The age- and sex-specific composition of atherosclerotic plaques in vascular surgery patients

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HIGHLIGHTS

- Older age is independently associated with rupture-prone iliofemoral plaque characteristics.
- Iliofemoral plaques derived from men show more rupture-prone characteristics compared to women.
- The sex differences in plaque composition are attenuated with an increase in age.

ARTICLE INFO

ABSTRACT

Background and aims: The sex- and age-related differences in the composition of iliofemoral atherosclerotic plaques are largely unknown. Therefore, the aim of the current study is to gain insight into plaque composition across strata of age and sex in a large cohort of vascular surgery patients.

Methods: Peripheral atherosclerotic plaques of patients who underwent iliofemoral endarterectomy (n = 790) were harvested between 2002 and 2014. The plaques were semi-quantitatively analyzed for the presence of lipid cores, calcifications, plaque hemorrhages (PH), collagen, macrophage and smooth muscle cell (SMC) content, and quantitatively for microvessel density. Patients were stratified by age tertiles and sex.

Results: Ageing was independently associated with rupture-prone iliofemoral plaque characteristics, such as higher prevalence of plaque calcifications (OR 1.52 (95%CI:1.03–2.24) p = 0.035) and PH (OR 1.46 (95%CI:1.01–2.09) p = 0.042), and lower prevalence of collagen (OR 0.52 (95%CI:0.31–0.86) p = 0.012) and SMCs (OR 0.59 (95%CI:0.39–0.90) p = 0.015). Sex-stratified data showed that men had a higher prevalence of lipid cores (OR 1.62 (95%CI:1.06–2.45) p = 0.025) and PH (OR 1.62 (95%CI:1.16–2.54) p = 0.004) compared to women. These sex-differences attenuated with increasing age, with women showing an age-related increase in calcifications (p = 0.002), PH (p = 0.015) and decrease in macrophages (p = 0.005). In contrast, men only showed a decrease in collagen (p = 0.043).

Conclusions: Atherosclerotic iliofemoral plaques derived from men display more rupture-prone characteristics compared to women. Yet, this difference is attenuated with an increase in age, with older women having more rupture-prone characteristics compared to younger women.

1. Introduction

Peripheral artery disease (PAD) is the main cause of chronic limb threatening ischemia (CLTI) and is caused by severe atherosclerosis. The buildup of atherosclerotic plaques can severely narrow and occlude arteries, thereby impairing tissue oxygenation [1]. Impaired blood flow in the lower extremities can lead to diminished walking distance, non-healing wounds, and tissue or limb loss [2]. Moreover, patients suffering from PAD have an increased risk for myocardial infarction, stroke, and vascular death, which reflects the systemic nature of
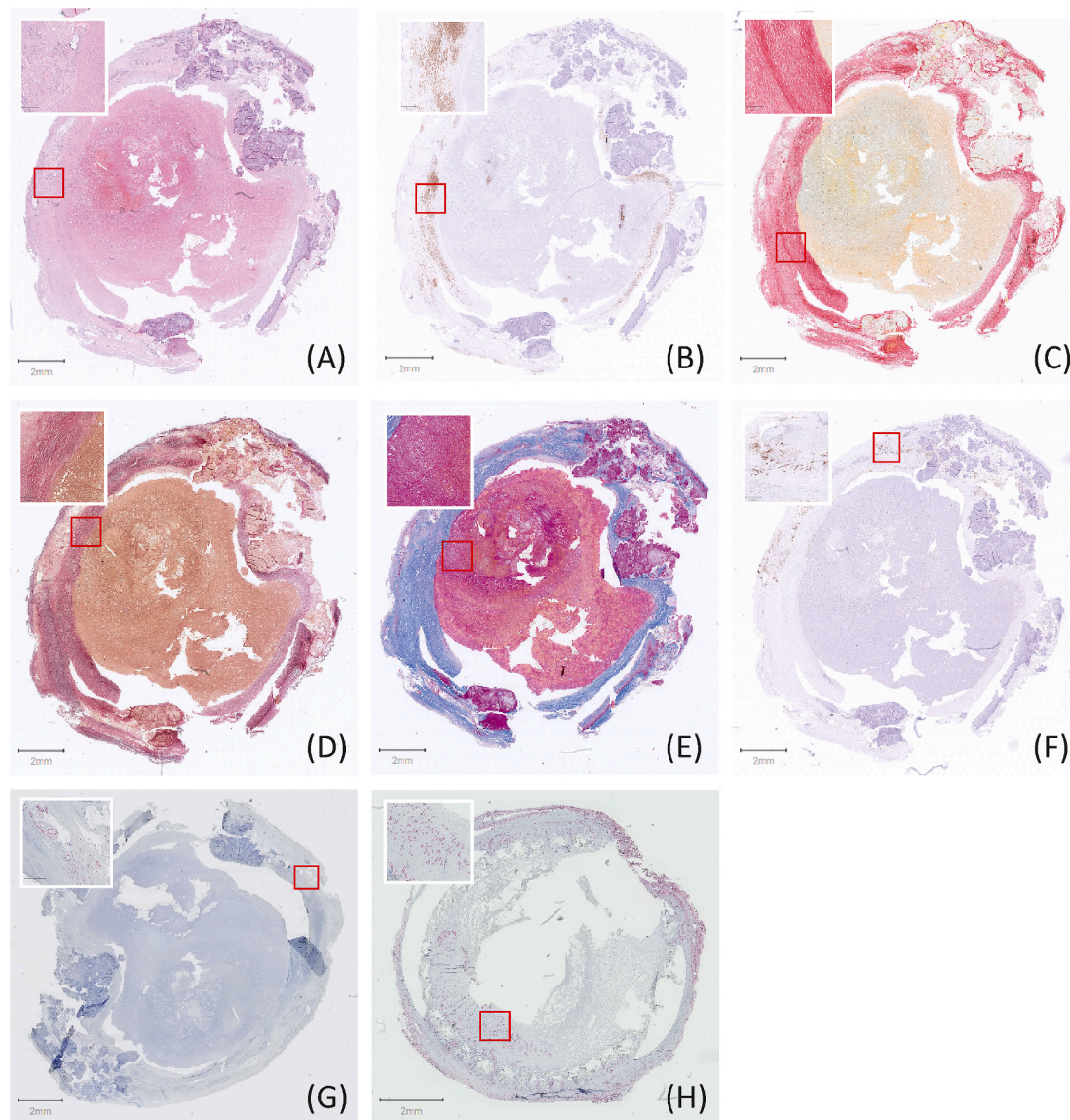
Fig. 1. Example of an atherosclerotic plaque stained for histological assessments.
(A-G) Example of one single culprit iliofemoral lesion stained for the presence of hematoxylin-eosin (A), α-smooth mucle actin (smooth muscle cells) (B), picrosirius red (C), elastin von Gieson (D), fibrin (E), CD68 (macrophages) (F), and CD34 (microvessel density) (G). (H) Example of a different culprit iliofemoral lesion stained with CD34, showing a higher microvessel density than the culprit lesion in panels A-G.

atherosclerosis [3,4].

