. Moxon JV, Liu D, Wong G, Weir JM, Behl-Gilhotra R, Bradshaw B, et al. Comparison of the Serum Lipidome in Patients with Abdominal Aortic Aneurysm and Peripheral Artery Disease. Circ Cardiovasc Genet. 2014;7:71-9.
4. Parr A, Jayaratne C, Buttner P, Golledge J. Comparison of volume and diameter measurement in assessing small abdominal aortic aneurysm expansion examined using computed tomographic angiography. Eur J Radiol. 2011;79(1):42-7.
5. Camino-Lopez S, Llorente-Cortes V, Sendra J, Badimon L. Tissue factor induction by aggregated LDL depends on LDL receptor-related protein expression (LRP1) and Rho A translocation in human vascular smooth muscle cells. Cardiovasc Res. 2007;73(1):208-16.
6. Wilson WR, Anderton M, Choke EC, Dawson J, Loftus IM, Thompson MM. Elevated plasma MMP1 and MMP9 are associated with abdominal aortic aneurysm rupture. Eur J Vasc Endovasc Surg. 2008;35(5):580-4.
7. Krishna SM, Seto SW, Moxon JV, Rush C, Walker PJ, Norman PE, et al. Fenofibrate increases high-density lipoprotein and sphingosine 1 phosphate concentrations limiting abdominal aortic aneurysm progression in a mouse model. Am J Pathol. 2012;181(2):706-18.
8. Moxon JV, Padula MP, Clancy P, Emeto TI, Herbert BR, Norman PE, et al. Proteomic analysis of intra-arterial thrombus secretions reveals a negative association of clusterin and thrombospondin-1 with abdominal aortic aneurysm. Atherosclerosis. 2011;219(2):432-9.

Supplementary Table 1: Independent risk factors for AAA in 498 community dwelling men screened for AAA.

Characteristic	Odds ratio	95% CI	P-value
Unadjusted			
Plasma LRP1 concentration	1.07	0.89-1.28	0.476
Adjusted for covariates			
Plasma LRP1 concentration	1.10	0.91-1.32	0.347
Waist:hip ratio	1.16	0.96-1.40	0.133
Ever smoking	3.44	2.12-5.58	<0.001
Hypertension	1.33	0.89-1.98	0.160
CHD	1.94	1.29-2.91	0.001

CHD: Coronary heart disease.

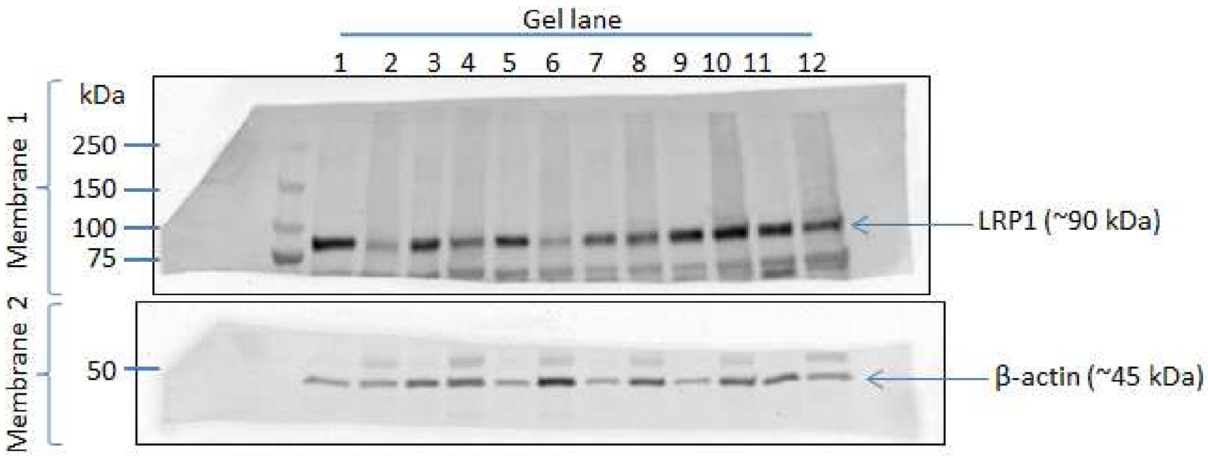
Odds ratios for plasma LRP1 concentration and waist:hip ratio refer to an increase of approximately 1 standard deviation (2.7 µg/ml and 0.06 respectively). For nominal characteristics, men with the risk factor were compared to those without.

Supplementary Table 2: Leave one out sensitivity analysis assessing the correlation between aortic LRP1 expression and AAA diameter.

