Genetically-determined NLRP3 inflammasome activation associates with systemic inflammation and cardiovascular mortality

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Abstract

Aims

Inflammation plays an important role in cardiovascular disease (CVD) development. The NOD-like receptor protein-3 (NLRP3) inflammasome contributes to the development of atherosclerosis in animal models. Components of the NLRP3 inflammasome pathway such as interleukin-1β (IL-1β) can therapeutically be targeted. Associations of genetically determined inflammasome-mediated systemic inflammation with CVD and mortality in humans are unknown.

Methods

We explored the association of genetic *NLRP3* variants with prevalent CVD and cardiovascular mortality in 538,167 subjects on individual participant level in an explorative gene-centric approach without performing multiple testing. Functional relevance of the SNP on NLRP3 inflammasome activation has been evaluated in monocyte-enriched peripheral blood mononuclear cells (PBMCs).

Results

Genetic analyses identified the highly prevalent (MAF 39.9%) intronic *NLRP3* variant rs10754555 to affect *NLRP3* gene expression. rs10754555 carriers showed significantly higher C-reactive protein and serum amyloid A plasma levels. Carriers of the G allele showed higher NLRP3 inflammasome activation in isolated human PBMCs. In carriers of the rs10754555 variant, the prevalence of coronary artery disease (CAD) was significantly higher as compared to non-carriers with a significant interaction between rs10754555 and age. Importantly, rs10754555 carriers had significantly higher risk for cardiovascular mortality during follow-up. Inflammasome inducers (e.g. urate, triglycerides, ApoC3) modulated the association between rs10754555 and mortality.

Conclusion

The *NLRP3* intronic variant rs10754555 is associated with increased systemic inflammation, inflammasome activation, prevalent CAD and mortality. This study provides evidence for a substantial role of genetically-driven systemic inflammation in cardiovascular disease and highlights the NLRP3 inflammasome as a therapeutic target.

Keywords

Cardiovascular diseases, Coronary artery disease, Inflammation, Inflammasome, NLRP3

One sentence summary

Genetically-determined NLRP3-mediated systemic inflammation associates with coronary artery disease and cardiovascular mortality.

Audio abstract

Inflammation represents a common hallmark of atherosclerotic cardiovascular diseases, which is mainly triggered by an activation of the innate immune system. The NLRP3 inflammasome is an important component of the innate immune system mediating processing of pro-interleukin-1β into mature interleukin-1β, thus, leading to a systemic proinflammatory response. The NLRP3 inflammasome plays a crucial role in the development atherosclerosis in several animal models and can be therapeutically targeted by canakinumab or colchicine. Here, we identified a highly-prevalent intronic variant in the *NLRP3* gene locus, which is associated with higher inflammasome activation in human monocytes, vascular injury in humanized mice, and systemic inflammation. Accordingly, this variant associates with a higher risk for coronary artery disease with a pronounced effect in younger individuals. Subsequently, in a meta-analysis on individual participant level comprising almost 500,000 subjects, this *NLRP3* variant is associated with a significantly higher cardiovascular mortality. This is enhanced in subjects with elevated triglycerides, apolipoprotein C3, or urate, which are known NLRP3 inflammasome activators. In general, this study provides evidence for a substantial role of genetically-driven systemic inflammation in cardiovascular disease and highlights the NLRP3 inflammasome as a therapeutic target.

Translational perspective

Inflammation plays crucial role in the development of atherosclerotic cardiovascular diseases (CVD). Interventional studies highlight the NLRP3 inflammasome as an important mediator of CVD. Here, we report that a genetic variant within the *NLRP3* gene locus refers to systemic pro-inflammatory state. This variant is associated with coronary artery disease risk and cardiovascular mortality predominately in younger subjects. Therefore, genetically-determined inflammation represents an important driver of atherosclerotic cardiovascular diseases. Identification of subjects at high inflammation-driven cardiovascular risk sets the stage for individualized treatments.

