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## **Leukocytes and complement in atherosclerosis**

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# a. Gender differences in leukocyte activation in subjects with and without coronary artery disease

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## ABSTRACT

**Introduction:** Leukocyte activation is linked to coronary artery disease (CAD). We evaluated the differences in leukocyte activation between men and women in relation to the existence and the severity of CAD.

**Methods:** Postmenopausal women (n=39) and men (n=59) scheduled to undergo coronary angiography were selected and their classical cardiovascular risk factors as well as CAD status were determined. The expression of leukocyte activation markers CD11b and CD66b were determined by flowcytometry using fluorescent labelled monoclonal antibodies.

**Results:** The most consistent gender differences in classical risk factors were higher HDL and plasma apoA1 in women, irrespective of the CAD status. In CAD+ women compared to CAD+ men, the expression of several markers was decreased. This was the case for neutrophil CD66b ( $6.60 \pm 0.35$  au vs.  $7.70 \pm 0.36$  au,  $P=0.05$ ) and CD11b ( $25.05 \pm 1.70$  au vs.  $32.12 \pm 1.68$  au,  $P=0.02$ ), and monocyte CD11b ( $30.09 \pm 1.59$  au vs.  $34.64 \pm 1.66$  au,  $P=0.05$ ) expression, whereas no differences could be demonstrated between CAD- men and women. CAD+ women also showed lower expression of monocyte CD11b ( $30.09 \pm 1.59$  vs.  $37.27 \pm 2.34$ ,  $P=0.01$ ) and neutrophil CD11b ( $25.05 \pm 1.70$  vs.  $37.03 \pm 3.94$ ,  $P=0.01$ ) than CAD- women. No differences were found for leukocyte activation markers between CAD- and CAD+ men. There was no relationship with the use of medication, cardiovascular risk factors and the severity of CAD. The variable most clearly associated with leukocyte activation was plasma triglycerides, but only in men.

**Conclusions:** Activation of neutrophils and monocytes is decreased in CAD+ postmenopausal women compared to men in contrast to the lack of gender difference in CAD- subjects.

## INTRODUCTION

Atherosclerosis is a progressive disease, in which inflammation plays a central role (1). Both, the “response to injury” hypothesis proposed by Ross and Glomset (2) and the more recent “response to retention” hypothesis (3) suggest that the earliest events in atherogenesis are part of an inflammatory response with an important role for leukocyte activation. It has been proposed that such a response is initiated by resident and recruited leukocytes in response to endothelial injury (4). Epidemiological studies have shown that leukocyte count is positively related to coronary artery disease (CAD) (5), as well as to traditional cardiovascular risk factors, such as smoking, hyperlipidemia, and insulin resistance (6,7).

It has been shown that lipoproteins (8,9) and glucose (10,11) can activate endothelial cells. Simultaneous activation of leukocytes, which is obligatory for the development of atherosclerosis, has also been described *in vitro* and *in vivo* (12-16). This leukocyte activation can be quantified by measuring the expression of neutrophil and monocyte integrins like CD11b and CD66b. CD11b (also termed MAC-1 or CR3) is one of the most important markers involved in early adhesion of leukocytes to the endothelium (17,18). CD66b (also termed CEACAM8) is a degranulation marker of neutrophils and is not expressed on lymphocytes or monocytes (19). Increased expression of these markers on fasting leukocytes in patients with CAD and diabetes has been described before (13,20-22).

Despite being one of the major causes of death in women at all ages, the prevalence of CAD in women is relatively low before menopause, only approaching equal prevalence rates for men and women in their seventh decade of life (23-25). There is a substantial gender-related variability in the prevalence and outcome associated with traditional cardiac risk factors such as lipids, hypertension, obesity, smoking and diabetes mellitus type 2 (T2DM) (25). So far, no studies have been published on gender-specific inflammatory characteristics in CAD.