Patient excluded*	**AAA diameter (mm)**	**LRP1 expression (RDU)†**	**Spearman's r**	**P-value**
1	49.0	1.01	0.600	0.350
2	60.0	1.78	0.700	0.233
3	65.0	0.75	0.900	0.083
4	60.5	1.67	0.700	0.233
5	110.0	4.62	0.300	0.683
6	68.9	2.65	0.300	0.683

* Patient ID is arbitrary

† Refers to LRP1 expression measured by Western blot (normalised to mean beta-actin expression for the whole group); RDU: Relative Density Units.



Supplementary Figure 1: Raw images of western blots detailing LRP1 (membrane 1) and β-actin (membrane 2) expression in aortic biopsies recovered from human organ donors (lanes 1, 3, 5, 7, 9 and 11) and AAA patients (lanes 2, 4, 6, 8, 10 and 12). Each lane contains 30µg protein and lane numbering is uniform for both membranes. After western blotting, membranes were cut between the 75 and 50 kDa markers, and probed with antibodies against LRP1 (membrane 1) or β-actin (membrane 2). As this experiment did not target medium-small (<35 kDa) proteins, portions of the membranes spanning this region were discarded.

After incubating in primary and secondary antibodies, immune complexes were visualised via chemiluminescence. Membranes were imaged separately (exposure times: 250 seconds (membrane 1) and 300 seconds (membrane 2)), and relative expression of each protein compared between samples via densitometry. No inter-membrane comparisons were made.

The Western blotting antibody (R&D Systems MAB6360) was selected after considering the following key factors:

1) The manufacturer specifically advises that the antibody is recommended for use in Western blotting analysis. Moreover, quality control data specifically state that no-cross reactivity was observed with LRP1 homologues. Collectively this suggested that the antibody displays high specificity for LRP1 under the experimental conditions employed for the current study.

2) The antibody is raised against an immunogen within the LRP1 intra-cellular domain, providing scope to specifically assess endogenous LRP1 expression within the aortic wall.

3) The antibody has been used in a recent high-impact publication,[1] indicating that it has been validated within the wider scientific community.

Reference:

1) Hayashi H, *et al.* (2012). A potential neuroprotetive role of apolipoprotein E-containing lipoproteins through low density receptor-related protein 1 in normal tension glaucoma. *J Biol Chem* 287(3) 25395-406

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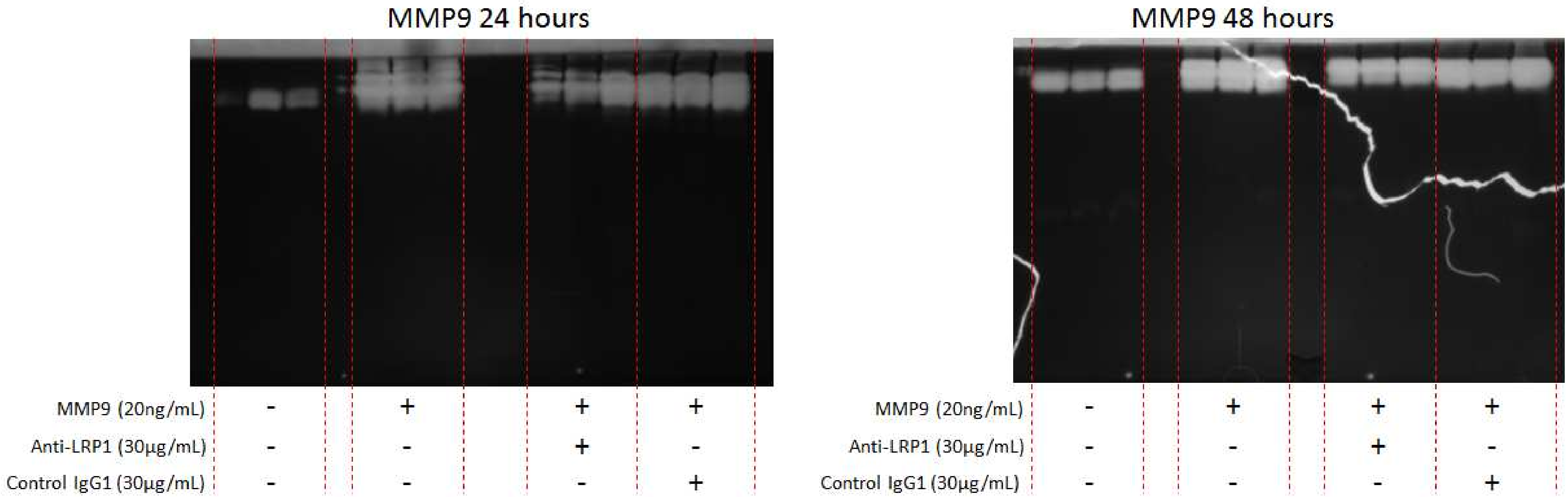

Supplementary Figure 2: Zymography gels showing MMP9 activity in conditioned media collected from VSMCs 24 and 48 hours after adding MMP9 to the culture. Details of reagents added to each culture are shown.