

# Loss of Y Chromosome in Blood Is Associated With Major Cardiovascular Events During Follow-Up in Men After Carotid Endarterectomy

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**Background**—Recent studies found an immune regulatory role for Y chromosome and a relationship between loss of Y chromosome (LOY) in blood cells and a higher risk of cancer and mortality. Given involvement of immune cells in atherosclerosis, we hypothesized that LOY is associated with the severity of atherosclerotic plaque characteristics and outcome in men undergoing carotid endarterectomy.

**Methods and Results**—LOY was quantified in blood and plaque from raw intensity genotyping data in men within the Athero-Express biobank study. Plaques were dissected, and the culprit lesions used for histology and the measurement of inflammatory proteins. We tested LOY for association with (inflammatory) atherosclerotic plaque phenotypes and cytokines and assessed the association of LOY with secondary events during 3-year follow-up. Of 366 patients with carotid endarterectomy, 61 exhibited some degree of LOY in blood. LOY was also present in atherosclerotic plaque lesions (n=8/242, 3%). LOY in blood was negatively associated with age ( $\beta=-0.03/10$  y;  $r^2=0.07$ ;  $P=1.6\times 10^{-7}$ ) but not with cardiovascular disease severity at baseline. LOY in blood was associated with a larger atheroma size (odds ratio, 2.15; 95% confidence interval, 1.06–4.76;  $P=0.04$ ); however, this association was not significant after correction for multiple testing. LOY was independently associated with secondary major cardiovascular events (hazard ratio=2.28; 95% confidence interval, 1.11–4.67;  $P=0.02$ ) in blood when corrected for confounders.

**Conclusions**—In this hypothesis-generating study, LOY in blood is independently associated with secondary major cardiovascular events in a severely atherosclerotic population. Our data could indicate that LOY affects secondary outcome via other mechanisms than inflammation in the atherosclerotic plaque. (*Circ Cardiovasc Genet.* 2017;10:e001544. DOI: 10.1161/CIRCGENETICS.116.001544.)

**Key Words:** atherosclerosis ■ cardiovascular diseases ■ cytokines ■ genetics ■ inflammation

Loss of the Y chromosome (LOY) in blood cells was already described in the 1960s and affects  $\approx 15\%$  of the male population of older age.<sup>1</sup> Only recently, LOY was associated with a higher risk of (nonhematological) cancer and overall mortality.<sup>2,3</sup> This relationship was speculated to be because of smoking and a disrupted tumor immunosurveillance.<sup>4</sup> Furthermore, LOY was associated with Alzheimer disease<sup>5</sup> and the occurrence of autoimmune diseases, such as primary biliary cirrhosis<sup>6</sup> and autoimmune thyroiditis.<sup>7</sup>

The Y chromosome exhibited an immunoregulatory function by acting as a global transexpression quantitative trait locus in mice.<sup>8</sup> The Y chromosome directly mediated changes in the transcriptome of CD4<sup>+</sup> T cells and macrophages, contributing to altered gene expression and alternative splicing. A role in global immune response was also found in the monocyte and macrophage transcriptome results of males with haplotype I that exhibited a 50% greater risk of myocardial infarction.<sup>9</sup> Comparison of gene expression data between haplotype I and other haplotypes revealed pathways that are related to inflammation and immunity, revealing downregulation of adaptive immunity and upregulation of inflammatory response in haplotype I carriers.

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Genetic variation on the Y chromosome has been associated with high blood pressure<sup>10</sup> and myocardial infarction,<sup>11</sup> independent of traditional cardiovascular risk factors, sex steroids, or aggression. Given the global immunoregulatory role of the Y chromosome and the involvement of immune cells in atherosclerosis together with its male predominance, we hypothesized that LOY is associated with more severe atherosclerosis leading to worse outcome in men undergoing carotid endarterectomy (CEA).

## Methods

### Patient Characteristics

The Athero-Express biobank study is an ongoing cohort study that includes atherosclerotic plaques and blood of patients undergoing either CEA or femoral endarterectomy in 2 large tertiary referral hospitals (University Medical Center Utrecht and St Antonius hospital Nieuwegein) in the Netherlands. Clinical data were obtained from medical files and standardized questionnaires. Age was determined as age at surgery. Current smoking was determined as patient-reported smoking in the past year. Hypertension and hypercholesterolemia were self-reported. Diabetes mellitus was considered present in any of the following cases: use of insulin or oral glucose inhibitors, self-reported diabetes mellitus in the patient questionnaire, or diabetes mellitus extracted from the medical file. A history of coronary artery disease was considered present if the patient had experienced a myocardial infarction or underwent a percutaneous coronary intervention or coronary artery bypass grafting surgery. Peripheral arterial occlusive disease was considered present if the patient either presented with an ankle-brachial index <0.7, claudication complaints, or underwent percutaneous or surgical intervention for peripheral arterial occlusive disease. Follow-up was obtained by questionnaires sent to the patients by mail 1, 2, and 3 years post-operatively. Major cardiovascular events ([sudden] cardiovascular death, hemorrhagic or ischemic stroke, myocardial infarction, fatal heart failure, or fatal aneurysm rupture) were validated using medical records. The medical ethics boards of both hospitals approved of the study, which is conducted in accordance with the declaration of Helsinki, and the subjects gave informed consent.

### Sample Collection

A detailed description of the sample phenotyping within the Athero-Express study can be found elsewhere.<sup>12</sup> In short, blood was obtained before surgery and subsequently stored at  $-80^{\circ}$ . Plaque specimens were immediately processed after removal during surgery. After identification of the area with the largest plaque burden (culprit lesion), the plaque was cut transversely into segments of 5 mm. The culprit lesion was fixed in 4% formaldehyde and subsequently decalcified and embedded in paraffin. Cross-sections were stained for histological examination. Remaining segments were stored at  $-80^{\circ}$  and used for the measurement of inflammatory cytokines and isolation of DNA.

### Histological Assessment of Specimens

Plaque specimens were stained using CD68 (macrophages),  $\alpha$ -actin (smooth muscle cells), picro-sirius red (collagen), and CD34 (microvessels). Furthermore, the presence of plaque thrombosis was determined using a combination of luminal thrombi, intraplaque hemorrhage, hematoxylin and eosin staining, and Mallory phosphotungstic acid-hematoxylin staining (fibrin). Either luminal thrombus, intraplaque hemorrhage, or both were considered presence of plaque thrombosis. Computerized analyses quantitatively assessed macrophages and smooth muscle cells as percentage of plaque area. Microvessels were identified morphologically and counted in 3 hotspots and subsequently averaged per slide. Collagen and calcifications were scored semiquantitatively into no (1), minor (2), moderate (3), or heavy (4) staining at  $\times 40$  magnification. These categories were grouped into bins (no/minor and moderate/heavy)

for the present analyses. The size of the lipid core was assessed using polarized light and cut off at an area of 10% and 40% of the plaque. All histological slides were assessed by the same dedicated technician.

### Cytokine Measurements of Specimens

To determine the effect of LOY on inflammatory phenotypes within the Athero-Express biobank, we analyzed the association between LOY and 7 different inflammatory cytokines: interleukin-6 and tumor necrosis factor- $\alpha$  as proinflammatory cytokines, interleukin-10 as an anti-inflammatory cytokine, regulated on activation, normal T cell expressed and secreted as a marker of T cell involvement, and monocyte chemoattractant protein-1, macrophage colony-stimulating factor, and Growth Differentiation Factor-15 as markers of macrophage involvement. Cytokines were measured by Luminex in plaque lysate (interleukin-6, tumor necrosis factor- $\alpha$ , interleukin-10, regulated on activation, normal T cell expressed and secreted, monocyte chemoattractant protein-1, and macrophage colony-stimulating factor) or citrate plasma (Growth Differentiation Factor-15) and normalized to protein content.