Introduction

Vascular inflammation is important in the initiation and progression of atherosclerotic vascular diseases (1). Inflammatory markers such as high-sensitivity C-reactive protein (hsCRP) and serum amyloid A (SAA) are associated with increased mortality in patients with manifest cardiovascular diseases (CVD) (2) and healthy subjects with elevated inflammatory markers are at increased risk for the development of CVD (3, 4). Inflammation in patients with CVD is characterized by activation of monocytes, which adhere to the endothelium and migrate into the sub-endothelial layer, where they are activated by endogenous mediators such as modified lipoproteins triggering an innate immune response (1, 5). These monocytes differentiate into tissue macrophages, acquire lipids and lipoproteins, and transform into foam cells contributing to atherosclerotic plaque formation (6, 7).

Interleukin-1β (IL-1β) represents one of the key cytokines released by activated monocytes and macrophages leading to vascular (micro)inflammation (1). The processing of pro-IL-1β into mature IL-1β is tightly regulated by a multimeric intracellular protein complex, the NOD-like receptor protein 3 (NLRP3) inflammasome (8). In addition to exogenous triggers, the NLRP3 inflammasome is activated by a variety of endogenous mediators such as urate, cholesterol crystals and oxidized low-density lipoprotein (oxLDL) (9). Moreover, we recently observed that lipoproteins such as the triglyceride-associated apolipoprotein C-III (ApoC3) directly mediate alternative NLRP3 activation in human monocytes leading to vascular injury *in vivo* (10). The CANTOS trial demonstrated that inhibition of IL-1β, the effector cytokine of the NLRP3 inflammasome, with the monoclonal antibody canakinumab reduced recurrent CV events in patients with previous myocardial infarction (MI) and elevated hsCRP >2 mg/L on top of maximally tolerated statin therapy (11). Recently, the COLCOT trial reported that colchicine, an anti-inflammatory agent for treatment of conditions such as gout, reduced a composite cardiovascular endpoint after MI by 23 % (12). Importantly, modulating NLRP3 inflammasome activity represents one mechanism by which colchicine reduces inflammation (12).

Despite growing experimental evidence for the NLRP3 inflammasome being a key driver of CVD and increased understanding of its molecular regulation, the clinical relevance of inflammasome activation in patients at risk for or with prevalent CVD is incompletely understood. In the present study,

we assessed the association of a gene variant affecting *NLRP3* gene expression and function with the prevalence of coronary artery disease (CAD) and CV mortality in 538,167 subjects.

Methods

Detailed description of the methods can be found in the Supplement.

Genetic association validation studies

The association between SNPs and all-cause as well as CV mortality was studied by genotype or in an additive genetic model. Since the current study is a gene-centric and not a genome-wide association study, it did not require genome-wide significance. Due to the explorative nature of the study, we did not account for the issue of multiple testing and thus report unadjusted p-values. Findings were validated in participants of ten studies comprising 526,091 participants. Study details are described in the Supplement.

Statistical analyses

Continuous variables are presented as mean ± standard deviation (SD) or mean ± 95% confidence intervals (CIs) for normally distributed variables or as median and interquartile ranges (IQR) for variables with skewed distributions. Categorical variables are presented as frequencies. Differences between continuous variables were assessed using one-way ANOVA or Kruskal Wallis test where appropriate. Differences between categorical variables were determined using χ^2-test. Generalized linear models were used to estimate age and sex adjusted marginal means of hsCRP or SAA according to rs10754555 SNP carrier status. In LURIC and GerMIFS, the association between rs10754555 genotype, CAD and severe CAD (only in LURIC) as well as mortality was assessed by logistic and Cox regression analyses. Severe CAD was defined as angiographically visualized ≥50 % stenosis. To study the effect of age, an interaction term between rs10754555 and age has been added to the respective models. Moreover, patients were divided into two groups at the age of 60 years corresponding to the first tertile of age in LURIC. Univariate and multivariable analyses were performed with adjustment for age, sex, diabetes mellitus, systolic blood pressure, BMI, smoking status, estimated glomerular filtration rate (eGFR), LDL-cholesterol (LDL-C), hsCRP, presence of CAD, and previous MI. In the experimental studies, one-way ANOVA followed by Dunnett's post-hoc tests were used to assess significant differences across rs10754555 genotype. Genotype distributions were tested for Hardy-Weinberg