The prevalence of PAD is strongly related to age and is over ten percent among patients between seventy and eighty years old [5]. Since this part of the population is growing fast, it is likely that an increasing number of patients with PAD will have to be treated in the near future [6]. Although traditional risk factors for PAD such as smoking and body mass index (BMI) decrease with age, advanced age is still an important predictor for adverse cardiovascular events after hospitalization [7]. In carotid and coronary artery patients, advanced age was associated with decreased plaque stability and rupture-prone plaques [8–10]. Nonetheless, the association between age and the underlying culprit of PAD, the atherosclerotic plaque, remains unknown.

To date, sex-related differences in cardiovascular disease (CVD) have gained increasingly more attention and literature evidence is growing. Nevertheless, sex-differences in PAD are still a matter of controversy. While traditional CVD risk factors are more prevalent in men, the disease burden of PAD seems to be higher in women than in men [5,11,12]. In addition, women show faster functional decline [13] and have poorer outcomes after lower extremity revascularization

procedures [14,15]. Among carotid endarterectomy (CEA) patients, women have an increased risk for restenosis, due to the stable nature of their plaques [16,17]. However, it is largely unknown if there are sex-related peripheral plaque differences that could help explain the differences in clinical characteristics of PAD between sexes.

In the current study, we provide detailed insight into the composition of the atherosclerotic plaque in men and women with PAD in different age strata using a histopathological analysis of nearly eight hundred plaques.

2. Patients and methods

2.1. Study population

Since 2002, all patients scheduled for revascularization of the ilio-femoral arteries in two large tertiary referral hospitals were invited to participate in the Athero-Express (AE) biobank study [18,19]. The AE study is an ongoing biobank, and extensive baseline characteristics, blood samples, and atherosclerotic plaque specimens are collected.

Clinical data were obtained from patient files and through standardized questionnaires. Questionnaires were completed at the screening visit prior to surgery and focused on medication use, cardiovascular risk factors, and medical history. Indications for iliofemoral endarterectomy (IFE) were reviewed by a multidisciplinary vascular team consisting of vascular surgeons, neurologists and an intervention radiologist. Patients that underwent an IFE were suffering from severe intermittent claudication, critical ischemia or tissue loss (Fontain classification IIb-IV) and presented with atherosclerotic lesions (TransAtlantic Intersociety Consensus [TASC] A to D) of the iliac and/or femoral arteries [20,21]. Preoperatively, patients were set on aspirin or clopidogrel, except for those taking anticoagulants for other comorbidities. Removal of the atherosclerotic plaques was performed by a team of experienced surgeons following standardized endarterectomy procedure. The AE study was approved by the medical ethics committees of both participating hospitals and is in accordance with the declaration of Helsinki. All patients provided written informed consent.

Between 2002 and 2014, a total of 1059 patients scheduled for revascularization of the iliofemoral arteries were enrolled in the AE study. We included the 790 (74.6%) IFE patients with complete data on atherosclerotic plaque histology in the present study.

2.2. Sample collection, processing, and histological assessment

Blood samples were collected prior to surgery and stored at −80° Celsius. The atherosclerotic plaques were transported to the laboratory and processed immediately after surgical removal. The plaques were dissected into 5-mm-thick cross-sectional segments along the longitudinal axis of the vessel by an experienced technician. Subsequently, the technician identified the culprit lesion, which is defined as the segment with the largest plaque burden. In the case of a total occlusion of the lumen, the segment with the largest plaque diameter was selected. The segment with the culprit lesion was then prepared and stored in 4% formaldehyde, decalcified, and embedded in paraffin for histological analysis. The rest of the plaque was snap frozen using liquid nitrogen and stored at −80° Celsius.

Histologic assessment of the atherosclerotic plaque specimens was performed according to a previously validated protocol, which was described in detail before [18,22]. In brief, cross-sections of the culprit lesion are stained (Fig. 1) and quantified for each patient. A hematoxylin-eosin (HE) staining was performed to obtain a general overview of the atherosclerotic plaque, including the lipid core and calcification. Picrosirius red was used for the assessment of collagen and alpha-smooth muscle actin (α-SMA) for smooth muscle cells. CD68 was stained to identify phagocytosis-active cells (e.g. macrophages). Plaque characteristics were scored semi-quantitatively at 40x magnification and grouped into no, minor, moderate and heavy staining. In the present study, these categories were binned into no/minor staining and moderate/heavy staining. Immunohistochemical staining for CD34 was performed to assess vessel density. Plaque microvessels were quantified in three hotspots (i.e. the area with the highest microvessel density) using a grid (100 × 100 μm) overlying these hotspots. Vessel density was determined by counting the number of vessels crossed by a bar of the grid within the selected hotspots. Subsequently, the average number of vessels per hotspot was calculated. Picrosirius red in combination with elastic-Van Gieson, HE and polarized light was used to visualize the lipid core. The size of the lipid core of the plaque was visually estimated as a percentage of the total plaque area, with a cutoff at 10% for iliofemoral plaques. The presence of plaque hemorrhage was determined using an HE staining and a fibrin staining. Plaque hemorrhage was defined as the composite of a hemorrhage at the luminal side of the plaque as a result of plaque disruption and intraplaque hemorrhage, which is observed as a hemorrhage within the plaque tissue. All plaque characteristics were scored and quantified with good intra- and inter-observer reproducibility (mean κ: 0.78 and 0.69, respectively) by two independent observers (GP and EV) [23].

2.3. Statistical analysis

Stratified analyses were performed based on sex and age of the patient. Patients who underwent IFE were stratified into age tertiles; T1: < 64.41 years, T2: 64.41–72.46 years and T3: ≥72.47 years. Chi-squared tests were used to compare categorical baseline characteristics across the three different age groups and between males and females. One-way ANOVA was used for the comparison of continuous, normally distributed variables across the three age groups and Kruskal-Wallis tests for continuous, non-normally distributed variables. Independent T-tests were used to compare continuous normally distributed variables between males and females and Mann-Whitney U tests for continuous, non-normally distributed variables.

