Genetic variation within the Y chromosome is not associated with histological characteristics of the atherosclerotic carotid artery or aneurysmal wall

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ABSTRACT

Background and aims: Haplogroup I, a common European paternal lineage of the Y chromosome, is associated with increased risk of coronary artery disease in British men. It is unclear whether this haplogroup or any other haplogroup on the Y chromosome is associated with histological characteristics of the diseased vessel wall in other vascular manifestations of cardiovascular diseases showing a male preponderance.
Methods: We examined Dutch men undergoing either carotid endarterectomy from the Athero-Express biobank (AE, n = 1217) or open aneurysm repair from the Aneurysm-Express biobank (AAA, n = 393). Upon resolving the Y chromosome phylogeny, each man was assigned to one of the paternal lineages based on combinations of single nucleotide polymorphisms of the male-specific region of the Y chromosome. We examined the associations between the Y chromosome and the histological characteristics of the carotid plaque and aneurysm wall, including lipid content, leukocyte infiltration and intraplaque haemorrhage, in all men.
Results: A majority of men were carriers of either haplogroup I (AE: 28% AAA: 24%) or haplogroup R (AE: 59% AAA: 61%). We found no association between Y chromosomal haplogroups and histological characteristics of plaque collected from carotid arteries or tissue specimens of aneurysms. Moreover, the distribution of frequency for all Y chromosomal haplogroups in both cohorts was similar to that of a general population of Dutch men.

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Conclusions: Our data show that genetic variation on the Y chromosome is not associated with histological characteristics of the plaques from carotid arteries or specimens of aneurysms in men of Dutch origin.

1. Introduction

Historically, the human Y chromosome was considered genomic wasteland. It was considered to be implicated only in sex determination and subject to rapid decline [1]. However, more recently, the Y chromosome gained interest, as it was found to contain dosage-sensitive regulators of gene expression and its loss was associated with smoking, cancer and death in different studies in men [2–4].

The major part of the Y chromosome (male-specific region, MSY) is inherited essentially unchanged from father to son. Phylogenetic analysis is a powerful tool to examine the ancient paternal ancestry of the Y chromosome [5,6]. The resulting chromosomal haplogroups are characterized by numerous specific genetic mutations and follow a distinct geographical distribution [6]. In Western Europe, haplogroups I and R and their subfamilies are among the most frequently observed Y chromosomal haplogroups although their prevalence differs between countries.

Genetic variation of the MSY was previously implicated in cardiovascular diseases (CVD) [7–11]. Common bi-allelic polymorphisms of MSY were associated with blood pressure, circulating concentrations of LDL-cholesterol, measures of a pro-atherogenic fraction of LDL-cholesterol and a paternal history of myocardial infarction [8,9]. A study in a Greek-Cypriot population found a higher risk for plaque presence in the carotid and femoral arteries of carriers of haplogroup K^{12}. In addition, men with haplogroup I showed a 50% increase in coronary artery disease (CAD) risk in two British populations [11,13]. Gene expression analysis revealed down-regulation of pathways of adaptive immunity together with up-regulation of pro-inflammatory response in the macrophages of haplogroup I carriers when compared to men from other Y chromosomal haplogroups [13]. However, in a recent Dutch effort, haplogroups did not show a predisposing effect on first or recurrent venous thrombosis [14].

The previous analyses were conducted primarily in relation to CAD or its modifiable risk factors [8,9,13]. Whether haplogroups are also related to the risk of other cardiovascular disorders, or the characteristics of the underlying atherosclerotic plaque is unclear. Only one study analyzed the relation between Y chromosomal haplogroups and plaque presence [12]. In addition, there is limited data on the association between Y chromosomal haplogroups and susceptibility to CVD in non-British cohorts.