equilibrium using exact tests (https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Meta-analysis on the association between rs10754555 SNP carrier status and cardiovascular mortality was performed by using hazard ratios and standard errors derived from multivariable adjusted Cox regression models at individual participant level provided by each study. Standard normal random-effects weighted meta-analysis was performed using the STATA package 'metan'. Between-study heterogeneity I^2 was determined as described previously (13). Small-study effects were excluded by using the Egger test provided within the STATA package 'metabias'. To study the effect of ApoC3, triglycerides, and urate, an interaction term with rs10754555 was introduced in the respective models. ApoC3, triglycerides, and urate were divided into two categories (quartile 1-3 vs. quartile 4). All other analyses were performed using SPSS version 25 and R version 3.3.3. The significance level was set at 0.05.

Results

NLRP3 genetic variants

We used GWAS data from the LURIC study comprising 3,061 patients referred for coronary angiography as cohort for single nucleotide polymorphism (SNP) preselection. Prioritization of a SNP with effects on expression of NLRP3 is shown in **Figure 1a** and identified rs10754555 as significant eQTL in the 'Blood eQTL browser' ($P=2.32*10^{-6}$) and the 'GTEX database' ($P=9.80*10^{-10}$, **Supplementary Table 1 and 2**). To validate rs10754555 as an eQTL of *NLRP3*, the association between rs10754555 and *NLRP3* mRNA expression in whole blood and peripheral blood mononuclear cells (PBMCs) was assessed in 36 cohorts comprising 31,556 samples included in the eQTLGen consortium (14) (**Figure 1b**). In these analyses, rs10754555 qualified as a significant eQTL of *NLRP3* (Z-score: 11.03, False-discovery rate (FDR) <0.05, $P=2.73*10^{-28}$). The allele and genotype frequencies of rs10754555 are consistent with Hardy-Weinberg equilibrium as shown in **Supplementary Table 3**. Data from the Roadmap Epigenomics project indicate that rs10754555 maps with promoter and enhancer histone marks and DNase hypersensitivity (**Supplementary Figure 1**). Importantly, heterozygous and homozygous rs10754555 carriers showed significantly higher levels of hsCRP (**Figure 1c**) and SAA (**Figure 1d**) as compared to non-carriers indicating that this variant is associated with a systemic pro-inflammatory state.

Biological relevance of rs10754555

The biological relevance of the rs10754555 variant was tested in monocyte-enriched PBMCs (**Figure 2a**, **Supplementary Table 4**, **Supplementary Figure 2a**), which revealed higher *NLRP3* mRNA expression in heterozygous and homozygous carriers of the G allele as compared to PBMCs from non-carriers (**Figures 2b**). Importantly, the plasma levels of IL-18 and IL-1β as NLRP3-dependent cytokines were also significantly higher in G allele carriers (**Figure 2c-d**). To directly assess NLRP3 inflammasome activation according to the rs10754555 variant carrier status, we quantified ASC specks in plasma. Notably, the rs10754555 G allele was associated with plasma ASC specks (**Figure 2e-g**). These findings confirm that carriers of the rs10754555 *NLRP3* G allele are characterized by greater inflammasome activation.

To corroborate these results, activation of the NLRP3 inflammasome was modeled by stimulating the isolated PBMCs with known inflammasome activators (i.e. LPS, ATP and Nigericin) and measuring the release of IL-1β into the cell culture supernatant. Upon stimulation with LPS, LPS+ATP, and LPS+Nigericin, PBMCs from heterozygous and homozygous *NLRP3* rs10754555 G allele carriers released significantly more IL-1β compared to cells from non-carriers (**Figures 3a-c**). Unstimulated monocytes did not release detectable concentrations of IL-1β. To determine the specificity of these findings, release of IL-6 and tumor necrosis factor (TNF) into cell culture supernatants was quantified (**Supplementary Figure 2b-g**), which did not differ according to rs10754555 variant carrier status. To prove the relevance of rs10754555 *in vivo*, we transplanted NOD-SCID mice with human PBMCs from non-carriers and homozygous rs10754555 carriers and subjected them to perivascular carotid injury, a mouse model for re-endothelialization, which we have recently shown to be NLRP3 dependent (15) (**Figure 3d-e**). Re-endothelialization was significantly impaired in humanized mice receiving PBMCs from homozygous rs10754555 carriers, in which NLRP3 protein expression was higher as compared to non-carriers (**Figure 3f**).