To assess the association between sex or age and the binary plaque characteristics, logistic regression analyses were performed, whereas continuous plaque characteristics were studied by linear regression. We applied univariable analyses and multivariable analyses for all plaque characteristics separately with correction for baseline characteristics associated with the plaque characteristic of interest ($p < 0.1$). These possible confounders per plaque characteristic can be found in Supplemental Table 1. Moreover, to study the sex-related age differences in iliofemoral plaque composition, stratified regression analyses based on the sex of the patient were performed. Regression analyses with the different age groups and with age as a continuous independent variable were performed with correction for the same possible confounders as in the primary analyses.

All analyses were performed in R version 3.4.1 and missing data were handled using the MICE package. A two-sided *p*-value < 0.05 was considered significant.

3. Results

3.1. Clinical characteristics of the study population

Fig. 2 depicts a flowchart of patient selection and stratification of patients who underwent IFE. The clinical characteristics of 790 patients included in the present study are presented in Table 1. In total, 577 males (73.0%) and 213 (27.0%) females with a mean age of 67.8 years undergoing IFE were included. Aspects of an unhealthy cardiovascular lifestyle, such as smoking (T1 55.8 *vs.* T3 29.8%, $p < 0.001$) and high alcohol intake (T1 48.3 *vs.* T3 27.7%, $p < 0.001$), are found more often in patients in the lower age groups (T1 and T2) compared to patients in the higher age group (T3). In contrast, other classical PAD risk factors are more prevalent in patients with increasing age, such as a lower estimated glomerular filtration rate (eGFR) (T1 89.4 *vs.* T3 65.7 mL/min/1.73 m², $p < 0.001$), an increase in the prevalence of hypertension (T1 65.0 *vs.* T3 77.4%, $p < 0.001$) and an increase in the prevalence of coronary artery disease (CAD) (T1 35.2 *vs.* T3 43.0%, $p = 0.003$) and stroke history (T1 3.2 *vs.* T3 9.2%, $p = 0.013$). Patients undergoing IFE were overall most likely to have a Fontaine IIb classification (53.8%). Fontaine classification did not differ between the different age groups. In the majority of patients, revascularization of the femoral artery (92.2%) was performed and the artery most often showed a total occlusion (64.5%). Thromboendarterectomy (66.7%) was more often performed than remote endarterectomy (REA) (21.7%). More specifically, plaque specimens from patients in the highest, more fragile age group (T2 and T3) were less likely obtained by REA (T1 32.0 *vs.* T3 23.2%, $p = 0.038$) and/or from the iliac artery (T1 13.6 *vs.* T3 3.8%, $p < 0.001$). In line with the increased incidence of hypertension across the different age groups, an increased use of antihypertensive-agents was found (T1 75.8 *vs.* T3 87.5%, $p < 0.001$). In addition, older patients (T3) used less antiplatelet medication compared to patients in the younger age groups (T1 and T2) (T1 85.6 *vs.* T3 76.8%, $p = 0.002$).

Baseline characteristics of patients stratified by sex are summarized in Table 2. Mean age did not differ between males and females. Nonetheless, male patients scored higher for some of the traditional
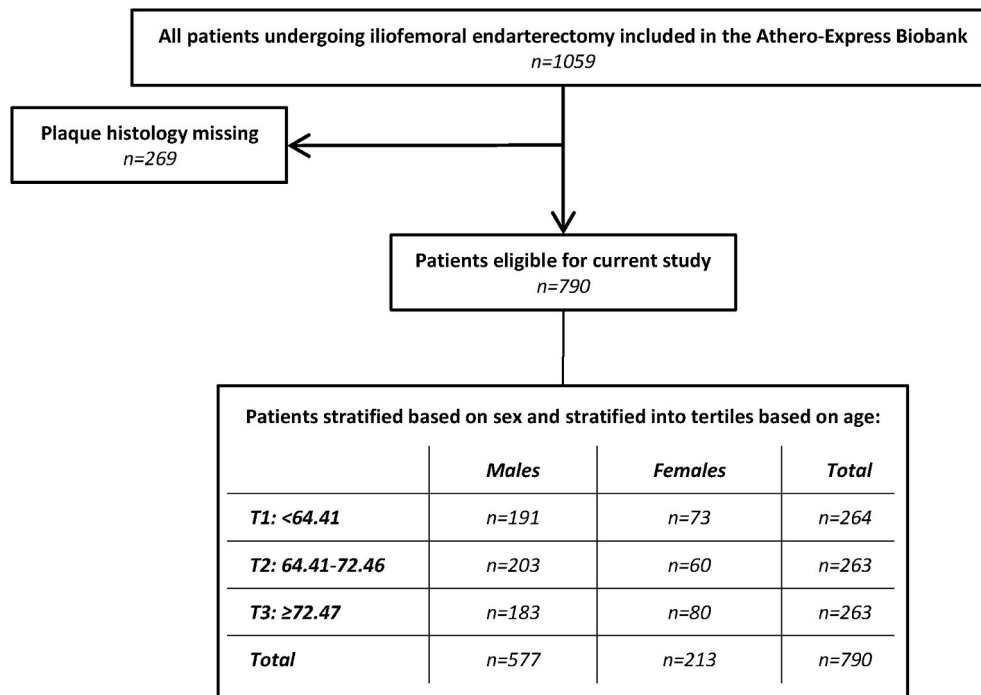
Fig. 2. Flowchart. Peripheral atherosclerotic plaques obtained from patients undergoing iliofemoral endarterectomy.

PAD risk factors compared to female patients, as they had higher BMIs (26.4 *vs.* 25.3, *p* = 0.001), were more often heavy alcohol consumers (45.7 *vs.* 22.4%, *p* < 0.001), had a higher median estimated number of pack years (28.0 *vs.* 15.0 years, p < 0.001), had lower levels of high-density lipoprotein cholesterol (1.11 *vs.* 1.30 mmol/L, *p* < 0.001), and presented more often with a positive history of CAD (45.2 *vs.* 35.7%, *p* = 0.016). Nonetheless, another risk factor was more prevalent in females, i.e. lower eGFR (78.5 *vs.* 72.0 mL/min/1.73 m^2, *p* = 0.003). The prevalence of current smokers and the incidence of hypertension, diabetes mellitus and hypercholesterolemia did not differ between the two sexes. The mean ankle-brachial index of patients was 0.60, regardless of sex. Relatively more males than females were classified as Fontaine IIb (57.7 *vs.* 43.2%) and in contrast, more females were classified as Fontaine III compared to males (36.2 *vs.* 22.9%) (*p* = 0.001). The prevalence of Fontaine IV classification was comparable in both groups.