### Genotyping Data and Quality Control

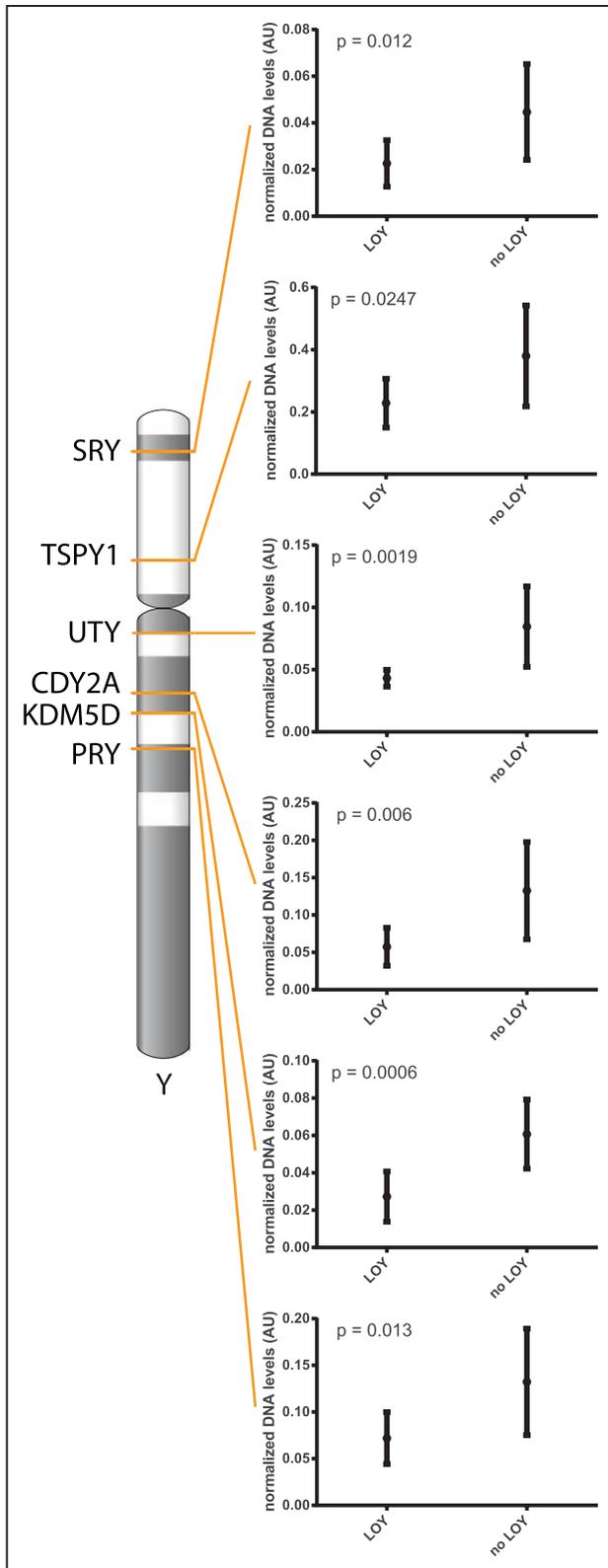
The methods of the Athero-Express Genomics Study have been described before.<sup>13</sup> Genome-wide single-nucleotide polymorphism genotyping data were collected in 1858 consecutive patients with CEA using DNA from blood or plaque (when no blood was available) and either the Affymetrix Genome-Wide Human SNP Array 5.0 (AEGS1) or the Affymetrix Axiom GW CEU 1 Array (AEGS2). The quality control pipeline consisted of first excluding samples with low average genotype calling and sex discrepancies based on Affymetrix Genotyping Console 4.0 Software metrics and thereafter filtering samples with a call rate >97%, variant call rate >97%, minor allele frequencies >3%, average heterozygosity rate  $\pm 3.0$  SDs, relatedness ( $\pi$ -hat >0.20), Hardy-Weinberg equilibrium ( $P < 1.0 \times 10^{-6}$ ), and based on population stratification (excluding samples >6 SDs from the average in 5 iterations during principle component analysis and by visual inspection).

After quality control, we kept 1640 samples for downstream analyses that were imputed using HapMap 2 CEU. For the current study, only the male samples of the AEGS2 ( $n=610$  total) could be used as the AEGS1 array does not contain Y chromosomal single-nucleotide polymorphisms.

### Determination of Loss of Y

To assess LOY, median log<sub>2</sub> ratios (observed intensity/reference intensity) were computed based on the raw intensity data from the male-specific Y chromosomal probes (mLRRY), excluding Pseudo-Autosomal Region 1 and Pseudo-Autosomal Region 2. Two blood samples were excluded because of outlying positive mLRRY values (defined as 1.5 interquartile ranges above the third quartile), leaving 366 blood samples and 242 plaque samples for analysis. We first calculated the peak of each mLRRY histogram using the density function in R for kernel density estimation as previously described.<sup>2</sup> Next, a noise distribution was derived to compute the cut-off value for LOY. To this end, the positive tail of the kernel density was mirrored over the distribution peak of the kernel density estimates (local median), generating a negative tail. The lower bound of the resulting distribution served as the cut-off value for LOY (Figure 1 in the Data Supplement).

As a validation, LOY was assessed by quantitative polymerase chain reaction of 6 Y chromosomal genes along the Y chromosome in 9 patients who exhibited dichotomous LOY and 8 patients who did not exhibit dichotomous LOY. Presence of 1 of the genes (TSPY1) was assessed by a commercially available kit (Y chromosome detection real-time polymerase chain reaction assay, Primerdesign Ltd). Primer design of the other 5 primers can be found in Table 1 in the Data Supplement. Detected DNA content between patients with and without LOY was compared using *t* tests and significant for all genes (Figure 1). Primers were first tested on a female control, and all yielded no DNA measurement in that sample.



**Figure 1.** Quantitative polymerase chain reaction of Y chromosomal genes. AU indicates arbitrary units; and LOY, loss of the Y.

### Replication Cohort

Replication of the Cox proportional hazards analysis on secondary cardiovascular events was performed in the AAA-Express.<sup>14</sup> The AAA-Express started as a spin-off of Athero-Express. AAA-Express is a biobank with patients who underwent open aneurysm repair in

the University Medical Center Utrecht and St. Antonius Hospital Nieuwegein between 2003 and 2013. Clinical characteristics, genotyping data (using Illumina Human Core Exome chip), and 3-year follow-up data on secondary cardiovascular events were present for 202 blood samples. Collection of data, including quality control of the single-nucleotide polymorphism data and determination of LOY in this cohort, was performed in the same way as in the Athero-Express cohort.