We have conducted a MSY phylogenetic analysis of 1610 Dutch men with available histological characteristics of the diseased vascular wall, obtained during carotid endarterectomy or open aneurysm repair. Both disorders show a “male disadvantage”, with men much more commonly affected than women [15]. Characteristics of the vessel wall have been shown to be associated with presenting symptoms, in occluded as well as in aneurysmatic vessels [16–18]. The use of histology has advantages over clinical diagnosis as it may shed light on the biological mechanism behind the observed increased CVD risk associated with haplogroup I.

2. Patients and methods

2.1. Athero-Express population

The Athero-Express biobank (AE) is a prospective ongoing cohort study including all patients undergoing carotid endarterectomy in two large tertiary referral hospitals in the Utrecht area of the Netherlands: the University Medical Center Utrecht in Utrecht and the St. Antonius Hospital in Nieuwegein [19]. Patient characteristics are collected through standardized questionnaires. Blood is collected preoperatively and stored together with the atherosclerotic plaque. Patients are asked to return a short follow-up questionnaire each year for three years. When they indicate a possible cardiovascular event, this is validated through health records kept by their general practitioner. Patients gave written informed consent and the study is approved by the ethics boards of both hospitals.

2.2. AAA-Express population

The AAA-Express biobank (AAA) started as a spin-off biobank of the AE, including patients undergoing open aneurysm repair in the same hospitals [20]. Questionnaires and follow-up were collected in the same fashion. Instead of atherosclerotic plaque, aneurysmal tissue was stored. Patients gave written informed consent and the study is approved by the ethics boards of both hospitals.

2.3. Processing of patient material in AE and AAA

The processing of patient material from the AE and AAA was described previously [19,20]. In short, atherosclerotic plaque and aneurysm tissue were immediately processed after removal. One segment, for AE the culprit lesion, was identified, stored in 4% formaldehyde, decalcified and embedded in paraffin for histological slide preparation. The remaining tissue was cut into fragments of 0.5 cm and stored at −80 °C. Using histology, we performed picrosirius red staining for collagen, CD68 staining for macrophages and α-actin staining for smooth muscle cells. For plaque histology, we additionally performed CD34 staining for the presence of microvessels. For the aneurysm wall histology, we also conducted CD3 staining for T-lymphocytes, CD20 staining for B-lymphocytes and CD138 staining for plasma cells. Plaque thrombosis was determined combining the presence of luminal thrombi or intraplaque haemorrhage, assessed by hematoxylin-eosin staining and Mallory's phosphotungstic acid-hematoxylin staining for fibrin. Collagen and calcifications were semi-quantitatively assessed at 40× magnification and grouped into no (1), minor (2), moderate (3) or heavy (4) staining. The categories were dichotomized into no/minor and moderate/heavy for the current study. For AAA, leukocyte infiltration was scored at 100× magnification, where <100 positively stained cells was considered minor staining and >100 positively stained cells was considered moderate/heavy staining. Lipid core size was cut off at an area of 40% of plaque size using polarized light. For AE, macrophages and smooth muscle cells were quantitatively assessed using computerized analysis and analyzed as percentage of plaque area. Microvessels were counted in three hotspots after

morphological identification and averaged per slide subsequently. A dedicated technician assessed all histological slides.

2.4. Haplogrouping of the Y chromosome

DNA was isolated following standardized protocols, as described previously, from blood, or if blood was unavailable, from atherosclerotic plaque tissue or aneurysm wall tissue [21]. LGC (LGC Genomics Ltd. United Kingdom) subsequently genotyped the AE patients following in-house protocols for eleven MSY SNPs (Supplemental Table 1), tagging 8 Y chromosomal lineages and subsets of the R haplogroup. Patients from the AE and AAA were additionally genotyped using the Y chromosomal probes of the Infinium HumanExome BeadChip v1.2 and Illumina HumanCoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of 68 MSY SNPs present on these Illumina BeadChips was used to discriminate between 65 possible haplogroups. Each individual was assigned to the haplogroup that best fitted their genotype, allowing for no more than one mismatch and 3% of missing genotypes. In cases where the two genotyping methods did not correspond (n = 9), the ExomeChip haplogroup was kept for further analyses, thus we combined the two datasets for the AE into one totaling to 1217 genotyped individuals. Haplogroup lineages were further grouped into haplogroup E, F, G, H, I, J, N, Q, R and T for the analyses.