Patients excluded from the present analyses due to missing plaque characterization data were, on average, one and a half year older (*p* = 0.021), more likely to be female (35.3% *vs.* 27.0%, *p* = 0.009) had a higher prevalence of diabetes (38.2 *vs.* 30.9%, *p* = 0.029) and a higher eGFR (84.6 *vs.* 76.7 ml/min/1.73 m^2, p = 0.001) compared to the patients included in the present analyses.

3.2. Iliofemoral plaque histology

The plaque specimens of patients in age group T2 (64.4–72.5 years) had significantly less moderate/heavy macrophage staining compared to the reference group (T1) (Supplemental Table 2). Patients in age group T3 (≥72.5 years) had a lower collagen and smooth muscle cell (SMC) content compared to the reference group. After correction for multiple confounders, the negative association between age group T3 and moderate/heavy collagen (OR 0.52 (95%CI 0.31–0.86) *p* = 0.012) and SMC (OR 0.59 (95%CI 0.39–0.90) *p* = 0.015) staining remained statistically significant. In addition, plaque specimens of patients in age group T3 had an increased presence of plaque hemorrhage (OR 1.46 (95%CI: 1.01–2.10) *p* = 0.042) compared to the reference group. The association between age group T2 and moderate/heavy macrophage staining was no longer significant, whereas the positive association of

T2 and moderate/heavy calcification (OR 1.52 (95%CI: 1.03–2.24) *p* = 0.035) remained statistically significant. Increasing age was not associated with the presence of a lipid core ≥10% in univariable or multivariable analyses. Vessel density, quantified as the mean number of microvessels per hotspot, was determined in a random subset of the PAD patients consisting of 240 plaque specimens and showed no association with advanced age.

The relation between male sex and the histological plaque characteristics is summarized in Supplemental Table 3. Men had a higher prevalence of plaque calcifications and plaque hemorrhages than women. After correction for multiple confounders, male sex was associated with the presence of a lipid core ≥10% (OR 1.62 (95%CI: 1.06–2.45) *p* = 0.025) and with the presence of plaque hemorrhage (OR 1.62 (95%CI: 1.16–2.25) *p* = 0.004).

3.3. Sex-age differences in iliofemoral plaques

The association between age and the different iliofemoral plaque characteristics, stratified by sex is presented in Supplemental Table 4 and visualized in Fig. 3. After correction for possible confounders, males and females showed different trends for moderate/heavy calcifications, the presence of plaque hemorrhage and moderate/heavy collagen and macrophage staining with increasing age. Namely, males showed a decrease in moderate/heavy collagen staining (OR T3 *vs.* T1 0.474) (*p* = 0.042) with advanced age, whereas females showed no association. In contrast, moderate/heavy calcifications (OR T3 *vs.* T1 4.016) (*p* = 0.002) and the presence of plaque hemorrhage (OR T3 *vs.* T1 2.343) (*p* = 0.015) increased with increasing age in females, but these plaque characteristics were not associated with age in males (*p* = 0.882 and *p* = 0.082, respectively). Despite the differences observed in trends between males and females for plaque hemorrhage, males had an increased presence of plaque hemorrhage compared to females across all age groups in multivariable analysis (data not shown). Lastly, the moderate/heavy macrophage staining decreased with advanced age (OR T3 *vs.* T1 0.235) (*p* = 0.005) in females, but showed no association in males (*p* = 0.988).

4. Discussion

In this study of 790 PAD patients, older age was associated with a more calcified and rupture-prone plaque, reflected by a higher prevalence of plaque hemorrhage, and a lower prevalence of collagen and smooth muscle cell content. In addition, men had a higher prevalence of lipid cores and plaque hemorrhage compared to women. Nonetheless, these sex-differences attenuated with an increase in age, with women showing an age-related increase in plaque calcifications, plaque hemorrhage and a decrease in macrophage content. In contrast, men only showed an age-related decrease in collagen content.

Increasing age is thought to predispose or accelerate CVD independent of other risk factors [24]. The increased susceptibility to atherosclerosis observed in the aging arterial wall is correlated with several, complex mechanisms that contribute to a pro-atherogenic environment [25]. For instance, *in vitro* experiments with human vascular SMCs demonstrated an increase in cell senescence and a reduction in cell proliferation with increasing age, which could explain the relatively low SMC content in plaques of elderly patients in the current study [26]. Since smooth muscle cells contribute to the formation of the protective fibrous cap, a decrease in SMC content is attributed to destabilization of the fibrous cap. Moreover, low smooth muscle content

Table 1
Clinical characteristics of study population.