Association between rs10754555 and the risk of coronary artery disease

Supplementary Tables 5-6 summarize the baseline characteristics of participants of the LURIC study population separated by rs10754555 genotype as well divided at age of 60 years. Minor allele frequency (G) for rs10754555 was 39.9 %. The prevalence of traditional CV risk factors such as age, sex, body mass index (BMI), smoking, hypertension, as well as lipid parameters did not differ between non-carriers and carriers of the rs10754555 *NLRP3* G allele. Moreover, there was no significant difference in the medication across different rs10754555 genotypes (**Supplementary Table 7**). Since there was a trend towards lower prevalence of hypertension and diabetes in homozygous rs10754555 carriers in LURIC, we assessed the association between rs10754555, blood pressure, and presence of hypertension in UKBiobank, which did not differ significantly between the groups (**Supplementary Table 8**), whereas the prevalence of diabetes was higher in rs10754555 G allele carriers. In homozygous rs10754555 G allele carriers, the risk for CAD and severe CAD was significantly higher as compared to non-carriers (**Figure 4a**, **Supplementary Table 9**). This association was present in participants below

60 years of age (odds ratio [OR] for prevalent CAD: 2.04, 95% CI 1.15-3.61; OR for severe CAD: 2.28, 95% CI 1.29-4.01), but not in those above 60 years (OR for prevalent CAD: 0.83, 95% CI 0.55-1.25; OR for severe CAD: 0.73, 95% CI 0.49-1.03) revealing an age-dependent association of rs10754555 with the development of atherosclerotic CVD. We confirmed these findings in the GerMIFS studies II-VII with individual patient data available. Importantly, also in GerMIFS, rs10754555 was associated with a higher risk for CAD in subjects aged below 60 years (OR 1.12, 95% CI 1.02-1.22, **Figure 4b**, **Supplementary Table 10**).

Association between rs10754555 and CV mortality

In LURIC, all-cause and CV mortality were significantly higher in heterozygous (hazard ratio [HR]: 1.26, 95 % CI: 1.08-1.45 and 1.22, 95 % CI: 1.01-1.47) and homozygous (HR: 1.31, 95 % CI: 1.08-1.59 and 1.35, 95 % CI: 1.07-1.72) rs10754555 variant carriers (**Supplementary Table 11**). There was no association between rs10754555 and other clinical endpoints such as fatal cancer or fatal infection (**Supplementary Table 12**). Interestingly, the percentage of rs10754555 G allele carriers decreased with increasing age (**Supplementary Table 13**). **Supplementary Figure 3** compares the effect of the rs10754555 genotype with other CV risk factors. Furthermore, we assessed the association between rs10754555 genotypes and CV mortality in ten prospective clinical trials enrolling 526,091 subjects with or without pre-existing CAD. Baseline characteristics for each individual study are shown in **Supplementary Tables 14-22**. Analyses were performed at an individual participant level. Additive genetic models show that the rs10754555 genotype is associated with significantly higher CV mortality in subjects from secondary prevention studies (HR 1.14, 95 % CI 1.07-1.21) and in subjects from primary prevention studies (HR 1.06, 95 % CI 1.01-1.11), without significant heterogeneity (I^2=22.2%, P=0.253 for secondary prevention studies and I^2=0.0%, P=0.999 for primary prevention studies, **Figure 5a-b**). Small-study effects were excluded using the Egger test (P=0.341 for meta-analysis on CV mortality in secondary prevention studies).