2.5. Statistical analyses

Differences in baseline characteristics were tested using ANOVAs and non-parametric Kruskall-Wallis tests, where applicable, for continuous variables. Categorical variables were compared using Chi-square tests. *Post-hoc* testing for ANOVA was performed using Tukey tests, for Chi-square test by observing the standardized residuals. Continuous plaque characteristics were log-transformed before analyses. They were analyzed at once for each cohort using MANOVA. Binary categorical plaque characteristics were analyzed using Chi-square tests. A multiple-testing corrected *p* value was considered significant. Multiple-testing correction was performed with the use of Bonferroni correction (for binary plaque characteristics in AE: 0.05/4 = 0.0125, for continuous plaque characteristics in AE: 0.05/5 = 0.01, for aneurysm characteristics in AAA: 0.05/11 = 0.0045). All statistical analyses were performed in SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

3. Results

3.1. Prevalence of Y chromosomal haplogroups

We included a total of 1610 male patients, from Athero-Express (AE, n = 1217) and AAA-Express (AAA, n = 393) in this study. Patients displayed a variety of Y chromosomal haplogroups (Supplemental Table 2). Most patients were carriers of haplogroup I (AE: 28% AAA: 24%) or haplogroup R (AE: 59% AAA: 61%). Because of low patient numbers in the other haplogroups, for all subsequent analyses, only patients with haplogroups E, G, I, J and R were included (Fig. 1).

3.2. Clinical characteristics

The men within the Athero-Express cohort exhibited characteristics of a severely cardiovascular compromised population (Table 1). They were on average 69 years old, 23.7% had a history of diabetes and 71.5% were hypertensive. Of all men, 26.4% presented with a stroke before undergoing CEA. After correction for multiple testing, there were no significant differences in any available clinical characteristics among carriers of the most common haplogroups of the Y chromosome in this population. Patient characteristics of the AE and AAA-Express can be found in Supplemental Tables 3 and 4.

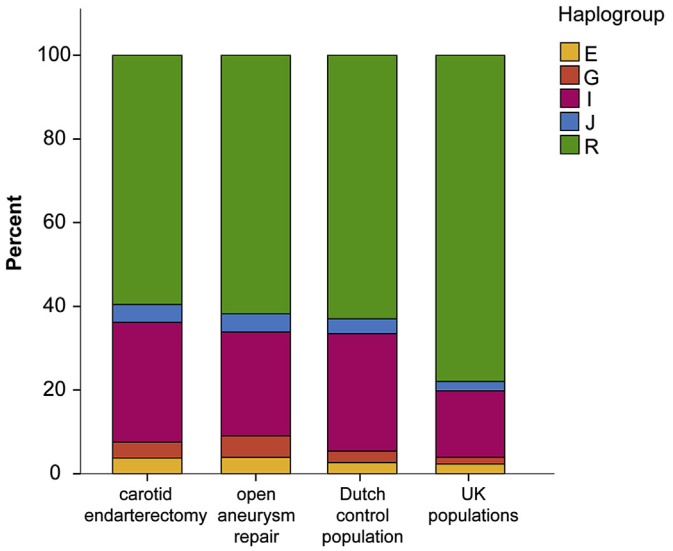

Fig. 1. Distribution of the five largest Y chromosomal haplogroups.
Distribution of the five largest Y chromosomal haplogroups in two Dutch CVD cohorts: carotid endarterectomy patients from the Athero-Express Biobank Study, aneurysm patients from the Aneurysm-Express Biobank Study, Dutch healthy controls of the Forensic Laboratory for DNA Research and the two UK populations (British Heart Foundation Family Heart Study (BHF-FHS) and West of Scotland Coronary Prevention Study (WOSCOPS) from Charchar et al. [13].