### Statistical Analyses

Binary LOY in blood was associated with baseline characteristics using  $\chi^2$  tests, *t* tests, and Wilcoxon signed-rank tests, where applicable, to determine possible confounders. The data were imputed using single imputation. All variables with a  $P < 0.1$  (age, body mass index, glomerular filtration rate, smoking, and hypertension) were put into a backstep multivariable model to determine their association with LOY. Remaining significant variables (age and smoking) were put into a multivariable model to assess whether LOY associates with severity of disease characteristics and box-cox transformed plaque phenotypes and inflammatory markers. A Cox proportional hazards model with all covariates that univariably associated with outcome (only age) was used to determine the association between LOY and major cardiovascular events during 3-year follow-up. Cox proportional hazards analysis in AAA included age as a covariate. Meta-analysis of the Athero-Express and AAA-Express cohorts was performed using inverse variance weighting on the models corrected for age. The proportional hazards assumption was assessed using scaled Schoenfeld residuals. Values  $P < 0.05$  were considered significant. The multiple testing threshold for plaque characteristics and inflammatory cytokines was set at 0.05/15 tests = 0.003. All statistical analyses were performed using the R computing platform, version 3.0.2.

## Results

### Loss of Y in Blood

We determined median log<sub>2</sub> ratios of Y chromosomal intensity (mLRRY) in 608 patients; in 366 patients, we used blood-derived DNA. Median log<sub>2</sub> ratios of Y chromosomal probes in these patients were negatively associated with age ( $\beta = -0.03/10$  y;  $r^2 = 0.07$ ;  $P = 1.6 \times 10^{-7}$ ; Figure I in the [Data Supplement](#)). Of the 366 patients, 61 (17%) exhibited dichotomous LOY chromosome in blood defined as mLRRY  $< -0.075$  (Table 1; Figure 1; Figure II in the [Data Supplement](#)). A trend was seen for more smoking, a lower body mass index, and less hypertension in the LOY group. No other baseline characteristics were found to differ between patients with and without LOY in blood (Table 1).

### Loss of Y in Plaque

Within 242 patients, we determined mLRRY in atherosclerotic plaque tissue. Median log<sub>2</sub> ratios of Y chromosomal probe intensity in plaque were also negatively associated with age ( $\beta = -0.02/10$  y;  $P = 5.02 \times 10^{-8}$ ; Figure I in the [Data Supplement](#)). Of the 242 patients, 8 (3%) exhibited dichotomous LOY in plaque defined as median log<sub>2</sub> value of Y chromosomal intensity  $< -0.129$  (Figure II in the [Data Supplement](#)). Because only 8 patients experienced LOY in plaque, we performed our analyses only on patients of whom we had blood-derived DNA.

### No Loss of Chromosome 21

LOY could be a sign of general intensity loss throughout the genome. We, therefore, determined whether we could find any

**Table 1. Baseline Characteristics of Patients With and Without LOY in Blood**

	Loss of Y (n=61)	No Loss of Y (n=305)	P Value
Age in years (IQR)	75 (69–79)	69 (62–75)	<0.001
BMI (IQR)	24.9 (23.5–27.0)	25.9 (24.1–28.4)	0.08
Current smoker, yes (%)	25/60 (42)	88/303 (29)	0.08
Diabetes mellitus, yes (%)	10/61 (16)	73/305 (24)	0.26
Hypertension, yes (%)	33/59 (56)	203/296 (69)	0.08
Hypercholesterolemia, yes (%)	31/53 (58)	187/281 (67)	0.33
History of coronary artery disease (%)	19/61 (31)	94/305 (31)	1
History of PAOD (%)	12/61 (20)	62/305 (20)	1
Use of antiplatelet therapy (%)	56/60 (93)	271/304 (89)	0.45
Use of lipid-lowering drugs (%)	44/61 (72)	244/305 (80)	0.23
Bilateral carotid stenosis (%)	17/48 (35)	129/266 (48)	0.13
GFR (MDRD) mL/min per 1.73 m <sup>2</sup> (SD)	68.7 (58.6–82.7)	74.5 (60.4–87.2)	0.12
LDL in mg/dL (IQR)	105 (86–127)	94 (70–124)	0.29
HDL in mg/dL (IQR)	41 (33–43)	39 (32–47)	0.52
Total cholesterol in mg/dL (IQR)	174 (148–186)	162 (135–200)	0.66
Triglyceride levels in mg/dL (IQR)	98 (80–148)	123 (89–177)	0.12
<b>Presenting symptoms (%)</b>			
Asymptomatic	4/60 (7)	42/302 (14)	0.27
TIA	39/60 (65)	172/302 (57)	
Stroke	17/60 (28)	88/302 (29)	

BMI indicates body mass index; GFR, glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; LOY, loss of the Y; MDRD, modification of diet in renal disease; PAOD, peripheral arterial occlusive disease; and TIA, transient ischemic attack.

evidence for loss of chromosome 21. We found a median log 2 ratio of intensity of chromosome 21 probes that was  $\approx 0$ , without any evidence for an association with age (Figure III in the [Data Supplement](#)).

### Association With Smoking

Previous studies point toward a role of smoking in LOY. Past smokers and current smokers exhibited a lower mLRRY than never smokers (Figure IV in the [Data Supplement](#)). We observed an association between mLRRY and smoking when corrected for age ( $\beta -0.02$  for current smokers compared with nonsmokers;  $P=0.03$ ). In a backward step model, age and smoking were found to be most predictive of LOY (Akaike information criterion for model with only age and smoking=307.25 versus Akaike information criterion for model with age, smoking, body mass index, glomerular filtration rate, and hypertension=310.79). Corrected for age, smoking was associated with dichotomous LOY (odds ratio, 2.83 [95% confidence interval, 1.50–5.35];  $P=0.001$ ).

### Association With Plaque Phenotypes

Because dichotomous LOY showed the largest effect on baseline characteristics, this measure was used to investigate the association between LOY and plaque characteristics and secondary cardiovascular outcome. To investigate whether

LOY in blood was associated with a more vulnerable plaque phenotype, we assessed the association between dichotomous LOY in blood and 7 classical plaque characteristics: amount of calcification, amount of collagen, atheroma size, presence of intraplaque hemorrhage, macrophage, smooth muscle cell content, and vessel density within the plaque. Furthermore, we assessed the association between dichotomous LOY in blood and specific inflammatory or anti-inflammatory cytokines within the atherosclerotic plaque. Corrected for age and smoking, dichotomous LOY in blood was nominally associated with a >10% atheroma size (odds ratio, 2.15 [1.06–4.76];  $P=0.04$ ; Table 2; Figure V in the [Data Supplement](#)).

