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 (β=-0.03/10 y; r=0.07; P=1.6×10⁻⁷) 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
Genetic variation on the Y chromosome has been associated with high blood pressure\(^{10}\) and myocardial infarction,\(^{11}\) independent of traditional cardiovascular risk factors, sex steroids, or aggression. Given the global immuneregulatory 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 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.\(^{12}\) In short, blood was obtained before surgery and subsequently stored at −80°C. 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°C and used for the measurement of inflammatory cytokines and isolation of DNA.

**Histological Assessment of Specimens**

Plaque specimens were stained using CD68 (macrophages), α-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 ×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-α 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 monocye chemotacttic 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-α, interleukin-10, regulated on activation, normal T cell expressed and secreted, monocye chemotacttic 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.\(^{13}\) 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±3.0 SDs, relatedness (pi-hat >0.20), Hardy–Weinberg equilibrium (P=1.0x10^{-4}), 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 2 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.\(^{2}\) 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 I 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 I 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.
Replication Cohort

Replication of the Cox proportional hazards analysis on secondary cardiovascular events was performed in the AAA-Express. 14 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 χ² 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 boxcox 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 2 ratios of Y chromosomal intensity (mLRRY) in 608 patients; in 366 patients, we used blood-derived DNA. Median log 2 ratios of Y chromosomal probes in these patients were negatively associated with age ($β$=-0.03/10 y; $r^2=0.07$; $P=1.6×10^{-7}$; 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 2 ratios of Y chromosomal probe intensity in plaque were also negatively associated with age ($β$=-0.02/10 y; $P=5.02×10^{-8}$; Figure I in the Data Supplement). Of the 242 patients, 8 (3%) 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).

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
evidence for loss of chromosome 21. We found a median log
2 ratio of intensity of chromosome 21 probes that was
≈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 (β−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 smok-
ing=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 dichoto-
mous 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 dichot-
omous 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 characteris-
tic \((P<0.1)\) that was also associated with major cardiovascular
end points. During 3 years of follow-up, men with dichoto-
mous LOY in blood had significant more major cardiovascular
end points (HR=2.28; 95% confidence interval, 1.11–4.67;
\(P=0.02\); Figure 2). We replicated the direction of this effect

| 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 m² (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   |
| Presenting symptoms (%) |               |                  |
| 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.
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

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

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

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 chemotactic protein; MCSF, macrophage colony-stimulating factor; NA, not applicable; RANTES, regulated on activation, normal T cell expressed and secreted; and TNF, tumor necrosis factor.

### Figure 2.

Cox proportional hazards model for major event-free survival. \( P = 0.02 \). Model corrected for age and current smoking.
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.\(^6\) 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.

## Sources of Funding
Dr Haitjema, D. Kofink, and S.W. van der Laan are supported by the EU’s Seventh Framework Programme project CVgenes@target (HEALTH-F2-2013-601456). S.W. van der Laan is funded through grants from the Netherlands CardioVascular Research Initiative (GENIUS, CVON2011-19) and the Interuniversity Cardiology Institute of the Netherlands (ICIN, 09.001). Dr Asselbergs is supported by a Dekker scholarship-Junior Staff Member 2014T001—Netherlands Heart Foundation and University College London Hospitals National Institute for Health Research Biomedical Research Centre.

## Disclosures
None.

## References


\(^{7}\) Persiani L, Bonomi M, Lleo A, Pasini S, Civardi F, Biachi I, et al. Increased loss of the Y chromosome in peripheral blood cells in male

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.
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Circ Cardiovasc Genet. 2017;10:e001544
doi: 10.1161/CIRCGENETICS.116.001544

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circgenetics.ahajournals.org/content/10/4/e001544

Data Supplement (unedited) at:
http://circgenetics.ahajournals.org/content/suppl/2017/08/02/CIRCGENETICS.116.001544.DC1

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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 log2 ratio of Y chromosomal probe intensity and age in blood and plaque.

Supplemental figure 3: Distribution of median log2 ratio of chromosome 21 intensity and its association with age in blood.

Supplemental figure 4: Median log2 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y chromosomal genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDY2A</td>
<td>CGGTGGTCTTTCAAGGTGACA</td>
<td>TGCGACATTAGTGGGTGCAT</td>
</tr>
<tr>
<td>KDM5D</td>
<td>ACAGGAAACGTTCGAGCTG</td>
<td>AGGACCCTAAAGGCTGTGG</td>
</tr>
<tr>
<td>PRY</td>
<td>AACAACAGGCTACTCTGCC</td>
<td>AGCAACCAAAGAAACCCCA</td>
</tr>
<tr>
<td>SRY</td>
<td>GATCCCGCTTCGGTACTCTG</td>
<td>GGTAAGTGCCCTAGCTGGT</td>
</tr>
<tr>
<td>UTY</td>
<td>ACTTGATGGAGCTTGCTTGACT</td>
<td>TGCCACAGCTAGTGACACTG</td>
</tr>
<tr>
<td><strong>Housekeeping genes</strong></td>
<td></td>
<td></td>
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<tr>
<td>P0</td>
<td>TGCACAATGGCAGCATCTAC</td>
<td>ATCCGTCCTCCACAGACAAGG</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>GATCGGCGGCTCCATCTCTG</td>
<td>GACTCGTCATACTCTGTGGC</td>
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</table>
Supplemental figure 1
Association between median log2 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 log2 ratio of chromosome 21 intensity and its association with age in blood.
Supplemental figure 4

Median log2 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