	PAD patients	T1: < 64.4 years	T2:64.4–72.5 years	T3:≥72.5 years	p-value[a]
	N = 790	n = 264	n = 263	n = 263	
Patient characteristics					
Age (years), mean (SD)	67.8 (9.1)				
Sex (male), n (%)	577/790 (73.0)	191/264 (72.3)	203/263 (77.2)	183/263 (69.6)	0.138
BMI, mean (SD)	26.1 (4.1)	26.0 (4.3)	26.6 (3.9)	25.8 (3.8)	0.117
eGFR (mL/min/1.71m^2), mean (SD)	76.7 (27.0)	89.4 (26.2)	75.1 (26.3)	65.7 (23.1)	< **0.001**
Current smoker, n (%)	312/781 (39.9)	145/260 (55.8)	90/263 (34.2)	77/258 (29.8)	< **0.001**
Estimated number of pack years, median [IQR]	24.5 [15.0–40.0]	24.8 [15.0–40.0]	29.6 [15.0–44.3]	20.0 [10.0–37.9]	**0.004**
Alcohol intake per week, n (%)					< **0.001**
None	143/702 (20.4)	31/236 (13.1)	46/242 (19.0)	66/224 (29.5)	
1-10	278/702 (39.6)	91/236 (38.6)	91/242 (37.6)	96/224 (42.9)	
> 10	281/702 (40.0)	114/236 (48.3)	105/242 (43.4)	62/224 (27.7)	
Diabetes mellitus[b], n (%)	244/790 (30.9)	73/264 (27.7)	81/263 (30.8)	90/263 (34.2)	0.264
Hypertension[c], n (%)	570/772 (73.8)	167/257 (65.0)	204/258 (79.1)	199/257 (77.4)	< **0.001**
Hypercholesterolemia[d], n (%)	503/711 (70.7)	167/234 (71.4)	182/242 (75.2)	154/235 (65.5)	0.065
Total cholesterol (mmol/L), mean (SD)	4.58 (1.17)	4.71 (1.14)	4.51 (1.13)	4.52 (1.22)	0.250
LDL cholesterol (mmol/L), mean (SD)	2.49 (0.89)	2.57 (0.88)	2.44 (0.86)	2.45 (0.94)	0.422
HDL cholesterol (mmol/L), mean (SD)	1.16 (0.39)	1.15 (0.40)	1.13 (0.40)	1.20 (0.34)	0.384
Triglycerides (mmol/L), median [IQR]	1.70 [1.25–2.33]	1.86 [1.30–2.41]	1.74 [1.30–2.54]	1.53 [1.10–2.14]	0.122
Patient history					
History of CAD, MI or coronary intervention, n (%)	337/790 (42.7)	93/264 (35.2)	131/263 (49.8)	113/263 (43.0)	**0.003**
History of stroke, n (%)	44/754 (5.8)	8/252 (3.2)	13/253 (5.1)	23/249 (9.2)	**0.013**
History of peripheral interventions, n (%)	391/787 (49.7)	119/263 (45.2)	137/262 (52.3)	135/262 (51.5)	0.208
Previous amputation, n (%)	34/740 (4.6)	7/246 (2.8)	10/249 (4.0)	17/245 (6.9)	0.083
Arterial characteristics					
Ankle-brachial index, mean (SD)	0.60 (0.21)	0.61 (0.20)	0.61 (0.21)	0.59 (0.19)	0.459
Fontaine classification, n (%)					0.193
Fontaine IIb	372/691 (53.8)	135/234 (57.7)	126/230 (54.8)	111/227 (48.9)	
Fontaine III	183/691 (26.5)	63/234 (26.9)	58/230 (25.2)	62/227 (27.3)	
Fontaine IV	136/691 (19.7)	36/234 (15.4)	46/230 (20.0)	54/227 (23.8)	
Operated artery, n (%)					< **0.001**
Femoral	720/790 (91.1)	228/264 (86.4)	239/263 (90.9)	253/263 (96.2)	
Iliac	70/790 (8.9)	36/264 (13.6)	24/263 (9.1)	10/263 (3.8)	
Restenosis, n (%)	118/710 (16.6)	36/239 (15.1)	50/231 (21.6)	32/240 (13.3)	**0.039**
Stenosis grade, n (%)					0.247
50–69%	32/567 (5.6)	6/201 (3.0)	14/181 (7.7)	12/185 (6.5)	
70–99%	164/567 (28.9)	56/201 (27.9)	56/181 (30.9)	52/185 (28.1)	
Occlusion	371/567 (65.4)	139/201 (69.2)	111/181 (61.3)	121/185 (65.4)	
Contralateral stenosis, n (%)					0.239
0–49%	137/340 (40.3)	39/103 (37.9)	56/121 (46.3)	42/116 (36.2)	
50–100%	203/340 (59.7)	64/103 (62.1)	65/121 (53.7)	74/116 (63.8)	
Type of surgery, n (%)					**0.038**
TEA	545/738 (73.8)	168/247 (68.0)	188/245 (76.7)	189/246 (76.8)	
REA	193/738 (26.2)	79/247 (32.0)	57/245 (23.3)	57/246 (23.2)	
Pre-operative medication use					
Use of statins, n (%)	589/790 (74.6)	195/264 (73.9)	206/263 (78.3)	188/263 (71.5)	0.188
Use of antiplatelets, n (%)	657/789 (83.3)	226/264 (85.6)	229/262 (87.4)	202/263 (76.8)	**0.002**
Use of anticoagulants, n (%)	132/790 (16.7)	36/264 (13.6)	42/263 (16.0)	54/263 (20.5)	0.097
Use of antihypertensives, n (%)	660/790 (83.5)	200/264 (75.8)	230/263 (87.5)	230/263 (87.5)	< **0.001**
Diuretics	351/790 (44.4)	100/264 (37.9)	115/263 (43.7)	136/263 (51.7)	**0.006**
Beta-blockers	356/790 (45.1)	95/264 (36.0)	137/263 (52.1)	124/263 (47.1)	**0.001**
Calcium channel blockers	258/790 (32.7)	68/264 (25.8)	100/263 (38.0)	90/263 (34.2)	**0.009**
ACE inhibitors	354/790 (44.8)	123/264 (46.6)	122/263 (46.4)	109/263 (41.4)	0.405

Table 1 (*continued*)

	PAD patients	T1: < 64.4 years	T2:64.4–72.5 years	T3: ≥72.5 years	*p*-value[a]
	N = 790	n = 264	n = 263	n = 263	
Angiotensin II receptor blockers	158/790 (20.0)	35/264 (13.3)	64/263 (24.3)	59/263 (22.4)	**0.003**
Use of anti-diabetics, n (%)	213/790 (27.0)	66/264 (25.0)	71/263 (27.0)	76/263 (28.9)	0.602
Insulin	38/790 (4.8)	14/264 (5.3)	14/263 (5.3)	10/263 (3.8)	0.578
Oral glucose inhibitors	129/790 (16.3)	38/264 (14.4)	39/263 (14.8)	52/263 (19.8)	0.367
Insulin and oral glucose inhibitors	46/790 (5.8)	14/264 (5.3)	18/263 (6.8)	14/263 (5.3)	0.718

PAD, Peripheral Artery Disease; BMI, Body Mass Index; eGFR, estimated Glomerular Filtration Rate; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; CAD, Coronary Artery Disease; MI, Myocardial Infarction; TEA, Thromboendarterectomy; REA, Remote Endarterectomy; PTA, Percutaneous Transluminal Angioplasty; ACE, Angiotensin-converting-enzyme. Data is presented as n/N (%), mean (SD) or median [IQR]. Bold values are considered statistically significant with a *p* < 0.05.

[a] Comparison of the three age groups by univariable analysis.

[b] Diabetes mellitus (type 1 and type 2) was defined as the presence of one of the following: (1) diabetes mellitus in medical history extracted from the patient file, or (2) use of either insulin or oral glucose inhibitors extracted from the patient file.

[c] Hypertension was defined as the presence of one of the following: (1) hypertension in medical history extracted from the patient file, or (2) use of anti-hypertensive medication extracted from the patient file.