In this study, we aimed to evaluate the differences in leukocyte activation markers in relation to CAD and gender in subjects undergoing diagnostic coronary angiography.

## MATERIALS AND METHODS

### Subjects

Male and female subjects who visited the outpatient clinics of the department of Cardiology and were scheduled to undergo diagnostic coronary angiography, were invited to participate. Only postmenopausal women were included based on a history of secondary amenorrhea of at least 1 year and not using hormone replacement therapy.

Exclusion criteria were: The presence of inflammatory disorders, e.g. rheumatoid arthritis, systemic lupus erythematosus and infections, CRP > 10 mg/L, disorders of kidney, liver and thyroid function.

The Institutional Review Board of the St. Franciscus Gasthuis Rotterdam and the regional independent medical ethics committee, Maasstad Hospital Rotterdam approved the study. The participants gave written informed consent.

## **Study design**

Shortly before the angiography, venous blood was obtained from a peripheral vein of the forearm. The subjects were divided into two different groups according to the results of the angiography. The first group consisted of subjects without any angiographical sign of CAD nor having a history of other atherosclerotic disease. The second group consisted of subjects with coronary atherosclerosis, ranging from wall irregularities to multi-vessel disease. Coronary angiography images were scored by an independent cardiologist.

## **Analytical methods**

All clinical chemistry measurements were performed on the same day as the diagnostic coronary angiography. Basic parameters for renal and liver function as well as glucose, CRP, total cholesterol, HDL cholesterol and TG were determined using a Synchron LX analyzer (Beckman Coulter, Brea CA, USA) according to standard procedures in our laboratory for clinical chemistry. LDL cholesterol values were calculated using the Friedewald formula. ApoAI and ApoB were determined by rate nephelometry using IMMAGE with kits provided by Beckman (Beckman Coulter, Brea CA, USA). Blood cell counts were determined using the LH analyzer (Beckman Coulter, Miami FL, USA). The leukocyte differentiation was determined as a five-part differentiation on the same instruments.

## **Leukocyte activation markers**

Blood samples for the measurement of leukocyte activation markers were collected in EDTA and were determined by flowcytometry on the same day. In order to differentiate leukocytes in lymphocytes, monocytes and neutrophils a CD45 (Immunotech Coulter, Marseille, France) versus SS gating strategy was used. Lymphocytes were defined as CD45 positive and low sideward scatter. Monocytes were defined as CD45 positive and intermediate sideward scatter. Neutrophils were defined as CD45 weak and high sideward scatter. The gates were set quite narrow for optimal differentiation of these cell populations rather than for completeness. For tube 1 twenty  $\mu$ L blood from an EDTA-anti-coagulated blood sample was added to 2.5  $\mu$ L of each CD66b FITC (Immunotech Coulter, Marseille, France), CD11b PE (Immunotech Coulter,

Marseille, France) and CD45 ECD (Immunotech Coulter, Marseille, France). Cells were incubated for 15 minutes in the dark at room temperature. Erythrocytes were lysed by adding 300  $\mu$ L of ice-cold isotonic erythrocyte lysing solution (NH<sub>4</sub>Cl 0.19M; KHCO<sub>3</sub> 0.01M; Na<sub>2</sub>EDTA·2H<sub>2</sub>O 0.12M, pH 7.2) for 15 minutes. A Coulter Epics XL-MCL flowcytometer with a 488nm Argon ion laser and EXPO 32 software was used for measurement and analysis. Cells were acquired during 2 minutes per sample. On average a total of 25.000 leukocytes per sample were measured. Fluorescence intensity of each cell was expressed as the mean fluorescence intensity (MFI), given in arbitrary units (AU). Additional experiments (data not shown) did not show significant differences between EDTA and heparin anti-coagulated blood for CD11b and CD66b expression. Furthermore we also did not find significant differences of CD11b and CD66b expression in a protocol in which we did not use ammoniumchloride for erythrocyte lysis (data now shown) .

