Loss of Y chromosome in blood is associated with major cardiovascular events during follow-up in men after carotid endarterectomy

Running title: Loss of the Y chromosome and cardiovascular events

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Abstract

Background:

Recent studies found an immune-regulatory role for Y, and a relation between loss of Y (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 (CEA).

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. Out of 366 CEA patients, 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=-0.03/10yr, r^2=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 (OR 2.15, 95% CI: 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 (HR = 2.28, 95% CI: 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.

Keywords: Genetics; Atherosclerosis; Inflammation; Secondary cardiovascular events

Introduction

Loss of the Y chromosome (Loss of Y, LOY) in blood cells was already described in the 60s and affects approximately 15% of the male population of older age[1] . Only recently LOY was associated with a higher risk of (non-haematological) cancer and overall mortality[2,3]. This relationship was speculated to be due to smoking and a disrupted tumor immunosurveillance[4]. Furthermore, LOY was associated with Alzheimer's disease[5]and the occurrence of auto-immune diseases such as primary biliary cirrhosis[6] and auto-immune thyreoiditis[7].

Indeed, the Y chromosome exhibited an immune-regulatory function by acting as a global trans-expression quantitative trait locus in mice[8]. The Y chromosome directly mediated changes in the transcriptome of CD4+ 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[9]. Comparison of gene expression data between haplotype I and other haplotypes revealed pathways that are related to inflammation and immunity, revealing down-regulation of adaptive immunity and up-regulation of inflammatory response in haplotype I carriers.

Genetic variation on the Y chromosome has been associated with high blood pressure[10] and myocardial infarction[11], independent from traditional cardiovascular risk factors, sex steroids or aggression. Given the global immune-regulatory 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 carotid endarterectomy (CEA) or femoral endarterectomy in two 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 hypercholesterolaemia were self-reported. Diabetes 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 was considered present if the patient had suffered 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 below 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 postoperatively. 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 prior to surgery and subsequently stored at -80 degrees. 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 degrees 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 haemorrhage, hematoxylin-eosin staining and Mallory's phosphotungstic acid-hematoxylin staining (fibrin). Either luminal thrombus, intraplaque haemorrhage 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 three hotspots and subsequently averaged per slide. Collagen and calcifications were scored semi-quantitatively into no (1), minor (2), moderate (3) or heavy (4) staining at 40x 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 seven different inflammatory cytokines: IL-6 and TNF-α as pro-inflammatory cytokines, IL-10 as an anti-inflammatory cytokine, RANTES as a marker of T-cell involvement and MCP-1, MCSF and GDF-15 as markers of macrophage involvement. Cytokines were measured by Luminex in plaque lysate (IL-6, TNF- α, IL-10, RANTES, MCP-1, MCSF) or citrate plasma (GDF-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 SNP genotyping data was collected in 1,858 consecutive CEA patients 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 GCOS4 metrics, and thereafter filtering samples with a call rate >97%, variant call rate >97%, minor allele frequencies >3%, average heterozygosity rate ± 3.0 standard deviations, relatedness (pi-hat >0.20), Hardy-Weinberg Equilibrium ($p < 1.0x10^{-6}$) and based on population stratification (excluding samples >6 standard deviations from the average in 5 iterations during principle component analysis and by visual inspection).

After quality control, we kept 1,640 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 SNPs.

Determination of Loss of Y

To assess LOY, median log2 ratios (observed intensity/reference intensity) were computed based on the raw intensity data from the male-specific Y chromosomal probes (mLRRY), excluding PAR1 and PAR2. Two blood samples were excluded due to 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 (Supplemental figure 1).