### Association With Secondary Cardiovascular End Points

To determine whether dichotomous LOY in blood has an influence on secondary cardiovascular endpoints during follow-up, we used a Cox proportional hazard model correcting for age because this was the only LOY-associated baseline characteristic ( $P<0.1$ ) that was also associated with major cardiovascular endpoints. During 3 years of follow-up, men with dichotomous LOY in blood had significant more major cardiovascular endpoints (HR=2.28; 95% confidence interval, 1.11–4.67;  $P=0.02$ ; Figure 2). We replicated the direction of this effect

**Table 2. Associations of LOY With Measures of (Inflammatory) Plaque Phenotypes**

Plaque Phenotype	$\beta$ of LOY (95% CI)	Odds Ratio of LOY (95% CI)	P Value
Atheroma size (>10%)	NA	2.15 (1.06 to 4.76)	0.04
Atheroma size (>40%)	NA	1.84 (0.98 to 3.41)	0.05
Calcification (major)	NA	0.86 (0.47 to 1.58)	0.62
Collagen (major)	NA	0.82 (0.39 to 1.64)	0.59
Intraplaque hemorrhage (present)	NA	0.87 (0.48 to 1.58)	0.65
Macrophage (increase of plaque area)	0.19 (−0.19 to 0.57)	NA	0.33
Smooth muscle cells (increase of plaque area)	0.05 (−0.33 to 0.42)	NA	0.81
Vessel density (increase per field)	−0.005 (−0.05 to 0.04)	NA	0.84
IL-6 in plaque (per pg/mL plaque lysate)	−0.37 (−1.81 to 1.08)	NA	0.61
IL-10 in plaque (per pg/mL plaque lysate)	−0.45 (−1.56 to 0.67)	NA	0.41
TNF- $\alpha$ in plaque (per pg/mL plaque lysate)	−0.32 (−1.33 to 0.69)	NA	0.52
MCSF in plaque (per pg/ $\mu$ g plaque lysate)	0.17 (−0.34 to 0.68)	NA	0.51
RANTES in plaque (per pg/ $\mu$ g plaque lysate)	−0.23 (−0.88 to 0.43)	NA	0.50
MCP-1 in plaque (per pg/ $\mu$ g plaque lysate)	0.14 (−0.18 to 0.46)	NA	0.39
GDF-15 in plasma (per SD pg/mL plasma)	0.11 (−0.11 to 0.34)	NA	0.33

Models corrected for age and current smoking. Continuous variables are box-cox transformed. CI indicates confidence interval; GDF, growth differentiation factor; IL, interleukin; LOY, loss of the Y; MCP, monocyte chemoattractant protein; MCSF, macrophage colony-stimulating factor; NA, not applicable; RANTES, regulated on activation, normal T cell expressed and secreted; and TNF, tumor necrosis factor.

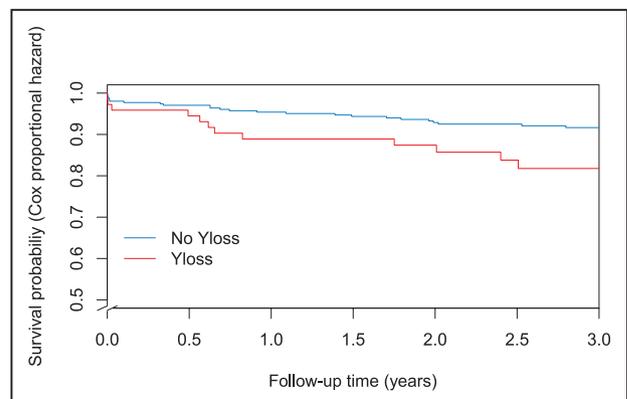
in the AAA-Express. Of the 202 patients, 29 exhibited LOY. During 3 years of follow-up, men with dichotomous LOY in blood had more major cardiovascular end points (HR=1.78 [0.54–5.85];  $P=0.34$ ; Figure VI in the [Data Supplement](#)). Meta-analysis of both cohorts confirmed the found effect (HR=2.13 [1.15–3.94];  $P=0.02$ ). Furthermore, we observed the same direction of effect when studying the association of mLRRY in Athero-Express and cardiovascular events during follow-up, corrected for age, although this did not reach statistical significance (HR=0.13 [0.01–1.33];  $P=0.09$ ). The effect was present in both smokers and nonsmokers (Figure VII in the [Data Supplement](#)). Atheroma size was not associated with major cardiovascular events during follow-up.

## Discussion

In this hypothesis-generating study in a population of male patients with CEA, LOY in blood was detectable in both peripheral blood, as well as in atherosclerotic lesions. Dichotomous LOY in blood was independently associated with a higher occurrence of major cardiovascular events during a 3-year follow-up period, and this effect was replicated in a second cohort of patients with cardiovascular disease. However, after correction for multiple testing, no associations were found between dichotomous LOY and systemic and local (plaque) inflammatory status, suggesting that alternate mechanisms may explain the association between LOY and outcome.

We hypothesized that LOY as an immunomodulating agent in the male genome would lead to a more severe type of cardiovascular disease by increased inflammation in the vascular wall, leading to a more unstable atherosclerotic

plaque phenotype, reflected by a macrophage-rich plaque phenotype with a larger lipid pool, more intraplaque hemorrhage, and more inflammatory cytokines. Although we found an increase in major cardiovascular events and some preliminary evidence pointing toward a larger lipid pool, we were unable to identify a more inflammatory atherosclerotic plaque in these patients bearing in mind correcting for the testing of 15 different inflammatory phenotypes. One of the reasons could be the different cell types in which we identified the LOY (blood) and in which we failed to observe an effect (plaque). However, both blood and plaque take part in the systemic inflammatory response in atherosclerotic disease and macrophages in the plaque derive from circulating monocytes. Furthermore, we also identified LOY in the atherosclerotic plaque itself. Interestingly, the amount of



**Figure 2.** Cox proportional hazards model for major event-free survival.  $P=0.02$ . Model corrected for age and current smoking.

patients with LOY in plaque was lower. Although we cannot be sure as to what cell type is responsible for the detectable LOY in plaque, this lower amount of LOY may possibly be because of the fact that the atherosclerotic plaque does not contain as many rapidly dividing cells as compared with peripheral blood. The difference between LOY in plaque and LOY in blood is also reflected by less variation of LOY between the plaque samples. It could also be because of the fact that the plaque is formed by invasion and division of cells during several decades, during which the Y chromosome is possibly not yet lost. In agreement, from experimental atherosclerosis studies, it has been established that plaque macrophages mostly derive from local proliferation rather than continuous infiltration.<sup>15</sup>

There are a few other possible explanations for the fact that we did not find any other association with plaque phenotype or inflammation. First, LOY could be so detrimental to the male body that all patients experiencing it die before they develop an operable form of atherosclerosis and thereby simply do not end up in our study. Second, LOY could influence atherosclerosis in an earlier phase of the disease, for example, affecting disease progression. Patients in the Athero-Express biobank experience severe end-stage disease and are, because of the operative guidelines, equally affected. Furthermore, a limitation of the current study is that it is limited in power to detect small but biologically relevant differences because of a relatively small sample size. With an event probability of 12%, to obtain 80% power for observing a hazard ratio of 2.0, one needs 1006 samples and we had only 366 (power of 29%).

A recent study found a relationship between LOY in blood and both (nonhematologic) cancer and overall mortality in healthy men from the longitudinal Uppsala Longitudinal Study of Adult Men cohort aged 71 to 84 years.<sup>2</sup> However, not all increased mortality risk during >40 years of follow-up could be attributed to malignant diseases. This leaves the question what is causing the other deaths unanswered. In a follow-up study, LOY was also associated with smoking, a risk factor for both cancer and death. Smoking, however, is also a major risk factor for cardiovascular disease. This increased risk is because of several factors, including inflammation but, for example, also coagulation, endothelial dysfunction, and adverse lipid profiles.<sup>16</sup> In our data, smoking was also significantly associated with mLRRY and with dichotomous LOY when corrected for age. Uncorrected, the absence of a significant association between smoking and dichotomous LOY may be explained by a lack of power (to obtain 80% power for observing a difference between 42% and 29%, 1 needs 580 samples [of which 20% LOY cases] and we had only 366). In a sensitivity analysis, we observed an effect in both smokers and nonsmokers. In summary, we found preliminary evidence to support the hypothesis that the association between LOY and mortality is through a higher risk of major cardiovascular events and that this association cannot be solely explained by smoking as a risk factor.

