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

Saskia Haitjema a, Jessica van Setten n, b, 1, James Eales c, 1, Sander W. van der Laan a, Ilaria Gandin d, Jean-Paul P.M. de Vries e, Gert J. de Borst f, Gerard Pasterkamp a, g, Folkert W. Asselberghs h, i, j, Fadi J. Charchar k, James F. Wilson l, m, Saskia C.A. de Jager a, Maciej Tomaszewski c, n, Hester M. den Ruijter a, *,

a Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht, The Netherlands
b Netherlands Heart Institute, Utrecht, The Netherlands
c Division of Cardiovascular Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom
d Department of Medical Sciences, University of Trieste, Trieste, Italy
e Department of Vascular Surgery, St. Antonius Hospital Nieuwegein, Nieuwegein, The Netherlands
f Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands
g Laboratory of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands
h Department of Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands
i Durrer Center for Cardiogenetic Research, ICIN-Netherlands Heart Institute, Utrecht, The Netherlands
j Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom
k Faculty of Science and Technology, Federation University Australia, Ballarat, Australia
l Centre for Global Health Research, Usher Institute of Population Health Sciences and Informatics, University of Edinburgh, Edinburgh, Scotland, United Kingdom
m MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh, Scotland, United Kingdom
n Division of Medicine, Central Manchester NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, United Kingdom

* Corresponding author. Laboratory of Experimental Cardiology, University Medical Center Utrecht, Room G.03.550, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.
E-mail address: h.m.denruijter-2@umcutrecht.nl (H.M. den Ruijter).

1 These authors contributed equally to this work.
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 open 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 aneurysmal 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 hemorrhage, 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 (LLC 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 CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22]. The combination of CoreExome BeadChip v1.1, respectively. In this case, we adhered to a protocol supplied by the Ygen consortium [22].

2.5. Statistical analyses

Differences in baseline characteristics were tested using ANOVAs and non-parametric Kruskal-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.

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

![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 (BHFFHS) and West of Scotland Coronary Prevention Study (WOSCOPS)) from Charchar et al. [13].](image-url)
occulsive 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 atherosclerotic plaques more macrophages are seen in the patients with a history of coronary artery disease in the AE 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, \( p \text{ value} = 0.004 \)). 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.

### Table 1

Patient characteristics of the Athero-Express biobank.

<table>
<thead>
<tr>
<th>n = 1206</th>
<th>E</th>
<th>n = 45</th>
<th>G</th>
<th>n = 46</th>
<th>I</th>
<th>n = 345</th>
<th>J</th>
<th>n = 52</th>
<th>R</th>
<th>n = 718</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>67.6 (10.9)</td>
<td>70.6 (7.6)</td>
<td>69.3 (9.5)</td>
<td>68.8 (9.7)</td>
<td>68.7 (8.7)</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>26.0 (3.7)</td>
<td>25.5 (2.4)</td>
<td>26.3 (3.4)</td>
<td>26.0 (3.2)</td>
<td>26.4 (3.4)</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR (MDRD), mean (SD)</td>
<td>72.2 (22.7)</td>
<td>73.6 (21.4)</td>
<td>73.4 (19.9)</td>
<td>73.6 (17.8)</td>
<td>73.6 (20.2)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>11/45 (24.4)</td>
<td>8/46 (17.4)</td>
<td>96/345 (27.8)</td>
<td>11/52 (21.2)</td>
<td>159/718 (22.1)</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>26/41 (63.4)</td>
<td>19/42 (45.2)</td>
<td>218/324 (67.3)</td>
<td>36/52 (96.9)</td>
<td>444/658 (67.5)</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>33/43 (76.7)</td>
<td>32/44 (72.7)</td>
<td>241/338 (71.3)</td>
<td>33/52 (63.5)</td>
<td>498/694 (71.8)</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of stroke</td>
<td>9/42 (21.4)</td>
<td>20/45 (44.4)</td>
<td>300/343 (87.5)</td>
<td>1.4 (1.2)</td>
<td>1.5 (1.2)</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presenting symptoms</td>
<td>32/45 (71.1)</td>
<td>36/78 (32.3)</td>
<td>57/339 (16.8)</td>
<td>10/52 (19.2)</td>
<td>97/715 (13.6)</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[J \neq J, 4.4\% \text{ for non-diseased vs. diseased cohorts, } p \text{ value } = \text{0.004}\]
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.

Financial support

The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007–2013 under grant agreement n° HEALTH-F2-2013-601456 (CVgenes-at-target). Sander W. van der Laan is funded through grants through the Netherlands CardioVascular Research Initiative (“GENIUS”, CVON2011–19) and the Interuniversity Cardiology Institute of the Netherlands (ICIN, 09.001). Folkert W. Asselbergs is supported by a Dekker scholarship-Junior Staff Member 2014T001 – Netherlands Heart Foundation and UCL Hospitals NIHR Biomedical Research Centre. Maciej Tomaszewski is supported by the British Heart Foundation (PG/16/49/32176).

Acknowledgements

We thank Eveline Altena and Peter de Knijff for generously making available the Dutch control population data from the Forensic Laboratory of DNA Research. Furthermore, we want to thank Arjan Schoneveld and Sander van de Weg for the help with the haplogrouping of the AE samples and Evelyn Velema for the histopathological phenotyping of the plaque and aneurysm samples. Lastly, we thank all patients included in the two cardiovascular cohorts for their participation.
References