As a validation, LOY was assessed by qPCR of six Y chromosomal genes along the Y chromosome in 9 patients that exhibited dichotomous LOY and 8 patients that did not exhibit dichotomous LOY. Presence of one of the genes (TSPY1) was assessed by a commercially available kit (Y-chromosome Detection real-time PCR assay, Primerdesign Ltd). Primer design of the other five primers can be found in Supplemental table 1. 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 that underwent open aneurysm repair in the UMC 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 was present for 202 blood samples. Patients in Aneurysm Express were genotyped using the Illumina HumanCore Exome chip. Collection of data, including quality control of the SNP 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 χ^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 value <0.1 (age, body mass index (BMI), glomerular filtration rate (GFR), 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 carried out using the R computing platform, version 3.0.2.

Results

Loss of Y in blood

We determined median log2 ratios of Y chromosomal intensity(mLRRY) in 608 patients; in 366 patients we used blood derived DNA. Median log2 ratios of Y chromosomal probes in these patients were negatively associated with age (beta=-0.03/10yr, r^2=0.07, p=1.6*10^{-7}, Supplemental figure 1). Of the 366 patients 61 (17%) exhibited dichotomous loss of the Y chromosome (LOY) in blood defined as mLRRY< -0.075 (Table 1, Figure 1, Supplemental figure 2). A trend was seen for more smoking, a lower BMI 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 log2 ratios of Y chromosomal probe intensity in plaque were also negatively associated with age (beta = -0.02/10yr, p=5.02*10^{-8}, Supplemental figure 1). Of the 242 patients 8 (3%) exhibited dichotomous LOY in plaque defined as median log2 value of Y chromosomal intensity < -0.129 (Supplemental figure 2). Because only eight patients suffered from 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 evidence for loss of chromosome 21. We found a median log2 ratio

of intensity of chromosome 21 probes that was around 0, without any evidence for an association with age (Supplemental figure 3).

Association with smoking

Previous studies point towards a role of smoking in loss of the Y chromosome. Past smokers and current smokers exhibited a lower mLRRY than never smokers (Supplemental figure 4). We observed an association between mLRRY and smoking when corrected for age (beta -0.02 for current smokers compared to non-smokers, p = 0.03). In a backward step model, age and smoking were found to be most predictive of LOY (AIC for model with only age and smoking = 307. 25 vs AIC for model with age, smoking, BMI, GFR and hypertension = 310.79). Corrected for age, smoking was associated with dichotomous LOY (OR 2.83(95% CI: 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 seven classical plaque characteristics: amount of calcification, amount of collagen, atheroma size, presence of intraplaque haemorrhage, macrophage and 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 larger than 10% atheroma size (OR 2.15 (1.06-4.76), p= 0.04, table 2, supplemental figure 5).

Association with secondary cardiovascular endpoints

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 as 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% CI 1.11-4.67, p = 0.02, figure 2). We replicated the direction of this effect 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 endpoints (HR = 1.78 (0.54-5.85), p = 0.34, Supplemental figure 6). 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 non-smokers (Supplemental figure 7). Atheroma size was not associated with major cardiovascular events during follow-up.

Discussion

In this hypothesis-generating study in a population of male carotid endarterectomy patients, loss of the Y chromosome 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 cardiovascular disease patients. However, after correction for multiple testing, no associations were found between dichotomous loss of the Y chromosome and systemic and local (plaque) inflammatory status, suggesting that alternate mechanisms may explain the association between LOY and outcome.

We hypothesized that loss of the Y chromosome 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 haemorrhage and more inflammatory cytokines. While we found an increase in major cardiovascular events and some preliminary evidence pointing towards 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 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 due to the fact that the atherosclerotic plaque does not contain as many rapidly dividing cells as compared to 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 due to the fact that the plaque is formed by invasion and division of cells over 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[15].