The mechanism by which the Y chromosome is lost remains elusive. A recent genome-wide approach identified *TCL1A* that is associated to hematological malignancies as a genetic susceptibility locus for LOY at chromosome 14.<sup>17</sup>

It might be that LOY chromosome reflects general genomic instability of which the small and last to be replicated Y chromosome is the first victim. Rapidly dividing cells might not take their time to replicate its telomeres, and this may lead eventually to loss of the entire chromosome. However, previous experiments blasting the Y chromosome apart have shown that it might be replicated and passed on to daughter cells, even when shattered into pieces even smaller than its original size.<sup>18</sup> Atherosclerosis might also accelerate genomic instability because of the formation of reactive oxygen species. However, we did not find a large proportion of LOY in the atherosclerotic plaque itself.

In our hypothesis-generating study, we found first preliminary evidence that LOY is independently associated with the occurrence of secondary major cardiovascular events in male patients after CEA. We replicated this effect in a cohort of male patients undergoing surgical aneurysm repair. More research is needed in a large sample of patients developing cardiovascular disease, preferably a cohort study that recorded cardiovascular disease incidence, to definitively answer the question how LOY is associated with adverse cardiovascular events and specify which events are most likely to be the cause of this association, whether or not smoking is the causative factor, and whether or not LOY is also associated with incidence or progression of cardiovascular disease.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

The Y chromosome was, until recently, considered genomic wasteland. However, recent studies found a much larger role for the Y chromosome than previously thought. One of the studied effects was loss of the Y chromosome (LOY) in peripheral blood cells. This LOY seems to be associated with a higher risk of Alzheimer disease, cancer, and overall mortality. Because of the found relationship with smoking and immune regulation, we speculated that there might be a role for LOY in cardiovascular disease too. We studied LOY in a population of patients operated on for carotid occlusive disease and studied the association with characteristics of the atherosclerotic plaque, as well as the occurrence of secondary cardiovascular events. We found no evidence for an association between LOY and characteristics of the plaque, but we observed an effect of LOY on secondary cardiovascular events (less Y chromosome=more events) independent of smoking status. We replicated this effect in a cohort of male patients undergoing surgical aneurysm repair. The mechanism of LOY and whether the observed effect of LOY on secondary cardiovascular events is causal remain elusive.

**Loss of Y Chromosome in Blood Is Associated With Major Cardiovascular Events During Follow-Up in Men After Carotid Endarterectomy**

Saskia Haitjema, Daniel Kofink, Jessica van Setten, Sander W. van der Laan, Arjan H. Schoneveld, James Eales, Maciej Tomaszewski, Saskia C.A. de Jager, Gerard Pasterkamp, Folkert W. Asselbergs and Hester M. den Ruijter

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## **SUPPLEMENTAL MATERIAL**

Supplemental table 1: Primers of the qPCR experiment

Supplemental figure 1: Determination of LOY cut off for blood and plaque samples

Supplemental figure 2: Association between median log<sub>2</sub> ratio of Y chromosomal probe intensity and age in blood and plaque.

Supplemental figure 3: Distribution of median log<sub>2</sub> ratio of chromosome 21 intensity and its association with age in blood.

Supplemental figure 4: Median log<sub>2</sub> ratio of Y chromosomal probe intensity for smoking history (past smoker, current smoker, never smoker)

Supplemental figure 5: Percentage of patients with and without LOY with small and large atheroma size

Supplemental figure 6: Association between LOY and major cardiovascular event-free survival in AAA-Express

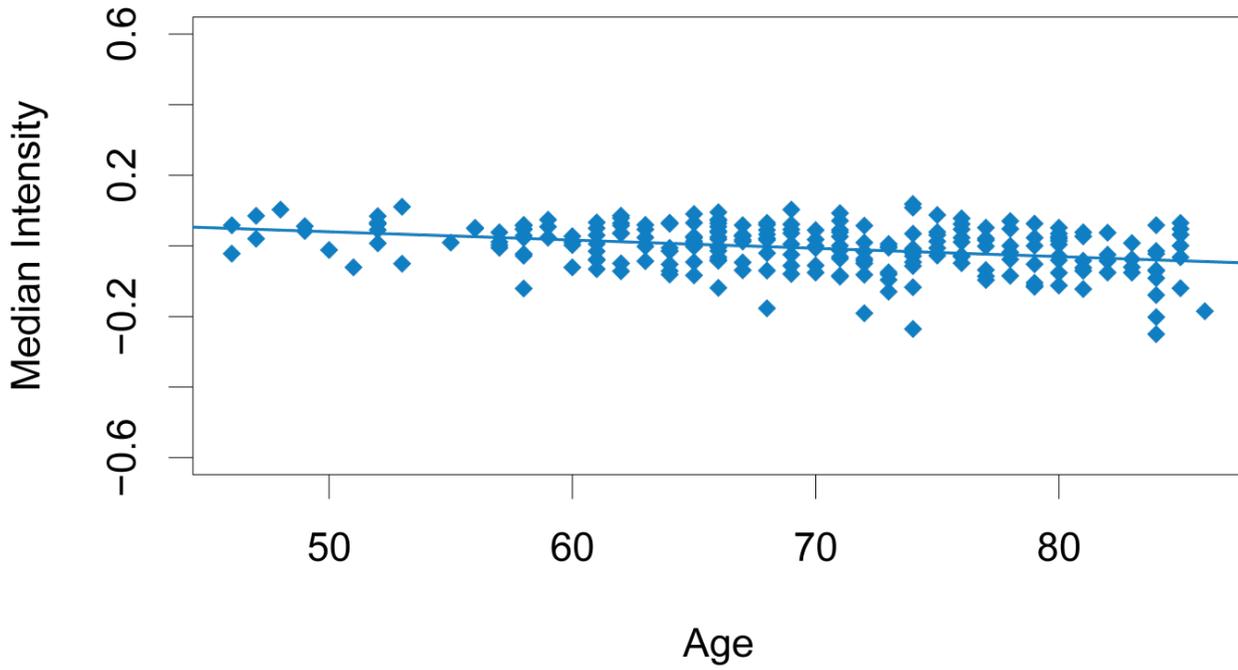
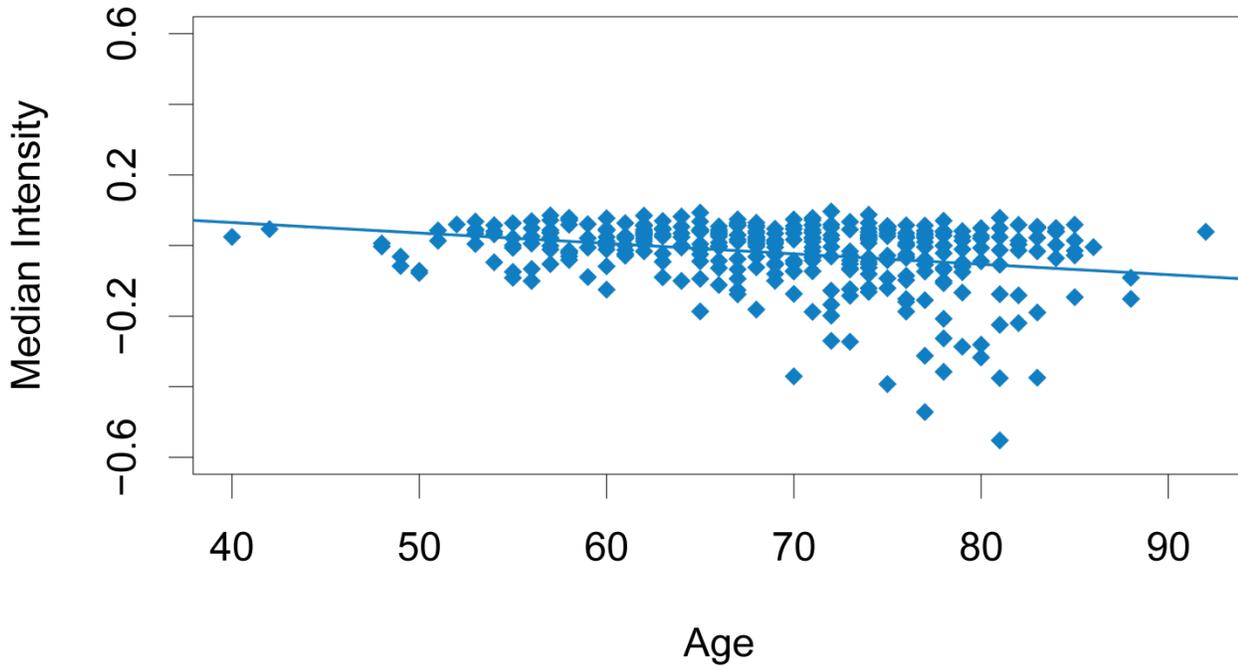
Supplemental figure 7: Association between LOY and major cardiovascular event-free survival in smokers (A) and non-smokers (B).