[d] Hypercholesterolemia was defined as the presence of one of the following: (1) hypercholesterolemia in medical history extracted from the patient file, or (2) use of lipid lowering medication extracted from the patient file.

potentially induces thinning of the fibrous cap of atherosclerotic plaques as smooth muscle cells produce most of the arterial interstitial collagen that provides strength to the fibrous cap [27]. The observed decrease in collagen content in lesions of older patients, could also be attributed to an increased expression of members in the matrix metalloproteinases (MMPs) family that occurs during aging [28]. MMP-13 levels play a key role in collagen degradation in mice and were strongly associated with collagen content in both early and established plaques of an atherosclerotic animal model [29,30]. Human MMP-1 and MMP-8 were associated with areas of cleaved collagen and are found in the shoulder regions of vulnerable plaques [31,32]. Moreover, in previous analyses from our laboratory, MMP-8 and MMP-9 levels were inversely associated with collagen content of carotid atherosclerotic lesion and positively associated with an unstable plaque phenotype [33]. The calcification process in atherosclerotic lesions appears to be associated with several changes that occur during vascular aging, such as endothelial damage, lipid oxidation and inflammatory responses [25]. Evidence obtained from human studies show that advanced age is associated with increased thickening of arterial walls, increased calcium deposition, and increased plaque instability [34]. However, these findings were mostly obtained in the coronary or carotid vascular bed. In a relative small study of patients with carotid artery stenosis, it has been reported that with increasing age, fat-content increased while smooth muscle cells content decreased [9]. A large cohort study showed an even more pronounced effect of advanced age on carotid plaque stability as they observed decreased smooth muscle cell content, large atheroma and heavy plaque calcifications in atherosclerotic plaques obtained from elderly patients [8]. In addition, a relation between increasing age and increasing atherosclerotic plaque burden, and loss of plaque stability was established in a large multi-center study with patients suffering from acute coronary syndrome [10].

Sex differences in CVDs have been of great interest over the last decade. Several studies provided evidence for a sex-related difference in atherosclerotic coronary and carotid plaque composition. For instance, a large, coronary computed tomography angiography-based study revealed that men had a higher proportion of calcified plaques and a higher prevalence of high-risk plaque features as compared to women [35]. Moreover, it was previously indicated that carotid atherosclerotic plaques obtained from male patients who underwent CEA revealed a higher prevalence of plaque hemorrhage compared to plaques obtained from female patients [36]. Still, data on the underlying culprit of PAD is scarce. A small single-center study reported that male sex was an independent predictor for plaque rupture in the iliofemoral arteries [37]. In line with these findings, male plaques had more rupture-prone

plaque features, such as lipid content and plaque hemorrhage in the present study. Moreover, we found that the sex differences in the presence of plaque hemorrhage were present across all age groups. Similarly, a small study using magnetic resonance imaging reported that the prevalence of intraplaque hemorrhage in carotid arteries of males was significantly higher compared to those of females for all ages [38]. In addition, this study showed that premenopausal women were not affected by carotid intraplaque hemorrhage, but that the presence of intraplaque hemorrhage increased with age in postmenopausal women. Hence, the difference between males and females in the presence of intraplaque hemorrhage decreased with advanced age. Results from the current study support these findings and provide strong evidence for a difference in trend with increasing age for the presence of various atherosclerotic plaque characteristics in women compared to men. For instance, calcifications, plaque hemorrhage and macrophage content in iliofemoral atherosclerotic plaques showed a trend with increasing age in women, but not in men. In contrast, collagen content was significantly associated with advanced age in men, but not in women. Likewise, an autopsy study of sudden coronary death patients indicated that calcification lags behind in women with severe coronary disease compared to men [39]. Another small autopsy study reported that atherosclerotic lesions in premenopausal women had a thicker fibrous cap compared to postmenopausal women and that plaque vulnerability increased as women advanced into their postmenopausal years [40]. Moreover, a recent study indicated that postmenopausal women show an increase in subclinical atherosclerosis, reflected by a greater intima-media thickness at the carotid, brachial and femoral arteries compared to premenopausal women [41]. Hence, it could be speculated that estrogen modifies the growth and composition of atherosclerotic lesions in women, resulting in plaque stabilization during their reproductive years and destabilization of the stable atherosclerotic plaque during their postmenopausal years. A recent study analyzed the *in vivo* response of rodent macrophages to estrogen and revealed that the female sex hormone acts as a proliferative signal for macrophages [42]. These findings could explain the decrease in macrophage content found in atherosclerotic plaques of women with advanced age in the current study. *In vitro* studies revealed that the estrogen-related plasticity of vascular smooth muscle cell is remarkable and can lead to the acquisition of many functionally distinct phenotypes (e.g. synthetic or contractile) with very different outcomes in disease progression [43]. However, whether such age- or sex-related phenotype transitions are present in the current study population requires further investigation. A postmortem study revealed that estrogen status correlates with the calcified-plaque burden of coronary atherosclerotic plaques in women,

Table 2
Clinical characteristics of male and female patients.

	Male	Female	p-value
	n = 577	N = 213	
Patient characteristics			
Age (years), mean (SD)	67.8 (8.6)	67.7 (10.3)	0.919
BMI, mean (SD)	26.4 (3.9)	25.3 (4.4)	**0.001**
eGFR (mL/min/1.71m^2), mean (SD)	78.5 (26.8)	72.0 (27.0)	**0.003**
Current smoker, n (%)	218/570 (38.2)	94/211 (44.5)	0.110
Estimated number of pack years, median [IQR]	28.0 [15.0–42.8]	15.0 [10.0–30.0]	**< 0.001**
Alcohol intake per week, n (%)			**< 0.001**
None	77/532 (14.5)	66/170 (38.8)	
1-10	212/532 (39.8)	66/170 (38.8)	
> 10	243/532 (45.7)	38/170 (22.4)	
Diabetes mellitus[a], n (%)	187/577 (32.4)	57/213 (26.8)	0.127
Hypertension[b], n (%)	411/562 (73.1)	159/210 (75.7)	0.468
Hypercholesterolemia[c], n (%)	367/524 (70.0)	136/187 (72.7)	0.488
Total cholesterol (mmol/L), mean (SD)	4.55 (1.13)	4.68 (1.25)	0.313
LDL cholesterol (mmol/L), mean (SD)	2.48 (0.85)	2.48 (1.00)	0.981
HDL cholesterol (mmol/L), mean (SD)	1.11 (0.34)	1.30 (0.47)	**< 0.001**
Triglycerides (mmol/L), median [IQR]	1.74 [1.25–2.37]	1.66 [1.25–2.29]	0.632
Patient history			
History of CAD, MI or coronary intervention, n (%)	261/577 (45.2)	76/213 (35.7)	**0.016**
History of stroke, n (%)	31/556 (5.6)	13/198 (6.6)	0.610
History of peripheral interventions, n (%)	283/574 (49.3)	108/213 (50.7)	0.727
Previous amputation, n (%)	28/547 (5.1)	6/193 (3.1)	0.252
Arterial characteristics			
Ankle-brachial index, mean (SD)	0.60 (0.20)	0.60 (0.21)	0.811
Fontaine classification, n (%)			**0.001**
Fontaine IIb	292/506 (57.7)	80/185 (43.2)	
Fontaine III	116/506 (22.9)	67/185 (36.2)	
Fontaine IV	98/506 (19.4)	38/185 (20.5)	
Operated artery, n (%)			0.378
Femoral	529/577 (91.7)	191/213 (89.7)	
Iliac	48/577 (8.3)	22/213 (10.3)	
Restenosis, n (%)	90/523 (17.2)	28/187 (15.0)	0.481
Stenosis grade, n (%)			0.102
50–69%	18/410 (4.4)	14/157 (8.9)	
70–99%	118/410 (28.8)	46/157 (29.3)	
Occlusion	274/410 (66.8)	97/157 (61.8)	
Contralateral stenosis, n (%)			0.493
0–49%	98/250 (39.2)	39/90 (43.3)	
50–100%	152/250 (60.8)	51/90 (56.7)	
Type of surgery, n (%)			0.710
TEA	409/542 (75.5)	136/196 (69.4)	
REA	133/542 (24.5)	60/196 (30.6)	
Pre-operative medication use			
Use of statins, n (%)	430/577 (74.5)	159/213 (74.6)	0.972
Use of antiplatelets, n (%)	475/576 (82.5)	182/213 (85.4)	0.319
Use of anticoagulants, n (%)	105/577 (18.2)	27/213 (12.7)	0.065
Use of antihypertensives, n (%)	490/577 (84.9)	170/213 (79.8)	0.086
Diuretics	260/577 (45.1)	91/213 (42.7)	0.557
Beta-blockers	258/577 (44.7)	98/213 (46.0)	0.745
Calcium channel blockers	204/577 (35.4)	54/213 (25.4)	**0.008**
ACE inhibitors	277/577 (48.0)	77/213 (36.2)	**0.003**
Angiotensin II receptor blockers	116/577 (20.1)	42/213 (19.7)	0.904
Use of anti-diabetics, n (%)	163/577 (28.2)	50/213 (23.5)	0.180
Insulin	26/577 (4.5)	12/213 (5.6)	0.188
Oral glucose inhibitors	104/577 (18.0)	25/213 (11.7)	0.101
Insulin and oral glucose inhibitors	33/577 (5.7)	13/213 (6.1)	0.289