There are a few other possible explanations for the fact that we did not find any other association with plaque phenotype or inflammation. Firstly, LOY could be so detrimental to the male body that all patients suffering from it die before they develop an operable form of atherosclerosis and thereby simply do not end up in our study. Secondly, LOY could influence atherosclerosis in an earlier phase of the disease, for example affecting disease progression. Patients in the Athero-Express biobank suffer from 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 relation between LOY in blood and both (non-hematological) cancer and overall mortality in healthy men from the longitudinal ULSAM cohort aged 71-84 years[2]. However, not all increased mortality risk during over 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 due to several factors, including inflammation but for example also coagulation, endothelial dysfunction and adverse lipid profiles[16]. 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%, one 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 non-smokers. 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 haematological malignancies as a genetic susceptibility locus for LOY at chromosome 14.[17]It might be that loss of the Y 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[18]. Atherosclerosis might also accelerate genomic instability due to 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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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, 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/1.73 m^2 (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 - TIA - Stroke	 4/60 (7) 39/60 (65) 17/60 (28)	 42/302 (14) 172/302 (57) 88/302 (29)	0.27

IQR: inter-quartile range, BMI: body-mass index, PAOD: peripheral arterial occlusive disease, GFR: glomerular filtration rate, MDRD: modification of diet in renal disease, SD: standard

deviation, LDL: low-density lipoprotein, HDL: high-density lipoprotein, TIA: transient ischaemic attack.

Table 2: Associations of LOY with measures of (inflammatory) plaque phenotypes

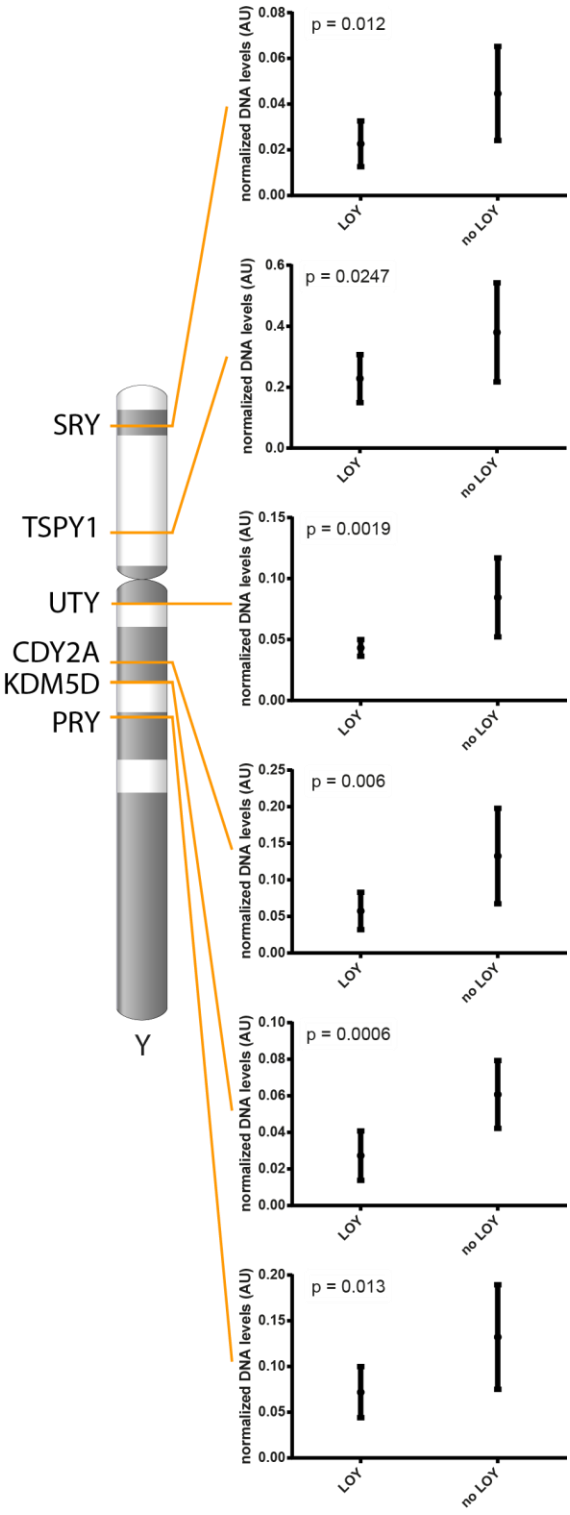
Plaque phenotype	Beta of LOY (95% CI)	Odds Ratio of LOY (95% CI)	p-value
Atheroma size (>10%)	*NA*	2.15 (1.06-4.76)	0.04
Atheroma size (>40%)	*NA*	1.84 (0.98-3.41)	0.05
Calcification (major)	*NA*	0.86 (0.47-1.58)	0.62
Collagen (major)	*NA*	0.82 (0.39-1.64)	0.59
Intraplaque haemorrhage (present)	*NA*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