**Supplemental table 1** Primers of the qPCR experiment

<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
<b>Y chromosomal genes</b>		
CDY2A	CGGTGGTCTTCAAGGTGACA	TGCGACATTAGTGGGTGCAT
KDM5D	ACAGGAACGTTCTCGAGCTG	AGGACCGTAAAAGGCTGTGG
PRY	AACAACAGGCCTACTCTGCC	AGCAACCACAAGAAACCCCA
SRY	GATCCCGCTTCGGTACTCTG	GGTAAGTGGCCTAGCTGGTG
UTY	ACTTGATGGAGCTTGCTTGACT	TGCCACAGCTAGATGACACTG
<b>Housekeeping genes</b>		
P0	TGCACAATGGCAGCATCTAC	ATCCGTCTCCACAGACAAGG
beta-actin	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC

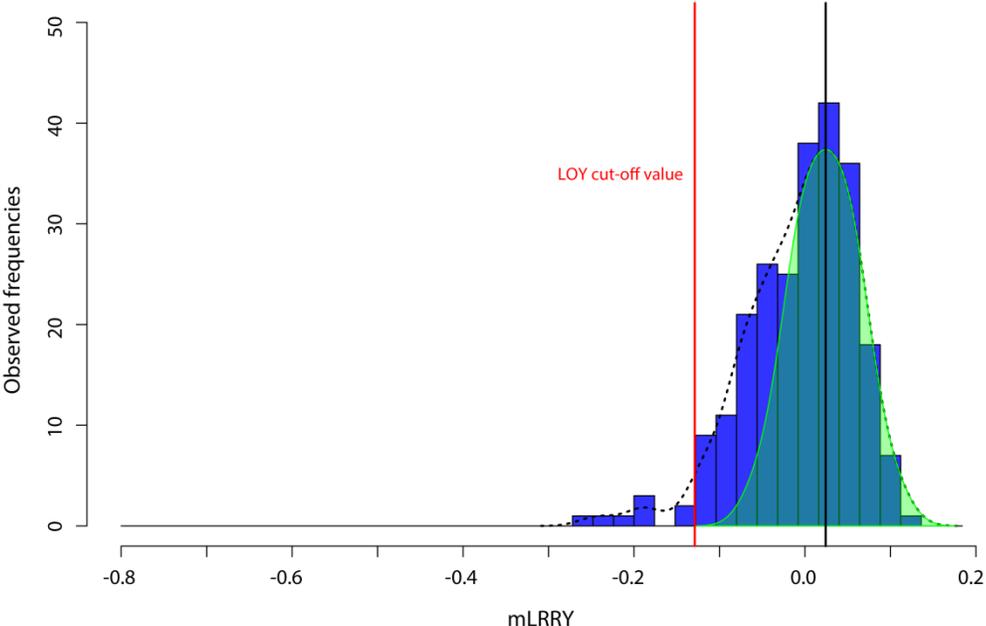
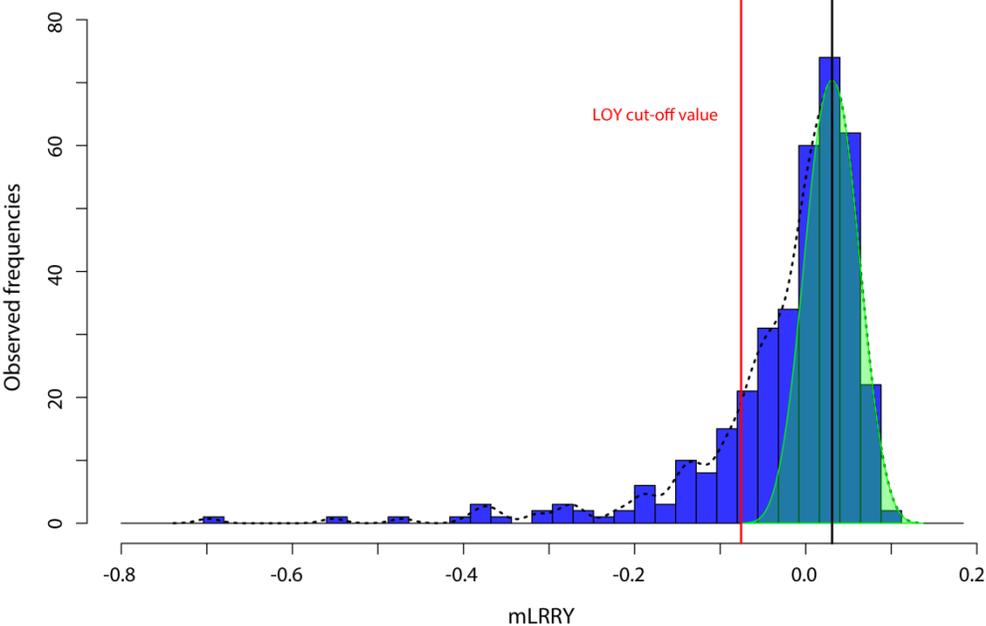
**Supplemental figure 1**

Association between median log<sub>2</sub> ratio of Y chromosomal probe intensity and age in blood (A) and plaque (B).