## Statistics

Data are given as mean $\pm$ SEM in the text, in the Tables and in the Figures. Baseline differences between the groups were tested by independent Students t-tests. The prevalence of CAD, medication use, smoking behavior and the prevalence of type 2 diabetes mellitus were tested by Chi-square tests. One way ANOVA with LSD test as post hoc test and Bonferroni correction for multiple comparisons were used to compare differences in the severity of CAD in different groups. Univariate regression analysis was carried out using Spearman correlation statistics. Data were analyzed in SPSS 16.0. Probability values less than 0.05 (2-tailed) were considered statistically significant.

## RESULTS

### Baseline cardiovascular and anthropometric characteristics (Table 1)

A total of 98 subjects were included of whom 59 were male and 39 female subjects. Table 1 shows the baseline characteristics of both women and men. HDL-cholesterol, apoAI and leukocyte counts were higher in women compared to men. The latter was due to higher neutrophil counts in women. The rest of the baseline cardiovascular and anthropometric determinants did not differ between the groups (Table 1).

Women used more beta-blockers (66.7% vs. 45.8%,  $P=0.03$ ) and diuretic medication (48.7% vs. 13.6%,  $P<0.0001$ ), and less statins (53.8% vs. 74.6%,  $P=0.03$ ) than men. Smoking behavior, the prevalence of T2DM and the use of aspirin, ACE-inhibitors, calcium channel antagonists, angiotensin II receptor blockers, nitrates, ezetimibe, metformin and SU-derivate did not differ by gender.

**Table 1.** Baseline characteristics in 59 male and 39 female subjects.

	Male (n=59)	Female (n=39)	P-value
Age (years)	62.69 (1.54)	65.28 (1.67)	0.27
BMI (kg/m <sup>2</sup> )	27.04 (0.56)	27.92 (0.72)	0.34
Waist circumference (m)	1.07 (0.02)	1.01 (0.03)	0.06
Systolic BP (mm Hg)	142 (3)	145 (4)	0.44
Diastolic BP (mm Hg)	80 (1)	82 (2)	0.33
Glucose (mmol/L)	6.91 (0.21)	6.48 (0.29)	0.23
Cholesterol (mmol/L)	4.70 (0.14)	5.09 (0.18)	0.09
HDL-C (mmol/L)	1.15 (0.03)	1.45 (0.07)	<0.001
LDL-C (mmol/L)	2.72 (0.13)	2.94 (0.15)	0.31
TG (mmol/L)	1.83 (0.12)	1.52 (0.16)	0.12
ApoA1 (g/L)	1.33 (0.03)	1.55 (0.06)	0.001
ApoB (g/L)	0.93 (0.04)	1.00 (0.05)	0.27
CRP (mg/L)	2.56 (0.31)	3.15 (0.41)	0.50
Leukocyte counts (10 <sup>9</sup> cells/L)	7.19 (0.23)	7.44 (0.30)	0.04
Monocyte counts (10 <sup>9</sup> cells/L)	0.62 (0.02)	0.55 (0.02)	0.77
Neutrophil counts (10 <sup>9</sup> cells/L)	4.46 (0.19)	4.56 (0.26)	<0.05
Lymphocyte counts (10 <sup>9</sup> cells/L)	1.89 (0.07)	2.14 (0.11)	0.25
Platelet counts (10 <sup>9</sup> cells/L)	226 (8)	240 (9)	0.24

Data are mean ( $\pm$  SEM). BP: blood pressure.

### Comparisons between genders with and without CAD (Tables 2 & 3)

CAD was found in 79.7% of the men, while relatively less female subjects had significant CAD (51.3%;  $P=0.003$ ). The number of vessels affected, a proxy for the severity of CAD, did not differ between women and men (25.0% vs. 21.2% for single-vessel CAD; 10.0% vs. 17% for 2-vessel CAD and 6.5% vs. 6.7% for 3-vessel CAD,  $P=0.75$ ).