3.3. Atherosclerotic plaque characteristics in Athero-Express

There were no significant differences in macrophage content, mast cell content, neutrophil content, smooth muscle cell content and vessel density in the atherosclerotic plaque, either together at once using MANOVA (Wilks' Lambda: 0.99, $p = 0.33$) or tested independently (Fig. 2, Supplemental Table 5) between the MSY haplogroups. No significant differences were observed between the MSY haplogroups in calcification, collagen, fat content and intraplaque haemorrhage (Fig. 3 and Supplemental Table 5).

3.4. Aneurysm characteristics in Aneurysm-Express

We set out to further explore the association between Y chromosomal haplogroups and vessel wall characteristics in AAA. No significant differences were observed comparing aneurysm wall characteristics of patients undergoing open aneurysm repair between the Y chromosomal haplogroups (Supplemental Table 6).

3.5. Comparison with the general population

We compared our two cardiovascular disease cohorts with a control cohort of 2067 healthy Dutch men from the Forensic Laboratory for DNA Research. The haplogroups followed the same distribution in the control cohort as in the cardiovascular disease cohorts with most of the men carrying haplogroup I or R (Fig. 1). A small difference was observed between the prevalence of haplogroup G in the control population (2.7%) *versus* haplogroup G in the diseased populations (AE: 3.8%, AAA: 5.1%, Supplemental Table 2). No other differences were found. Based on this finding, an association between one of the haplogroups and risk of carotid

Table 1
Patient characteristics of the Athero-Express biobank.

n = 1206	E n = 45	G n = 46	I n = 345	J n = 52	R n = 718	*p* value
Age, mean (SD)	67.6 (10.9)	70.6 (7.6)	69.3 (9.5)	68.8 (9.7)	68.7 (8.7)	0.49
BMI, mean (SD)	26.0 (3.7)	25.5 (2.4)	26.3 (3.4)	26.0 (3.2)	26.4 (3.4)	0.33
GFR (MDRD), mean (SD)	72.2 (22.7)	73.6 (21.4)	73.4 (19.9)	73.6 (17.8)	73.6 (20.2)	1
Diabetes mellitus	11/45 (24.4)	8/46 (17.4)	96/345 (27.8)	11/52 (21.2)	159/718 (22.1)	0.25
Hypercholesterolemia	26/41 (63.4)	19/42 (45.2)	218/324 (67.3)	36/52 (69.2)	444/658 (67.5)	0.06
Hypertension	33/43 (76.7)	32/44 (72.7)	241/338 (71.3)	33/52 (63.5)	498/694 (71.8)	0.68
Current smoking	9/44 (20.5)	20/45 (44.4)	103/342 (30.1)	23/52 (44.2)	230/708 (32.5)	0.04
History of coronary artery disease	8/45 (17.8)	15/46 (32.6)	116/345 (33.6)	18/52 (34.6)	266/718 (37.0)	0.11
History of stroke	9/42 (21.4)	7/44 (15.9)	77/328 (23.5)	10/48 (20.8)	151/677 (22.3)	0.85
Peripheral arterial occlusive disease	8/45 (17.8)	11/46 (23.9)	69/345 (20)	12/52 (23.1)	169/718 (23.5)	0.68
Total cholesterol, median (IQR)	4.4 (3.7–5.3)	4.7 (3.8–5.5)	4.5 (3.8–5.2)	4.7 (3.7–5.2)	4.5 (3.8–5.3)	0.94
LDL cholesterol, median (IQR)	2.5 (2.0–3.5)	2.9 (2.2–3.3)	2.6 (2.0–3.2)	2.9 (2.0–3.3)	2.6 (2.0–3.3)	0.97
HDL cholesterol, median (IQR)	1.1 (0.9–1.2)	1.0 (0.8–1.5)	1.1 (0.9–1.3)	1.0 (0.9–1.3)	1.1 (0.9–1.3)	0.94
Triglycerides, median (IQR)	1.3 (1–1.9)	1.3 (1.1–1.8)	1.4 (1–2.1)	1.5 (1–2.3)	1.5 (1.0–2.1)	0.68
Antiplatelet use	41/45 (91.1)	40/45 (88.9)	300/343 (87.5)	46/52 (88.5)	625/718 (87)	0.94
Statin use	32/45 (71.1)	36/46 (78.3)	264/345 (76.5)	39/52 (75)	558/718 (77.7)	0.86
Presenting symptoms						0.19
Asymptomatic	2/45 (4.4)	5/46 (10.9)	46/339 (13.6)	5/52 (9.6)	118/715 (16.5)	
Ocular	24/45 (53.3)	19/46 (41.3)	135/339 (39.8)	26/52 (50)	323/715 (45.2)	
TIA	13/45 (28.9)	12/46 (26.1)	101/339 (29.8)	11/52 (21.2)	177/715 (24.8)	
Stroke	6/45 (13.3)	10/46 (21.7)	57/339 (16.8)	10/52 (19.2)	97/715 (13.6)	
Contralateral stenosis >50%	17/40 (42.5)	22/45 (48.9)	135/305 (44.3)	23/48 (47.9)	303/641 (47.3)	0.89