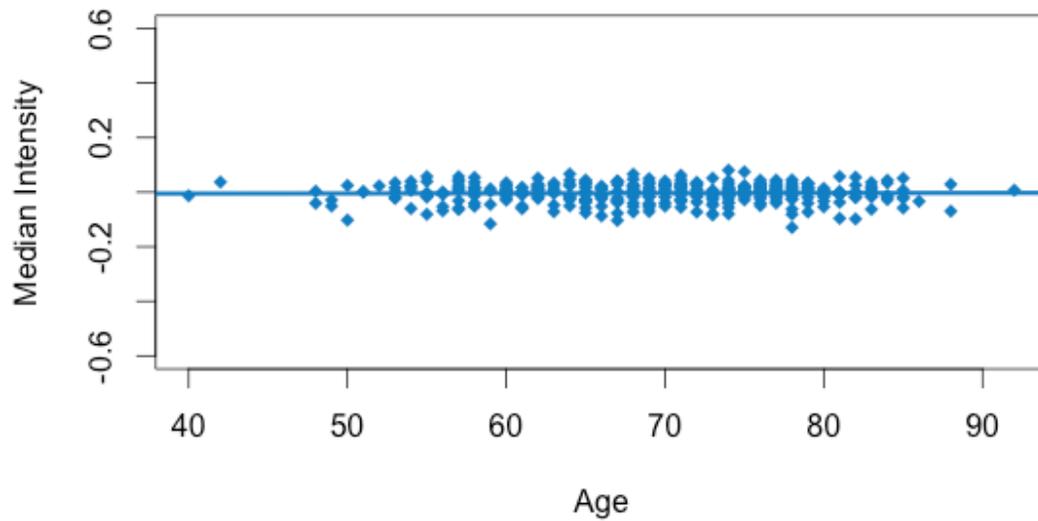
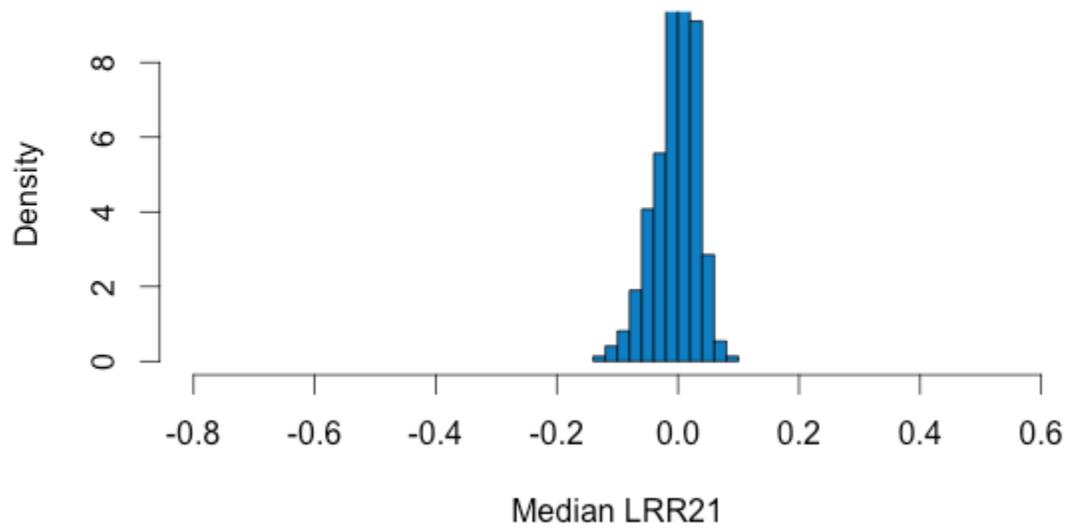
# Supplemental figure 2

Determination of cut off for A) blood and B) plaque samples.



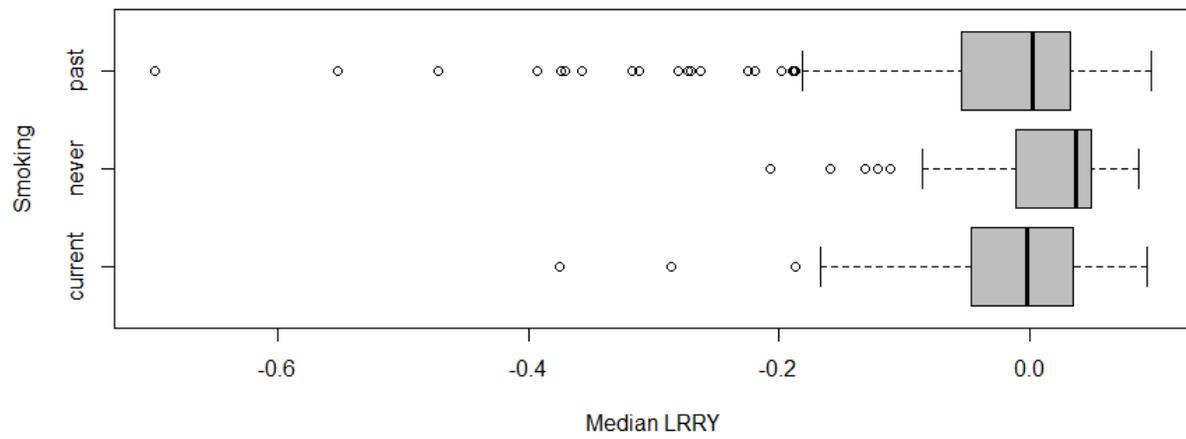
### Supplemental figure 3

Distribution of median log<sub>2</sub> ratio of chromosome 21 intensity and its association with age in blood.



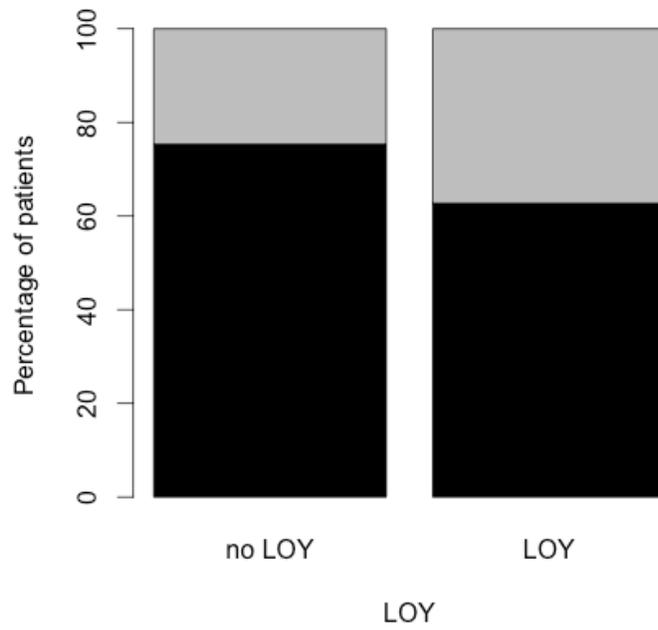
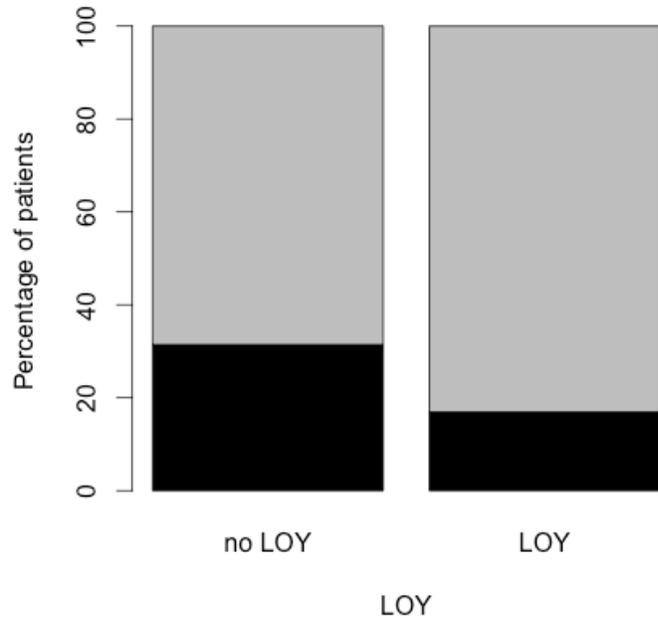
### Supplemental figure 4