Table 2 shows the differences between male and female subjects without (CAD-) and with CAD (CAD+). CAD- women were older than CAD- men. Their HDL, apoA1 and platelet counts were higher when compared to CAD- men (Table 2). Table 2 also shows that CAD+ women had significantly higher HDL and apoA1 concentrations when compared to CAD+ men; other variables did not differ by gender.

There were no differences in the presence of T2DM, smoking behavior and the use of medication between CAD- women and men (Table 3).

CAD+ women used more beta-blockers and diuretics when compared to CAD+ men, with no differences for T2DM, smoking behavior and the use of other medication (Table 3).

### Comparisons within genders (Tables 2 & 3)

CAD+ women had lower total cholesterol, LDL and platelet counts than CAD- women. LDL was lower in CAD+ men than in CAD- men (Table 2).

**Table 2.** Baseline characteristics in 12 male and 19 female subjects without coronary artery disease (CAD-), and 47 male and 20 female subjects with coronary artery disease (CAD+)

	CAD-			CAD+		
	Male (n=12)	Female (n=19)	P-value	Male (n=47)	Female (n=20)	P-value
Age (years)	53.25 (2.82)	60.74 (2.11)	0.04	65.11 (1.62)	69.60 (2.21)	0.12
BMI (kg/m <sup>2</sup> )	26.55 (1.92)	27.35 (1.01)	0.72	27.14 (0.57)	28.60 (1.03)	0.20
Waist circumference (m)	1.06 (0.07)	0.97 (0.03)	0.30	1.07 (0.02)	1.07 (0.06)	0.92
Systolic BP (mm Hg)	135 (5)	138 (4)	0.71	143 (3)	152 (6)	0.14
Diastolic BP (mm Hg)	85 (3)	85 (3)	0.93	79 (1)	80 (2)	0.68
Glucose (mmol/L)	6.32 (0.42)	6.63 (0.51)	0.68	7.05 (0.24)	6.34 (0.32)	0.10
Cholesterol (mmol/L)	5.18 (0.28)	5.46 (0.24)	0.45	4.58 (0.16)	4.73 (0.24)*	0.59
HDL-C (mmol/L)	1.20 (0.08)	1.51 (0.10)	0.02	1.14 (0.04)	1.40 (0.09)	0.003
LDL-C (mmol/L)	3.26 (0.26)	3.34 (0.19)	0.80	2.58 (0.15)*	2.53 (0.21)**	0.85
TG (mmol/L)	1.62 (0.18)	1.36 (0.16)	0.30	1.89 (0.14)	1.68 (0.27)	0.46
ApoA1 (g/L)	1.40 (0.05)	1.61 (0.07)	0.04	1.32 (0.04)	1.50 (0.08)	0.03
ApoB (g/L)	1.01 (0.07)	1.08 (0.07)	0.55	0.92 (0.04)	0.93 (0.06)	0.85
CRP (mg/L)	2.50 (0.79)	2.84 (0.61)	0.73	2.57 (0.33)	3.45 (0.55)	0.17
Leukocyte counts (10 <sup>9</sup> cells/L)	6.69 (0.40)	7.53 (0.55)	0.29	7.32 (0.27)	7.37 (0.28)	0.92
Monocyte counts (10 <sup>9</sup> cells/L)	0.63 (0.05)	0.53 (0.03)	0.09	0.62 (0.03)	0.57 (0.03)	0.26
Neutrophil counts (10 <sup>9</sup> cells/L)	3.96 (0.34)	4.74 (0.49)	0.26	4.59 (0.22)	4.39 (0.23)	0.58
Lymphocyte counts (10 <sup>9</sup> cells/L)	1.97 (0.12)	2.09 (0.17)	0.61	1.87 (0.08)	2.19 (0.14)	0.05
Platelet counts (10 <sup>9</sup> cells/L)	208 (19)	258 (11)	0.02	231 (8)	222 (12)*	0.58

Data are mean ( $\pm$  SEM). \*:  $P < 0.05$  and \*\*:  $P < 0.01$  vs. subjects without CAD (CAD-) within same gender. BP: blood pressure.