BMI, Body Mass Index; eGFR, estimated Glomerular Filtration Rate; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; CAD, Coronary Artery Disease; MI, Myocardial Infarction; TEA, Thromboendarterectomy; REA, Remote Endarterectomy; PTA, Percutaneous Transluminal Angioplasty; ACE, Angiotensin-converting-enzyme. Data is presented as n/N (%), mean (SD) or median [IQR]. Bold values were considered statistically significant with a $p < 0.05$.

[a] Diabetes mellitus (type 1 and type 2) was defined as the presence of one of the following: (1) diabetes mellitus in medical history extracted from the patient file, or (2) use of either insulin or oral glucose inhibitors extracted from the patient file.

[b] Hypertension was defined as the presence of one of the following: (1) hypertension in medical history extracted from the patient file, or (2) use of antihypertensive medication extracted from the patient file.

[c] Hypercholesterolemia was defined as the presence of one of the following: (1) hypercholesterolemia in medical history extracted from the patient file, or (2) use of lipid lowering medication extracted from the patient file.
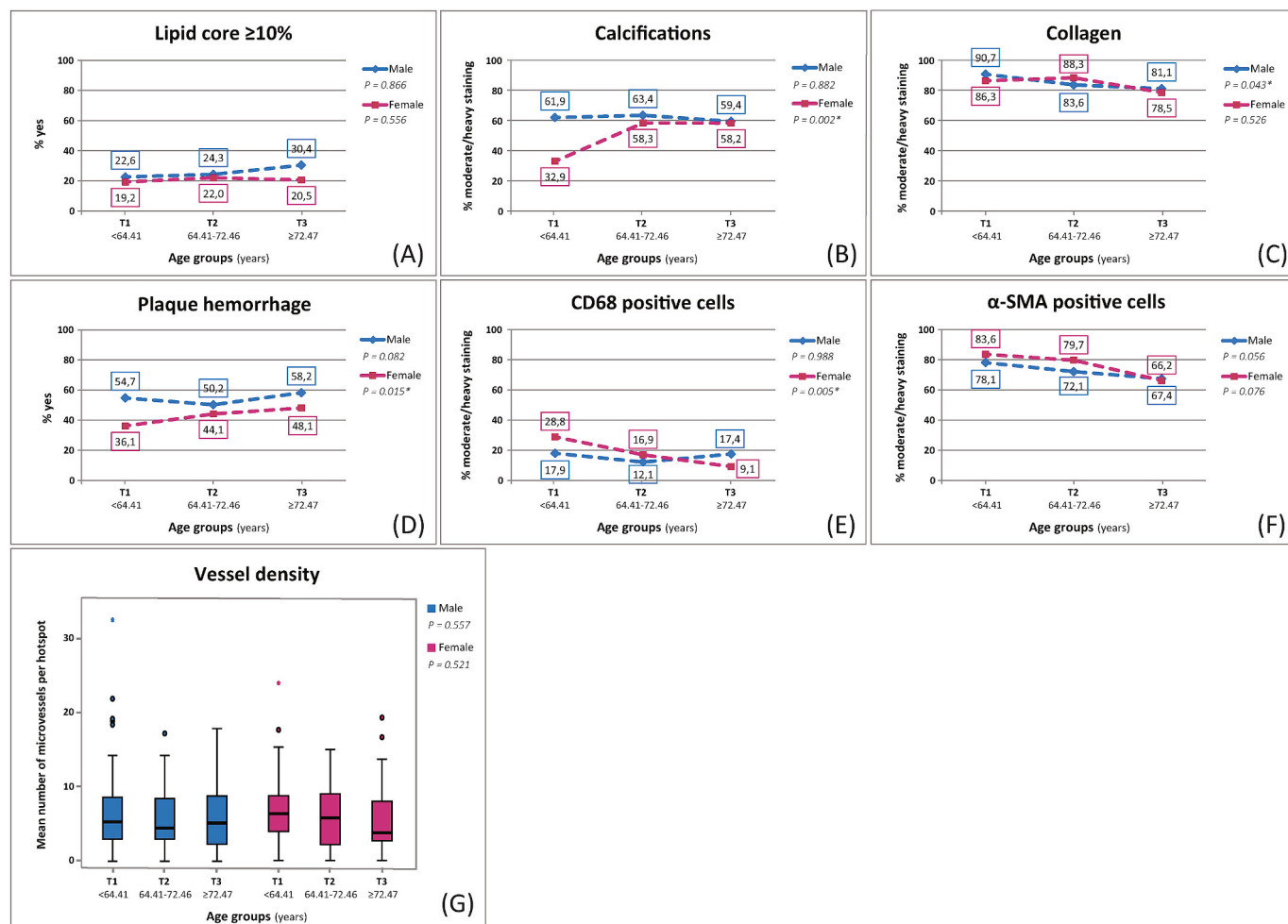
Fig. 3. Iliofemoral plaque characteristics across the different age groups in men and women.
The semi-quantitative atherosclerotic plaque characteristics (A–F) are presented separately for males (blue) and females (pink). The iliofemoral plaque characteristics are presented as the percentage of atherosclerotic plaques scored positive for the presence of a lipid core ≥10% (A) or for the presence of a plaque hemorrhage (D), and the percentage of atherosclerotic plaque quantified with a moderate/heavy staining for calcification (B), collagen (C), CD68 positive cells (E) and α-smooth mucle actin (α-SMA) positive cells (F). The iliofemoral plaque characteristic vessel density is quantified as the mean number of microvessels per hotspot (G) and is presented separately for males (blue) and females (pink). *Statistical significant ($p < 0.05$) association between age as a continuous independent variable and the plaque characteristic of interest stratified for males and females. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