AE, Athero-Express; SD, standard deviation; BMI, body mass index; GFR, glomerular filtration rate; LDL, low density lipoprotein; HDL, high density lipoprotein.

occlusive disease or aneurysm development is unlikely. Compared to previously described populations from the United Kingdom, all Dutch populations showed more haplogroup I carriers and less haplogroup R carriers (Fig. 1 and Supplemental Table 2).

4. Discussion

In our study in 1610 Dutch men, we found no association of Y chromosomal haplogroups with histological characteristics of the diseased vessel wall. Moreover, we found no difference in distribution of Y chromosomal haplogroups in the general Dutch population *versus* our patients with severe atherosclerotic cardiovascular disease.

Previous research in two British cohorts found an association between haplogroup I and coronary artery disease [13]. We did not observe an association between MSY haplogroups and characteristics of the diseased vessel wall in the Dutch cohorts. There are several explanations for this apparent discrepancy. First of all, we studied different diseases, namely carotid occlusive disease and aneurysm formation. While atherosclerosis is a shared pathophysiological mechanism in all three cardiovascular diseases, their genetic background and disease mechanism are known to be only partially overlapping [23]. For example, the inflammation in aneurysms is driven mainly by infiltration of B and T lymphocytes, whereas in atherosclerotic plaques more macrophages are seen [24–26]. In addition, we investigated cohorts with only diseased patients, in contrast to the British cohorts that included both patients with CAD and apparently healthy controls. If haplogroups would account for the increased risk via the shared pathophysiological background of the three cardiovascular diseases, one would expect a different frequency distribution of haplogroups in our diseased cohorts. Increased risk of CAD that translates into higher rates of cardiovascular mortality in haplogroup I could account for a lower prevalence of patients with haplogroup I in our cohorts that only included patients suitable for surgery. On the contrary, increased risk of CAD due to a higher atherosclerosis risk could also have led to a higher prevalence of patients with haplogroup I in our cohorts, as patients were included based on overt atherosclerotic disease. However, we found the same haplogroup I frequency and distribution of other haplogroups in the Dutch cohorts of patients with vessel disease when compared to a large cohort of healthy Dutch men. Another explanation could be the differences in genetic background of the British and Dutch. Indeed, the distribution of Y haplogroups in Dutch men was different from the one observed in the cohorts from the United Kingdom. In the latter, there were more carriers of haplogroup R and less carriers of haplogroup I. Moreover, we were unable to observe the effect of haplogroup I on the susceptibility of CAD in our cohort of Dutch men undergoing vascular surgery. We found a similar percentage of carriers of haplogroup I in the patients with a history of coronary artery disease in the AE (116/423, 27.4%) and men recruited from the general Dutch population (571/2067, 27.6%). In AAA, there were fewer men with haplogroup I and a history of CAD (20/120, 16.7%) than men with haplogroup I in the general population. These findings may suggest differences in the effect of the Y chromosome on CAD risk between the Dutch men described above and the British cohorts published before. However, these results must be interpreted with caution. Clinically confirmed diagnosis of CAD was not the key phenotype in men recruited in the Dutch cohorts, thus neither confirmation or exclusion of CAD was as rigorous as in previous studies that focused primarily on defining CAD outcomes. Moreover, the current data come exclusively from cross-sectional studies with all limitations inherent to this design, whereas the previously reported study showed incident coronary artery disease during a long follow-up. Future, large prospective studies of CAD in non-British cohorts will be necessary to gain additional insight into the association between haplogroup I and CAD. Comparing the Y chromosomal haplogroup distribution of the diseased cohorts to the Dutch control population, we observed a slight enrichment for haplogroup G in the Dutch diseased cohorts (2.7 *vs.* 4.4% for non-diseased *vs.* diseased cohorts, p value = 0.004). This could point towards an increased disease risk for haplogroup G carriers compared to other haplogroups. However, we did not observe differences in patient or disease characteristics for carriers of haplogroup G. An alternative explanation could be that our patients were included mainly in the larger cities in the Netherlands, where people may have a more diverse genetic background whereas the control population participants were included from smaller towns and villages.