CI: confidence interval, IL: interleukin, MCP-1: monocyte chemotactic protein, MCSF: macrophage colony-stimulating factor, RANTES: regulated on activation, normal T cell expressed and secreted, TNF: tumor necrosis factor

Models corrected for age and current smoking.

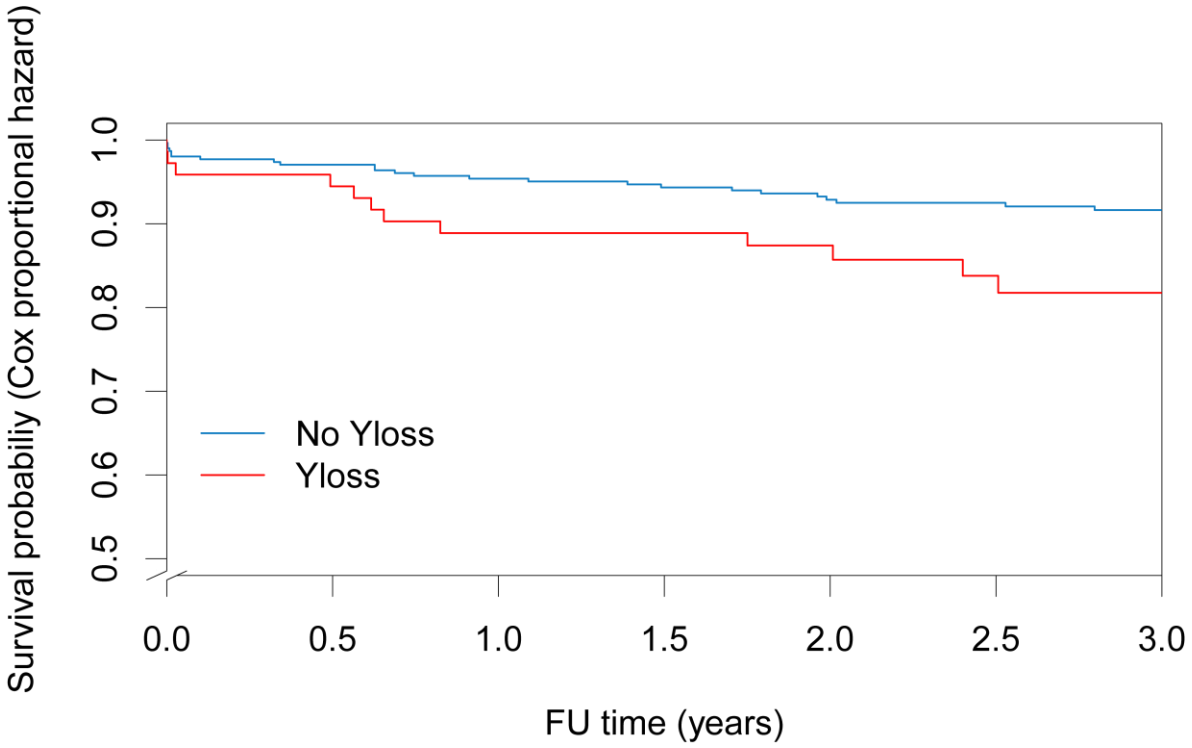
Continuous variables are box-cox transformed.

Figure 1: qPCR of Y chromosomal genes



AU: arbitrary units

Figure 2: Cox proportional hazards model for major event-free survival



P = 0.02

Model corrected for age and current smoking