The presence of T2DM was comparable between CAD+ and CAD- women (26.3% vs. 25.0% diabetics, respectively,  $P=0.61$ ). CAD- women used less statins and aspirin (42.1% vs. 75.0% users,  $P=0.04$ , Table 3) than CAD+ women. There were no differences for the use of other medication, the presence of T2DM and smoking behavior.

There was a trend for a lower prevalence of T2DM in CAD- men compared to CAD+ men. The use of statins and aspirin (Table 3) was lower in CAD- men. Smoking behavior and the use of other medication did not differ between men without and with CAD.

### Leukocyte activation markers (Figures 1 & 2)

In general, there were no differences between male and female subjects for the expression of monocyte CD11b ( $35.42 \pm 1.58$  au vs.  $33.68 \pm 1.51$  au,  $P=0.45$ ), neutrophil CD11b ( $32.82 \pm 1.55$  au vs.  $31.04 \pm 2.34$  au,  $P=0.51$ ) and CD66b ( $7.62 \pm 0.33$  au vs.  $6.82 \pm 0.49$  au,  $P=0.16$ ).

No differences were found between CAD- women and men for the expression of these leukocyte activation markers (Figures 1 & 2).

When compared to CAD+ men, CAD+ women had a lower expression of neutrophil CD11b ( $25.05 \pm 1.70$  au vs.  $32.12 \pm 1.68$  au,  $P=0.02$ , Figure 2A), with a trend for CD66b ( $6.60 \pm 0.35$  au vs.



**Table 3.** The use of medication in 12 male and 19 female subjects without coronary artery disease (CAD-), and 47 male and 20 female subjects with coronary artery disease (CAD+)

	CAD-			CAD+		
	Male (n=12)	Female (n=19)	P-value	Male (n=47)	Female (n=20)	P-value
Type 2 diabetes mellitus	8.3	26.3	0.23	34.0 <sup>§</sup>	25.0	0.33
statins	50.0	31.6	0.26	80.9*	75.0*	0.41
aspirin	25.0	42.1	0.28	83.0**	75.0*	0.33
beta blockers	33.3	57.9	0.17	48.9	75.0	0.04
diuretics	16.7	42.1	0.14	12.8	55.0	0.001
ace-inhibitors	25.0	36.8	0.39	34.0	20.0	0.20
angiotensin II receptor blockers	8.3	26.3	0.18	25.5	20.0	0.44
calcium channel antagonists	8.3	26.3	0.18	36.2	35.0	0.58
long-acting nitrates	0	10.5	0.37	14.9	25.0	0.26
ezetimibe	0	5.3	0.61	10.6	10.0	0.65
Metformin	0	10.5	0.37	17.0	15.0	0.58
Su-derivates	0	5.3	0.61	14.9	10.0	0.46

\* Data are % of the medication users. <sup>§</sup>:  $P=0.08$ , \*:  $P<0.05$  and \*\*:  $P<0.001$  vs. subjects without CAD (CAD-) within same gender.

7.70±0.36 au,  $P=0.05$ , Figure 2B) and for monocyte CD11b (30.09±1.59 au vs. 34.64±1.66 au,  $P=0.05$ , Figure 1).