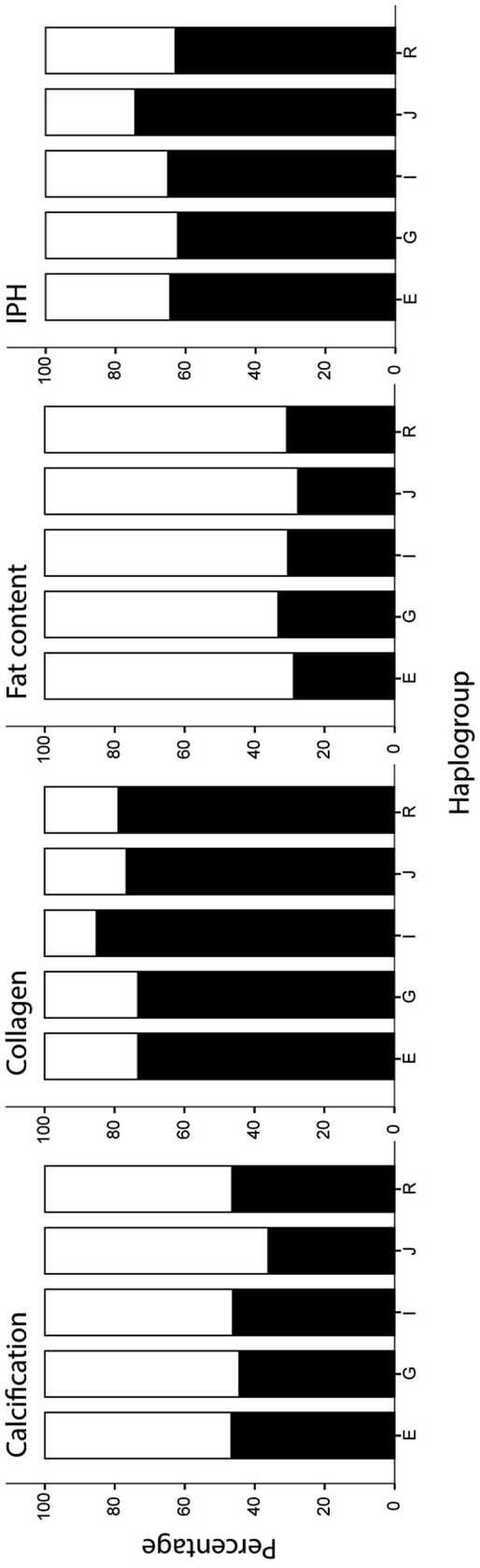

Fig. 2. Binary plaque characteristics of the Y chromosomal haplogroups in Athero-Express.
Black bars: moderate/heavy staining for calcification and collagen, >40% fat content, presence of IPH; white bars: no/minor staining for calcification and collagen, <40% fat content, absence of IPH.