Median log<sub>2</sub> ratio of Y chromosomal probe intensity for smoking history (past smoker, current smoker, never smoker)



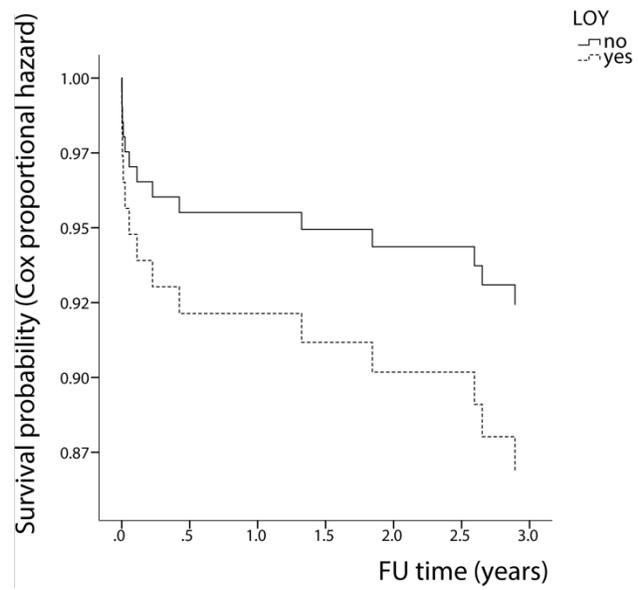
**Supplemental figure 5**

Percentage of patients with small atheroma size (black) and large atheroma size (grey) for cutoff values of 10% (A) and 40% atheroma size (B).



### Supplemental figure 6

Association between LOY and major cardiovascular event-free survival in AAA-Express

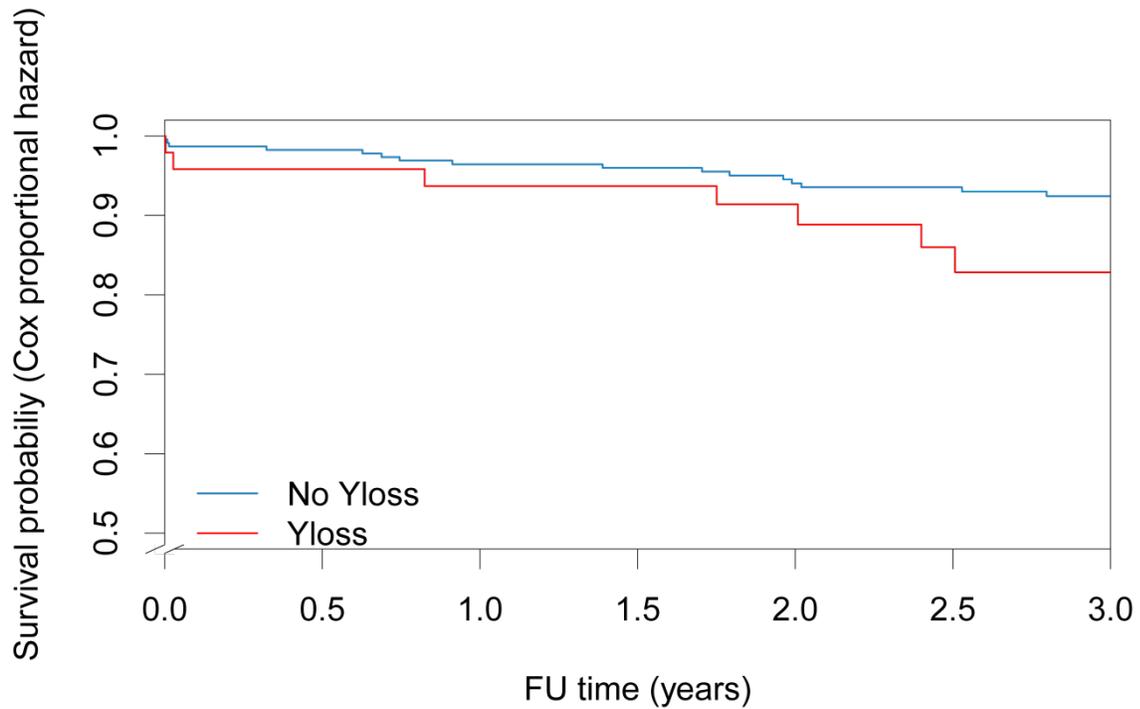
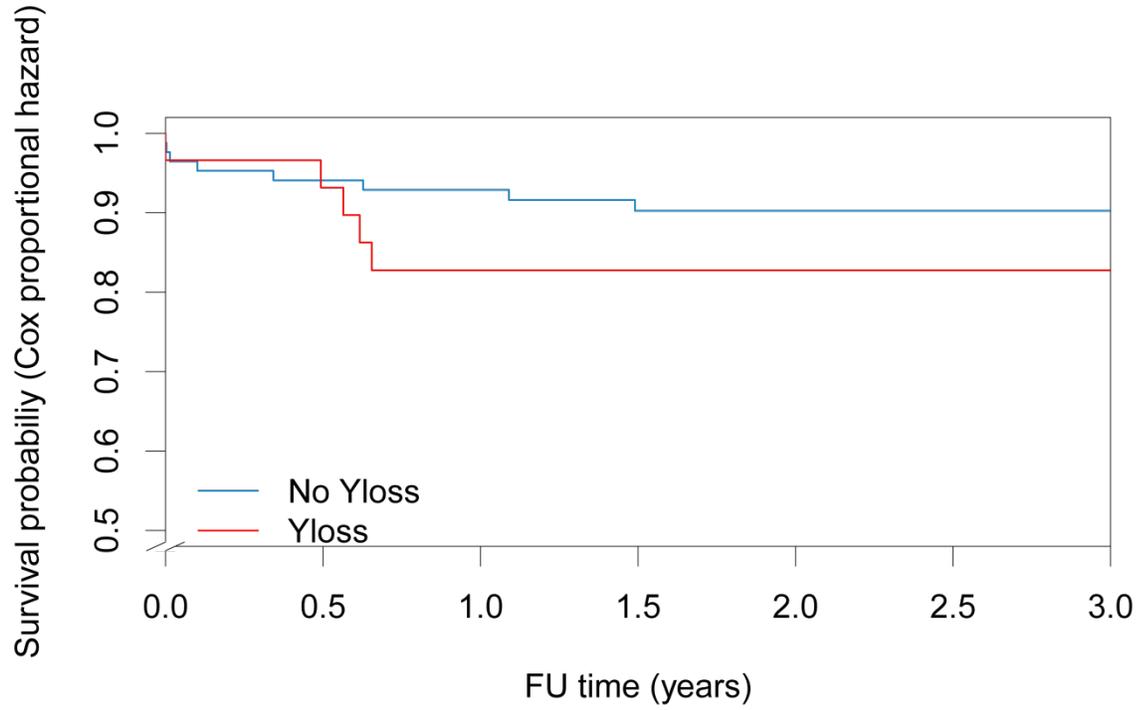


$P = 0.34$

*Model corrected for age*

**Supplemental figure 7**

Association between LOY and major cardiovascular event-free survival in smokers (A) and non-smokers (B).



*Models corrected for age*