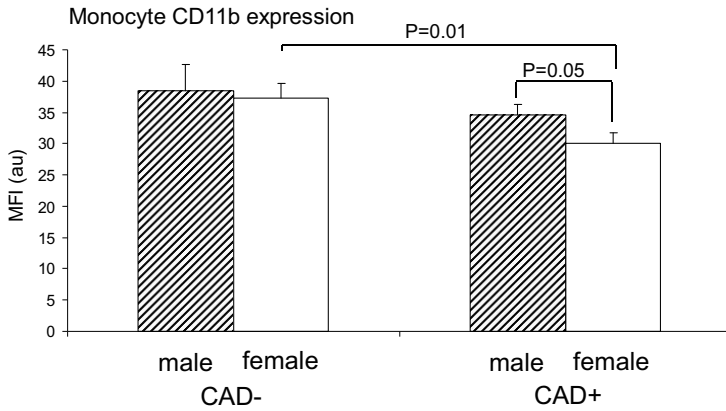
Comparing CAD- with CAD+ men, no differences were found for the leukocyte activation markers (Figures 1 & 2). CAD+ women had a lower expression of monocyte CD11b (30.09±1.59 au vs. 37.27±2.34 au,  $P=0.01$ , Figure 1) and neutrophil CD11b (25.05±1.70 au vs. 37.03±3.94 au,  $P=0.01$ , Figure 2A) than CAD- women.

We also compared male and female subjects with and without T2DM, and on and off therapy to correct for these parameters. These drugs included statins, aspirin, beta blockers and diuretics. The differences did not change the results we found for the whole group (Figures 1&2) and the differences between the genders remained (data not shown).

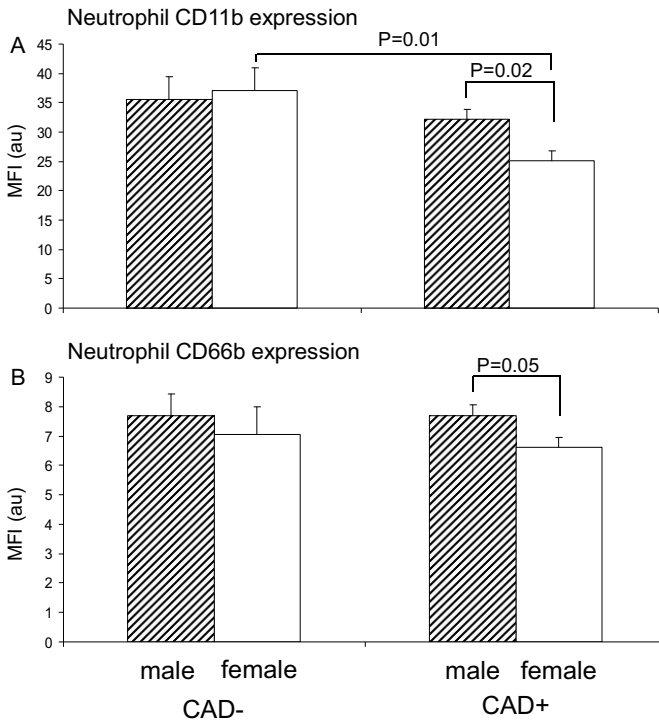
The expression of monocyte CD11b and neutrophil CD66b among all patients correlated only with plasma TG ( $r=0.22$ ,  $P=0.03$  and  $r=0.20$ ,  $P<0.05$ , respectively). Neutrophil CD11b correlated positively with plasma TG ( $r=0.33$ ,  $P=0.001$ ), apoB ( $r=0.28$ ,  $P=0.006$ ) and total cholesterol ( $r=0.23$ ,  $P=0.03$ ). Neutrophil CD11b correlated negatively with HDL ( $r=-0.20$ ,  $P<0.05$ ). Stratified for gender, all these correlations were due to men, as none of the classical cardiovascular risk factors in women correlated significantly with leukocyte activation markers.

## DISCUSSION

In this study, we have shown that in a group of subjects undergoing coronary angiography, women with coronary artery disease had a lower expression of leukocyte activation markers when compared to men with CAD. This gender difference was consistent for the monocyte



**Figure 1.** Mean±SEM expression of monocyte CD11b in male and female subjects, without (CAD-) and with (CAD+) angiographically proven coronary artery disease (CAD). CAD was defined as at least wall irregularities observed during the coronary angiography. Expression of monocyte CD11b is shown as Mean Fluorescence Intensity (MFI).



