Materials and Methods

Serum sortilin associates with aortic calcification and cardiovascular risk in men

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Cohort

The Structure of the Aging Men's Bones (STRAMBO) study is a single center prospective community-based cohort study on skeletal fragility and its determinants in men. The study is performed as a collaboration between Institut National de la Santé et de la Recherche Médicale and Mutuelle de Travailleurs de la Région Lyonnaise (MTRL), France.[1] The study was approved by the Local Ethics Committee and is conducted in agreement with the Helsinki Declaration of 1975 and 1983. Letters inviting participation were sent to randomly selected men of 20–87 years old from the MTRL list living in Greater Lyon. During the recruitment in 2006–2008, 1169 volunteers replied to the invitation and provided written informed consent. Men who provided informed consent, answered the questionnaire, and participated in the medical exams, were included in the study. No exclusion criteria were applied. Given low prevalence of abdominal aortic calcification (AAC) in young men, measurements of serum sortilin were performed in 897 men aged 50 and older. Twenty men were excluded from analysis due to missing values of various biological measurements or due to non-assessable DXA scans (e.g. due to severe scoliosis leading to the projection of abdominal aorta on the vertebral bodies) (**Supplementary Figure II**). They did not differ from other 877 men. The distribution of the measured sortilin values was highly skewed with a small subgroup of 47 men who were excluded from analysis, as their serum sortilin levels were higher than 1.5-fold interquartile range above the third quartile (>125 ng/mL). We also performed log-transformation, that showed an "upward curve" at $ln_{sortilin}$ of 4.8, that corresponds to 125 ng/ml. All baseline characterstics were not significant different between the excluded 47 men and 830 men who were included in the main analysis **(Supplementary Table I)**.

Assessment of the AAC score

At baseline, Dual-energy X-ray absorptiometry (DXA) was performed using the Hologic Discovery A device equipped with a rotatory C-arm (Hologic Inc., Waltham, MA, USA) as reported previously.[2] Lateral single-energy scans of the spine were obtained in the dorsal decubitus position. Lumbar AAC was assessed on the lateral DXA scans using a 24-point semi-quantitative score described by Kauppila.[3] Calcifications in the abdominal aorta adjacent to the first four lumbar vertebrae were assessed in the posterior and anterior aortic walls using the midpoint of the intervertebral space above and below the vertebra as boundaries (eight segments). Individual severity scores for each segment (0–3) were added to yield AAC scores ranging from 0 to 24. Intra- and inter-rater reproducibility was assessed by two experienced readers using 76 scans as previously described.[2] Intra- and inter-rater agreement (assessed by intra class correlation coefficient) for AAC score (continuous scale) was 0.95 (95% confidence interval (95%CI: 0.91–0.99) and 0.90 (95%CI: 0.86–0.94) respectively. Intra- and inter-rater agreement assessed using the κ score for AAC score >5 vs. 0-5 (a cut-off used in this study) was κ=0.94 (95%CI: 0.89–0.98) and κ=0.87 (95%CI: 0.76–0.99), respectively. Whole body composition was assessed using the same device.

Epidemiological questionnaire

At baseline, all participants responded to an interviewer-administered questionnaire. Smoking status (current, former or never smoker, daily number of cigarettes, duration of smoking) and alcohol intake (average amount of alcohol consumed weekly) were self-reported. Total calcium intake included dietary intake (estimated by a food frequency questionnaire)[4] and pharmacological supplements. Time spent outdoors was calculated based on the overall amount of time spent walking, gardening and practicing outdoor leisure sport activity (e.g., cycling, jogging, skiing, and hiking). Seasonal activities were averaged on the entire year. Occupational physical activity was evaluated according to a self-reported four-level scale (low, moderate, relatively high, and high) corresponding to the longest period during the professional activity. Pharmacologically-treated co-morbidities present at baseline (ischemic heart disease, diabetes mellitus, Parkinson's disease) and history of stroke were self-reported, dichotomized (yes/no), and not further ascertained. Blood pressure was measured using a sphygmanometer after 5 minutes rest in a reclining position. Medications (angiotensin II [ATII] receptor antagonists, angiotensin converting enzyme [ACE] inhibitors, beta-blockers, calcium channel blockers, statins, fibrates, diuretics, vitamin K antagonists [VKA], and glucocorticoids) were self-reported, verified using medical prescriptions and dichotomized (yes/no). The physical performance was assessed by clinical tests as described previously.[5]

Measurement of serum Sortilin

Non-fasting serum was collected at baseline and stored at -80°C. Sortilin was assessed in a blinded manner using aliquots which had not been thawed. Sortilin was measured by a sandwich ELISA (Aviscera Bioscience, Santa Clara, CA, USA; SK00472-01) based on polyclonal antibodies raised against human sortilin extracellular domain (78-765). Samples were measured using a microtiter plate reader at 450 nm. The detection limit is 300 pg/mL, and the dynamic range is 1.56 to 200 ng/mL. The intra- and interassay coefficients of variation (CV) were 4% and 9%, respectively. The ELISA was validated by spike assessments using recombinant sortilin from R&D systems (3154-ST; NS0-derived Ser78-Asn755).