Known NLRP3 inflammasome activators and the association between rs10754555 and mortality

Several endogenous NLRP3 inflammasome activators have been identified, of which ApoC3, triglycerides, and urate are of particular importance in CV diseases. The release of IL-1β from PBMCs stratified according to the rs10754555 genotype was modulated by baseline triglyceride or urate concentrations (**Supplementary Figure 4a-f**, **Supplementary Table 23-24**). Furthermore, PBMCs from heterozygous or homozygous rs10754555 carriers released significantly higher concentrations of IL-1β after stimulation of ApoC3 or monosodium urate (**Supplementary Figure 5**). Therefore, we assessed the association between rs10754555 and CV mortality with respect to the ApoC3, triglyceride, or urate plasma levels. In LURIC, rs10754555 was only associated with CV mortality in subjects with high ApoC3 and triglyceride plasma levels (i.e. in the 4th quartile, **Figure 6a-b**, **Supplementary Tables 25-26**). This was confirmed in subjects of the UKBiobank and was independent of age and also present in subjects with elevated triglycerides due to SNPs in the *APOC3* gene locus (**Figure 6b**, **Supplementary Tables 27-30**). Vice versa, in UKBiobank, triglyceride plasma levels were associated with higher CV mortality (HR 1.17, 95 % CI 1.08-1.26) in the total population, with the strongest effect in homozygous rs10754555 carriers (**Supplementary Table 31**, HR 1.57, 95% CI 1.30-1.90). Similar results were obtained when participants of LURIC and UKBiobank were dichotomized according to urate plasma levels or carriers of SNPs associated with higher urate (**Figure 6c**, **Supplementary Tables 32-37**).

Discussion

The main and novel finding of this study is that genetically-determined sterile inflammation mediated by a specific cellular pathway (i.e. NLRP3) associates with higher prevalence of CAD and higher CV mortality. These associations are particularly prominent in the younger population, in which the influence of genetic predisposition likely predominates over life-style and environmental risk factors for (premature) CVD. Moreover, these findings highlight the NLRP3 inflammasome as a pathophysiologically important pathway and a potential therapeutic target.

Sterile inflammation is a hallmark of patients with atherosclerotic CVD (1), with experimental data showing a pivotal role of the NLRP3 inflammasome. In *NLRP3*- and in *IL1B*-deficient mice, atherosclerotic lesion formation was markedly reduced (9, 16). Nevertheless, the effect of NLRP3 on atherosclerosis is dependent on the experimental atherosclerosis model, the type of atherogenic diet, and the gender of the mice (17). NLRP3 inflammasome activation and subsequently enhanced IL-1β production has been linked to maladaptive vascular remodeling after injury and adverse endothelial activation (18, 19). Acceleration of atherosclerosis by clonal hematopoiesis is partially mediated by NLRP3-dependent IL-1β secretion (20, 21) and attenuated in subjects with genetic IL-6 signaling deficiency due to missense mutations of the IL-6 receptor (22).

Our study links genetically-driven inflammation with CVD prevalence and outcomes. An intronic variant within the *NLRP3* locus has been identified, which is not associated with other CV risk factors or alterations of lipids, but appears to specifically increase systemic (micro)inflammation. rs10754555 represents an intronic *NLRP3* variant, which is scored as *NLRP3* eQTL by the provided evidence. Moreover, rs10754555 maps with promoter and enhancer histone marks, and with DNAse I-sensitive regions. This indicates that rs10754555 might indeed associate with increased *NLRP3* mRNA transcription. Accordingly, rs10754555 was identified as *NLRP3* eQTL in whole blood and PBMCs in the eQTLGen consortium. Importantly, our experimental studies show that the rs10754555 genotype is associated with higher *NLRP3* mRNA expression, higher IL-18 plasma levels, increased ASC speck formation and inflammasome activation in human monocyte-enriched PBMCs, which represent a major inflammatory effector cell type in blood (1). Moreover, we have shown that PBMCs from rs10754555 G allele carriers suppressed re-endothelialization in humanized mice. The release of IL-6 and TNF from

monocytes treated with known NLRP3 activators was not linked to the rs10754555 carrier status. This indicates that this genetic variant is not associated with unspecific pro-inflammatory cell activation, but specifically with NLRP3 inflammasome activation.