**Figure 2.** Mean±SEM expression of neutrophil CD11b (2A) and CD66b (2B) in male and female subjects, without (CAD-) and with (CAD+) angiographically proven coronary artery disease (CAD). CAD was defined as at least wall irregularities observed during the coronary angiography. Expression of the markers is shown as Mean Fluorescence Intensity (MFI).

CD11b and neutrophil CD11b and CD66b expression. However, CAD- subjects did not show any differences in these markers. Furthermore, we found that CAD+ women had lower activation markers than CAD- women, whereas such differences were not observed in men.

Our data may suggest that leukocyte activation in postmenopausal women with CAD seems to be compromised. These women used more beta-blockers and diuretics than their male counterparts, and they used more statins and aspirin than women without CAD. Some (third-generation) beta-blockers (combined with alpha-inhibitors) have been shown to prevent endothelial adhesiveness of human mononuclear cells suggesting less leukocyte activation (26) and also to prevent infiltration of inflammatory cells into the sub-endothelial space (27). There are no data available on the effects of diuretics on leukocyte activation. Previous studies showed that statins have no effect, reduced or even increased CD11b expression on leukocytes (20,28-30). Despite the anti-platelet effects, aspirin does not influence the leukocyte activation marker CD11b (31). If statins and aspirin would have been responsible for the gender differences of leukocyte activation, we would have expected similar results in CAD+ men since they used more statins and aspirin than CAD- men. The same applies to beta-blockers and diuretics, as no differences were found between CAD+ and CAD- women. Moreover, we corrected for these medication by comparing male and female subjects on and off therapy and still, the results remained in favour of a lower leukocyte activation status in women. However, we included small number of patients and all these drugs have anti-inflammatory effects and we cannot rule out that their concerted action can affect leukocyte activation markers in atherosclerotic women.

A clustering of risk factors is common in postmenopausal women, notably obesity, hypertension, and dyslipidemia, possibly related to gender-specific metabolic differences exacerbated by hormonal imbalances (25). In our study, most CAD+ women were on standard drug therapy and had higher HDL and apoA1 concentrations than men with CAD. Moreover, these women had lower total cholesterol, LDL and platelet counts than CAD- women. However, it is unlikely that the gender differences can be explained by these parameters since the differences between genders were not consistent.

The severity of CAD was not related to gender differences neither, since no differences were found for 1-, 2- or 3-vessel disease between women and men with CAD.

Taking all these findings into account, we do not have a satisfactory explanation for the gender differences. The fact that the cardiovascular risk factors, in contrary to men, did not correlate with the leukocyte activation markers in women is illustrative and suggests that other, not yet found, variables affect leukocytes in women. The main question is whether lower leukocyte activation markers in CAD+ women is a protective or risk enhancing factor. As mentioned before, there is a large body of evidence linking CD11b and CD66 expression in leukocytes to the existence of atherosclerosis (13-16,20-22). So these markers have been proposed as surrogate measures for CAD. One explanation could be that the leukocyte activation in the

peripheral blood is underestimated due to increased CD11b and CD66b expression in the atherosclerotic plaque in women. Further studies are needed to explain our data.

Finally, plasma TG remains the strongest factor influencing the leukocyte activation markers in men. We have previously shown that triglycerides are able to induce leukocyte activation by direct interaction with leukocytes, and the generation of oxidative stress (15).

In conclusion, we show for the first time that leukocyte activation markers CD11b and CD66b in monocytes and neutrophils are compromised in CAD+ women, and that despite differences, neither the classical cardiovascular risk factors, severity of CAD, nor the use of anti-inflammatory drugs can explain this new phenomenon. Furthermore, our study supports that TG show the strongest correlation with leukocyte activation in men.

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### **Disclosures**

None declared.

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