independent of other risk factors. Premenopausal women and post-menopausal women receiving estrogen replacement therapy had a significantly lower calcium content and plaque area compared to post-menopausal women not receiving estrogen therapy [44]. The calcium content of atherosclerotic plaque in women increases with advanced age in the current study, despite their postmenopausal age (68 ± 10 years). Since atherosclerosis is a slow, progressive disease the protective effects of estrogen on plaque calcification would still be reflected in the atherosclerotic plaque composition of women shortly after menopause. Over time these protective effects wear off and the atherosclerotic plaque composition of females destabilizes and becomes equivalent to those of males, as observed for several plaque features of older women in the present cohort as compared to younger women. Nonetheless, it should be kept in mind that the vascular health of females is multi-factorial and cannot be linked solely to estrogen reduction [45].

The increase in knowledge in sex- and age-related trends in the pathological substrate of atherosclerotic disease is of great value, and to the best of our knowledge, this study represents the first study on sex-specific aging effects in PAD so far.

4.1. Future perspectives

The results of the current study are of interest in light of different treatment modalities applied to treat iliofemoral stenosis. As it has been previously described, the outcomes of different treatment strategies are influenced by the type of lesions treated [16,46,47]. For example, atherosclerotic plaques obtained during IFE with a high collagen and smooth muscle cell content were associated with an increased occurrence of restenosis [46]. Low macrophage infiltration and low lipid content of atherosclerotic plaques predicts increased restenosis rates in patients who underwent CEA [16]. Moreover, it is known that periph-eral vascular interventions such as balloon angioplasty and stenting are less successful in patients with heavily calcified plaques and often result in high restenosis rates [48], stent fracture and low consecutively vessel patency [49]. Moreover, endovascular techniques have largely sub-stituted open surgery as the most commonly used technique in the treatment of PAD. Henceforth, non-invasive imaging techniques are becoming more important in revealing plaque characteristics that could pertain important information regarding lesion specifics. Different atherosclerotic lesions likely show different success rates to either plain balloon, drug eluting balloon or drug eluting stent angioplasty. In

addition, plaque hemorrhage has been previously associated with increased risk of future cardiovascular events [22]. Hence, plaque characteristics could be valuable in the selection of high-risk patients that may benefit from more intensified medical therapy. Effective, but expensive add-on therapies such as PSCK-9 inhibitors could provide further reduction of residual cardiovascular risk and are recommended for secondary prevention for very high risk patients [50]. Incorporating this information in our treatment plans will bring us one step further to personalized medicine.

The composition of the pathological substrate of iliofemoral atherosclerotic disease plays an important role in the outcome of medical, endovascular and surgical treatment procedures. Unravelling the atherosclerotic plaque and factors that influence the plaque composition, such as sex and age, may allow for improved risk stratification and treatment selection.

4.2. Limitations

First, a total of 1059 PAD patients scheduled for revascularization were enrolled in the AE study and 790 atherosclerotic plaque specimens were available for histological assessment. The 269 patients that were excluded in the current analyses due to missing plaque characterization data were on average older, more often female, had a higher prevalence of diabetes and a higher eGFR compared to the patients included in the present study. However, it should be noted that availability of the atherosclerotic plaque specimens was not depend on age, sex or other comorbidities of the patient, but was attributed to logistical difficulties. Second, the surgical intervention for the treatment of PAD is often preceded by a period of conservative therapy, i.e. supervised exercise training combined with medical and lifestyle management. Therefore, the iliofemoral atherosclerotic plaques included in the present analysis are most likely end-stage atherosclerosis and no statements can be made about earlier stages in the disease process. In addition, since previous evidence indicate that medical treatment, such as statin therapy, affects atherosclerotic plaque morphology [51], medication use was included in the multivariable models as possible confounder. However, we did not have specific information about the compliance and duration of medicine intake prior to surgery. Moreover, it would be of great interest to stratify analysis for pre/postmenopausal status and to investigate its associations with plaque morphology. Unfortunately, only eight of the included women reported to still have a menstruation. In addition, considering the mean age of menopause is 51 years old, only 13 (7%) of the 213 women who underwent IFE had a pre-menopausal age (< 51 year). We are therefore underpowered to stratify pre- and post-menopausal women and provide relevant information. Furthermore, plaque erosion (e.g. thrombus on an intact plaque surface) and/or de-endotheliazation are gaining interest as a female-specific pathophysiologic mechanism in peripheral arterial disease patients. Due to both surgical techniques and ex vivo processing of the atherosclerotic plaque, we were not able to score the presence of thrombus on an intact plaque surface (i.e. plaque erosion) or de-endotheliazation. Instead, we refer to features associated with the stability of atherosclerotic plaques such as plaque hemorrhage, smooth muscle cells, inflammatory cells etc. Nonetheless, this study still observes large differences in plaque composition between sexes and different age groups of patients who underwent IFE.

4.3. Conclusion

Atherosclerotic iliofemoral plaques derived from men display more rupture-prone characteristics compared to women. Yet, this difference was attenuated with an increase in age, with older women having more rupture-prone characteristics as compared to younger women.

CRediT authorship contribution statement

Marie de Bakker: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Nathalie Timmerman:** Data curation, Writing - review & editing. **Ian D. van Koeverden:** Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Dominique P.V. de Kleijn:** Validation, Writing - review & editing. **Gert J. de Borst:** Conceptualization, Methodology, Data curation, Writing - review & editing. **Gerard Pasterkamp:** Conceptualization, Methodology, Validation, Data curation, Writing - review & editing. **Eric Boersma:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision. **Hester M. den Ruijter:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2020.07.016.

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