rs10754555 was only associated with higher risk for CAD in subjects aged below 60 years. This SNP-age interaction was confirmed in the GerMIFS studies and by applying the same age cutoff. SNP-environment interactions and in particular SNP-age interactions were reported for CVD-relevant SNPs but also for SNPs in genes involved in inflammation such as *IL1RL1* (23-26). This observation points to an interaction between age and NLRP3 activation. Although the NLRP3 inflammasome is associated with a functional decline in aging (27, 28), *NLRP3* gene expression and NLRP3 inflammasome activation have been reported to decline with age (29, 30). Moreover, we found that the percentage of heterozygous and homozygous rs10754555 carriers decreased with increasing age, which could explain the lack of association between rs10754555 and CAD in the elderly.

Importantly, rs10754555 is associated with CV mortality, but not mortality related to infection or cancer. Our validation cohorts comprise a wide range of different patient populations including patients with prevalent CAD as well as subjects from the general population. Across these studies, the rs10754555 *NLRP3* variant was consistently associated with increased CV mortality. Moreover, the high frequency of the risk allele (MAF 39.9 %) indicates that increased NLRP3 inflammasome activity might contribute substantially to CV mortality on the population level. In agreement with the pre-clinical data on the association between NLRP3 inflammasome activation and atherosclerosis (9), our study shows that the rs10754555-mediated increase in all-cause mortality is mainly driven by CV deaths. These data highlight an important role of the innate immune system in the pathophysiology of CVD. Similar to NLRP3, gain-of-function mutations within the interleukin-6 receptor locus were found to be associated with increased risk for CAD (31, 32).

Indeed, in animal studies, inhibition of the NLRP3 inflammasome by the selective, small-molecule inhibitor MCC950 reduced experimental autoimmune encephalomyelitis and myocardial infarction (33, 34). Compelling evidence for the benefit of therapeutically targeting NLRP3-dependent pathways is provided by studies using the monoclonal, IL-1β-targeting antibody canakinumab. In patients after MI with persistently elevated hsCRP, canakinumab lowered the rate of recurrent CV events

by 15 %, when 150 mg of canakinumab were administered (11). Posthoc analyses of the CANTOS trial revealed persistently elevated levels of the NLRP3-dependent cytokine IL-18, which are unaffected by canakinumab treatment and still associated with future CV events (35). Therefore, inhibition of the NLRP3 inflammasome or its assembly in contrast to the specific inhibition of one effector cytokine, could potentially provide a stronger reduction of CV events. Nevertheless, IL-1β release can also be induced by other inflammasome sensors such as Absent in melanoma 2 (AIM2), which is activated by double-stranded DNA by exogenous pathogens and also during tissue damage (36). In addition to canakinumab, treatment with colchicine, which modulates the NLRP3 inflammasome, reduces CV events in patients post MI with a stronger effect as compared to canakinumab (12). Since treatment with canakinumab or colchicine are associated with potential serious adverse events, strategies to select patients for targeted treatment are necessary. Screening for genetic variants associated with NLRP3 activation and subsequently elevated IL-1β and IL-18 may help to identify subjects with increased risk for CV events – especially at young age - as a result of sustained (micro)inflammation particularly when plasma levels of known inflammasome activators such as ApoC3, triglycerides, or urate are elevated.

Some limitations of our study should be considered. Although our results highlight the NLRP3 inflammasome as a potential factor promoting CV mortality, further studies are needed to prove that in particular carriers of the rs10754555 *NLRP3* variant benefit from a specific anti-inflammatory treatment. In the present study, the *NLRP3* variant rs10754555 is linked to mortality. Based on the study designs, we cannot show an association between the mutant carrier status and non-fatal CV events. The age-rs10754555 on CAD risk could not have been validated in CARDIOGRAM due to limited access to individual patient data. Therefore, this interaction was validated in the GerMIFS studies (N=6,389 CAD cases and N=5,687 controls), which are part of the CARDIOGRAM consortium.

In conclusion, this is the first study to demonstrate the association between genetically-driven inflammation and CV diseases by engaging a specific pro-inflammatory pathway (i.e. the NLRP3 inflammasome). These findings set the stage for individualized treatments in subjects with inflammation-driven high CV risk.