There are some limitations to this study. Some Y chromosomal lineages (e.g. F, N, T) were excluded from the analysis because they were only present in few men in the studied populations. Moreover, the subgroups of Y chromosomal haplogroups were binned into larger groups to increase the power of the analysis (e.g. R1, R1a, R1b and R1b1b2 were binned into haplogroup R). We, therefore, cannot exclude the possibility of an association of any subgroup or smaller haplogroup with cardiovascular disease. In addition, we had low power to detect differences in some lineages that were included in the analysis (e.g. E, J and G) and we cannot exclude with certainty a possible association between those haplogroups and cardiovascular disease. Haplogrouping was performed on several batches of genotyped data. However, for AE, we found excellent overlap in haplogroups comparing the ExomeChip haplogroup and the haplogroup determined by genotyping of individual SNPs. We observed some nominally significant associations between haplogroups and baseline characteristics. However, the number of positive associations was low and within the expected range for the number of tests we performed, we, therefore, considered them false positives.

Since the publication of the association of haplogroup I and coronary artery disease, the cardiovascular research community has become interested in the Y chromosome. Replication of the association has been lacking, and publications of other groups outside of the United Kingdom are scarce [14]. We included two different Dutch cardiovascular cohorts and found no association between haplogroups and histology of the diseased vessel wall. The contribution of genetic content on the Y chromosome to human health and disease seem to be more complex than previously thought and possibly depends on the interaction with the other chromosomes of the genome. Large efforts might shed more light on the relation between Y chromosomal haplogroups and cardiovascular disease.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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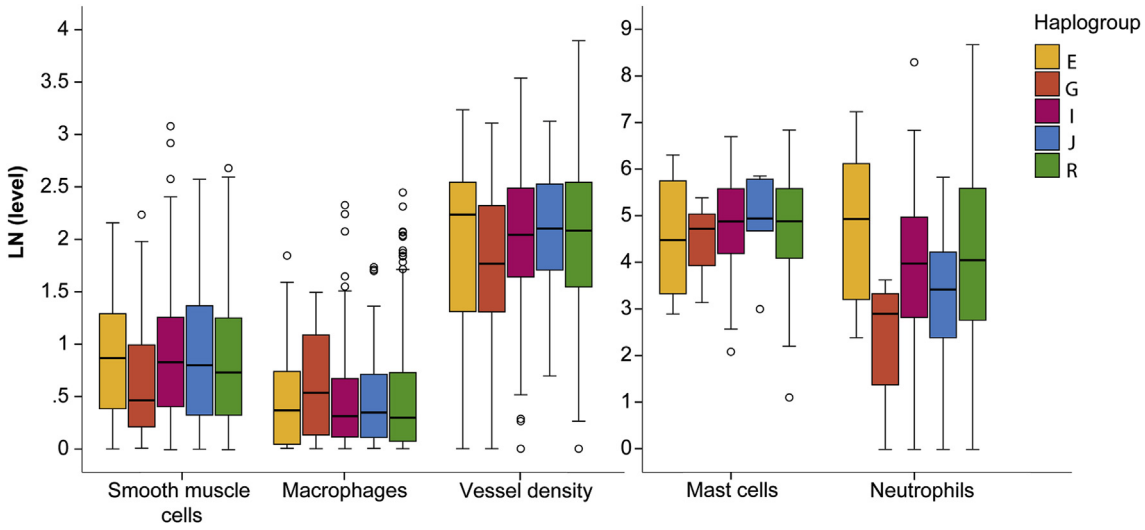

Fig. 3. Continuous plaque characteristics of the Y chromosomal haplogroups in Athero-Express.

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