Other biological measurements

Low Density Lipoprotein Cholesterol (LDL-C) was determined by an FDA-approved homogenous direct method from Roche Diagnostics (Indianapolis, IN) at the Department of Laboratory Medicine, Children's Hospital Boston, MA as previously described.[6] Serum osteoprotegerin (OPG) was measured by ELISA (Biomedica Medizinprodukte GmbH & Co. Kg, Vienna, Austria) as described previously.[7] Testosterone was measured by tritiated RIA after diethylether extraction with a detection limit of 0.06 nmol/L as previously described.[7] Serum parathyroid hormone (PTH) was measured using a human-specific two-site immunochimiluminescence assay (ELECSYS; Roche, Indianapolis, IN, USA) as described previously.[1] Serum 25-hydroxycholecalciferol (25OHD) was measured with RIA (DiaSorin, Stillwater, MN, USA) after acetonitril extraction.[1] High-sensitivity C-reactive protein (hsCRP) was measured by immunoturbidimetric latex CRP assay (Roche Diagnostics, Mannheim, Germany).[8] Serum calcium, phosphorus, and cholesterol were measured using standard laboratory methods. Serum creatinin was measured by the compensated Jaffé's method. Glomerular Filtration Rate (GFR) was calculated using the CKD-EPI equation.[9]

Definition of incident events and endpoints

Men aged 60 and over at baseline (n=745) were followed up prospectively for up to 8 years. The major aim of the STRAMBO project is to study the determinants of fragility fractures in men. Such fractures mainly occure in men aged over 60, therefore only men aged 60 and over were followed up prospectively.

Epidemiological questionnaire was sent every year by mail regarding major adverse cardiovascular events (MACE) and major adverse cerebro-cardiovascular events (MACCE)

during the preceding year. When a participant or a proxy reported a MACE/ MACCE, the corresponding medical records (discharge letter from the hospital, copy of a letter sent by a physician from the hospital to the GP in charge of the participant) were requested. We have used exclusively the self-reported events, which were reviewed and confirmed by a health professional in charge of the participant. The rate of missing replies between recruitment and the end of the individual follow-up was 1.16%.

MACE was defined as a composite of cardiovascular death, non-fatal ST-elevation or non-ST-elevation myocardial infarction and unstable angina. Acute coronary syndrome was diagnosed when one of the following terms was mentioned in the medical records of an emergency hospitalization: 1) increased cardiac troponin, 2) significant electrocardiogram changes of ST-segment/T-wave or pathological Q wave accompanied by significant coronary artery stenosis confirmed by coronary angiography. MACCE was defined as MACE plus non-fatal stroke. Stroke was diagnosed when head computed tomography (CT) scans showed cerebral infarction or intracerebral hemorrhage.

Coronary artery disease without emergency state (such as stable angina with or without elective (not emergent) percutaneous coronary intervention) and sudden cardiac death (defined as natural, rapid, and unexpected death not related to anaphylaxis, asphyxia or trauma) was not counted as events. Information on death was obtained from a proxy or from a physician indicated by the participant at recruitment.

Statistics

Statistical analyses were performed using the SAS 9.3 software (SAS, Cary, NC). For the descriptive analyses, continuous variables with Gaussian distribution are presented as mean and standard deviation, continuous variables with skewed distribution as median and interquartile range, and class variables as number and percentage per class. Unadjusted comparisons were made using the analysis of variance for variables with Gaussian distribution and the Kruskal-Wallis test for those with skewed distribution. Age-adjusted comparisons were performed using the analysis of covariance (after log-transformation for variables with skewed distribution). For categorical variables, unadjusted comparisons were performed using chi-square test. The age-adjusted comparisons of the dichotomized variables were performed using the Cochran-Mantel-Haenszel's test. For continuous variables trend was assessed using linear regression. For categorical variables trend was assessed using the chi-square test for trend. Simple and age-adjusted correlation coefficients were calculated using Pearson's correlation coefficient. The cut-points for sortilin were data derived.

The association between baseline sortilin levels (continuous, classes) and risk of MACCE was assessed using the Cox model. The model was adjusted progressively for the variables which were significant in the bivariable models or are associated with cardiovascular risk: 1) age; 2) fat mass, alcohol intake, smoking (current, former, never), occupational physical activity, time spent outdoors; 3) co-morbidities (self-reported ischemic heart disease, systolic blood pressure, prior stroke, self-reported pharmacologically treated diabetes mellitus, AAC) and treatments (angiotensin receptor antagonists, ACE inhibitors, diuretics, statins, beta-blockers, fibrates, VKA); 4) biological assays (GFR, serum testosterone, OPG, 25OHD, LDL-C and hsCRP). At each step, the variables that had $p<0.20$ and/or changed hazard risk (HR) for sortilin by ≥5% were retained. The proportional hazard risk was checked with the Schoenfeld residues. The models assessing the associations of sortilin and other factors (e.g. diabetes) with the risk of MACCE were adjusted for the same confounders as those in the final model above. The models of the link of sortilin with MACE and stroke were adjusted as the final model above. Then, the alternative models were adjusted for Framingham risk factors[10] (age, systolic blood pressure, anti-hypertensive treatment, diabetes, LDL-C cholesterol, and current smoking).

Then, the univariable association between the risk of MACCE and sortilin level was studied using the LOESS method. Based on the visual analysis of the curve, two threshold values were selected

arbitrarily and the multivariable analyses were repeated using the group with the lowest sortilin level (<60 ng/mL) as the reference. Youden's index was calculated based on the sensitivity and specificity values obtained using the unadjusted ROC curve analysis.
The association between sortilin levels (continuous or quartiles) and AAC severity (5 vs 0-5) was assessed using logistic regression. The final model included variables that had p<0.20 in the final model or changed odds ratio (OR) for sortilin by ≥5% . These variables are age, fat mass, height, current smoking, self-reported coronary heart disease, blood pressure, self-reported pharmacologically treated diabetes mellitus, calcium intake, vitamin D and calcium supplements, VKA, calcium channel blockers, phosphorus, calcium and interaction between calcium and phosphorus.

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