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Figure legends

Figure 1. Identification of SNPs regulating *NLRP3* expression.

a Variant prioritization approach. **b** Expression quantitative trait locus (eQTL) meta-analysis for rs10754555 in whole blood or PBMCs in the eQTLGen consortium comprising 31,556 samples from 36 cohorts. **c** Age and sex adjusted least square means of high sensitivity C-reactive protein (hsCRP) and **d** serum amyloid A (SAA) in 3,061 participants of the LURIC study (mean±95 % CI).

Figure 2. Functional effects of rs10754555 on expression of *NLRP3* and inflammasome activation in freshly isolated human PBMCs.

a Experimental work-flow. **b** mRNA expression of *NLRP3* in freshly isolated PBMCs. **c** Plasma levels of IL-18 and **d** and IL-1β according to rs10754555 genotype. **e** Representative fluorescence microscopy of Alexa Fluor-488 labeled ASC specks from plasma and GFP-ASC in the supernatant of THP-1 cells (Representative of three independent experiments). **f** Mean fluorescence intensity (MFI) of ASC specks in plasma samples according to rs10754555 genotype. **g** Representative flow cytometry images of ASC speck quantification in plasma. Each dot represents an individual patient, whiskers of the box plots represent 5 and 95 percentiles.

Figure 3. Modulation of NLRP3 inflammasome response by rs10754555 in freshly isolated human PBMCs and humanized mice.

a-c Concentration of IL-1β in the supernatant of freshly isolated PBMCs stimulated with lipopolysaccharide (LPS, 10 ng/ml, 3 hrs), LPS (3 hrs) and ATP (5 mM, 1 hr), LPS (3 hrs) and Nigericin (1 µM, 1 hr). Each dot represents an individual patient, whiskers of the box plots represent 5 and 95 percentiles. **d** Experimental outline of the murine perivascular carotid injury model in NOD-SCID mice transplanted with human PBMCs (i.e. humanized mice). **e** Re-endothelialized area 72 hrs after carotid injury in humanized mice and representative microphotographs. **f** Western blot of NLRP3 protein expression in transplanted PBMCs from nine individual donors. Mean ± 95% CI.

Figure 4. Risk of coronary artery disease (CAD) as a function of rs10754555 genotype.

a Odds ratios for prevalent CAD and severe CAD (visual stenosis ≥50 % in coronary angiography) according to rs10754555 genotype in 3,061 participants of the LURIC study divided into two groups at age of 60 years (first tertile of age) and **b** in a meta-analysis of 12,076 participants with individual patient data available included in GerMIFS. Results are adjusted for age and sex.

Figure 5. Risk of cardiovascular mortality as a function of rs10754555 genotype.

Random-effects meta-analysis on cardiovascular mortality associated with rs10754555 genotype in **a** secondary and **b** primary prevention cohorts/studies. Shown are the hazard ratios for cardiovascular mortality associated with rs10754555 *NLRP3* variant in 33,488 participants from 8 studies comprising patients with prevalent CAD (i.e. secondary prevention), and in 492,603 participants from 2 studies from the general population. Analyses from each individual study were adjusted for age and gender.

Figure 6. ApoC3, triglycerides, and urate modulate the association between rs10754555 and cardiovascular mortality.

a Association between rs10754555 genotype and cardiovascular mortality in 3,061 participants of the LURIC study divided in subjects with low (≤17.3 mg/dL, quartile 1-3) and high (>17.3 mg/dL, quartile 4) ApoC3 plasma levels. **b** Association between rs10754555 genotype and cardiovascular mortality in LURIC study and in 483,258 participants of UKBiobank divided in subjects with low (≤201 mg/dL, quartile 1-3) and high (>201 mg/dL, quartile 4) triglyceride plasma levels. **c** Association between rs10754555 genotype and cardiovascular mortality in LURIC and in UKBiobank divided in subjects with low (≤5.1 mg/dL, quartile 1-3) and high (>5.1 mg/dL, quartile 4) urate plasma levels. Interaction refers to the interaction term between ApoC3, triglycerides, or urate and rs10754555 included in the Cox regression models.