# Circulating cells as biomarkers in cardiovascular disease : the difference between men and women

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# III

# CD4 and CD8 T cell subsets associate with coronary artery disease in sex-specific fashion

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Manuscript submitted for publication

## Abstract

Background- While a limited number of epidemiologic studies have addressed the biomarker potential of circulating T cells for coronary artery disease (CAD) none have looked at sex-specific differences in populations of T cells. The aim of the current study was to determine sex-specific associations of a wide range of T cell subsets with CAD.

Methods and Results- The cohort consisted of 550 CAD patients from the *Circulating Cells* study that were referred for coronary angiography. Flow cytometry analysis of blood samples was used to determine circulating T cell numbers. At baseline a significant 25% higher $CD3^+$ T cell count was observed in female ($p<0.001$) but not in male unstable CAD compared to stable CAD patients. Furthermore, in females we observed large significant differences in a number of T cell subsets while in males only a few small differences were found in T cell subsets between stable CAD and unstable CAD patients.

Prospective analysis showed that none of the T cell subsets could independently predict major adverse CAD events in the female cohort. In the male cohort the $CD4^+CD127^-CD46^+$ and $CD4^+CD46^+CD25^{high}$ T cell subset showed an age adjusted odds ratio of 2.54 (1.14-6.06 CI95%, p=0.02) and 2.32 (1.05-5.47 CI95%, p=0.04).

Conclusion- We show that the number of circulating T cells are sex-specific in CAD patients. Only females showed significant differences in major T cell subsets between stable CAD and unstable CAD patients, but only in males T cell subsets may have potential value as a prognostic biomarker.

## Introduction

Both innate and adaptive immunity have a leading role in modulating the progression of atherosclerosis. In human lesions the ratio of macrophage to T cell is between 4:1 and 10:1, the dominant cell type in the plaque is therefore the macrophage but T cells are present at all stages of atherosclerosis[1]. The first experimental evidence that underlined the important role of adaptive immunity in atherosclerosis came from atherosclerosis prone apolipoprotein E deficient mice that were also lymphocyte deficient. In these double knockout mice a marked reduction in atherosclerotic plaque formation was shown[2], while reconstitution of these mice with $CD4^+$ T cells aggravated atherosclerosis development[3]. Subsequent studies have focused on the specific contribution of T cell subsets to the development of atherosclerosis.

Th1 cells are the predominant T helper cell observed in the atherosclerotic plaque. IFNγ is the characteristic cytokine produced by Th1 cells, studies which show that deletion of IFNγ or its receptor attenuates experimental atherosclerosis implicate the Th1 cell subset in the progression of atherosclerosis[4,5]. In addition, in a mouse model deficient for the transcription factor Tbet, which mediates the differentiation of Th1, atherosclerosis formation is significantly decreased[6]. In line with these observations is the finding that treatment with the Th1 inducing cytokine IL-12 aggravates atherosclerotic lesion development, while blockade of IL-12 using a vaccination approach inhibits lesion development. As IL-12 is one of the primary cytokines driving Th1 differentiation this was further evidence that Th1 cells have a pro-atherogenic effect[7,8].

In contrast to Th1 cells, studies on Th2 cells show contradictory findings concerning their role in atherosclerosis. The characteristic cytokine IL-4 produced by Th2 lineage cells is rarely detected in plaques, in mice nor in humans[9,10]. Contrary to this observation some studies have found reduced lesion formation in mice deficient for IL-4. Others found increased lesion formation or that IL-4 deficiency had no influence on lesion formation[11–14].

Compared to the other two major T helper cell subsets, Th17 is a relatively

newly described subset and the role of Th17 in atherosclerosis is under active debate. Studies in experimental models for atherosclerosis have found aggravated inflammation and a pro-atherogenic effect of IL-17 and its receptor, whereas others describe an athero-protective role for Th17 and IL-17[15].

Regulatory T cells (Treg) can suppress proliferation of effector T cells by cytokine secretion and direct cell-to-cell contact. Initial studies of the group of Mallat show that Tregs reduce atherosclerosis development[16]. Subsequent research showed that induction of oral tolerance to several antigens, including oxidized LDL-C, induces an athero-protective response mediated by Tregs[17] via production of IL-10 and TGFβ[18–20].

The role of other T cell subsets is less well defined and the role of CD8 T cells in atherosclerosis is, despite their relatively large numbers in human atherosclerotic lesions, not fully understood[21,22].

Studies in humans are mostly restricted to epidemiological studies and have focused at associations between cardiovascular disease manifestations such as acute coronary syndrome, stable and unstable angina and T cell subset counts in peripheral blood[23–31]. In contrast, only two prospective studies have been performed on T cell subsets for biomarker discovery[32,33]. These studies show that circulating Tregs and Th2 cells could potentially be predictive for future cardiovascular events.

Research has shown that there are intrinsic differences between the immune system of males and females and this has consequences for the development of immune mediated diseases such as autoimmune diseases[34]. Also in coronary artery disease (CAD) there are large differences in symptoms, risk factors and the underlying pathology of CAD between men and women. New insights into sex-specific differences in cardiovascular disease have led to adaptations in diagnostic testing and treatment regiments[35]. Cardiovascular disease development in women generally lags 7 to 10 years behind compared to men in the expression of clinical symptoms[36]. It is not fully understood what causes this delay but it is suggested that sex steroids have a protective effect on atherosclerotic plaque development during the pre-menopausal years of a woman's life[37].

Research into novel prognostic blood based biomarkers has not found any new biomarkers with substantial additive value over the classic biomarkers such as LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and hsCRP. Sex-specific factors have shown to influence CAD development and progression, we hypothesize that these factors influence lipid and inflammatory processes leading to differences in CAD biomarker expression between sexes. In this paper, a cohort of 550 patients scheduled for coronary angiography was analyzed by flow cytometry for sex-specific T cell subset distribution in blood both for the purpose of diagnosing CAD as well as for predicting future major adverse cardiac events (MACE).

## Methods

### Study population

The study cohort consists of a total of 550 CAD patients between the age of 31 and 83 from the *Circulating Cells* Study cohort. The T cell flow cytometry panel was performed for logistic reasons only on part of the *Circulating Cells* Study cohort, therefore the study cohort is smaller than the whole *Circulating Cells* Study cohort. In brief, *Circulating Cells* is a multi-center study in which CAD patients scheduled for coronary angiography were included. Exclusion criteria were age < 18 years, inability to give informed consent, suspected drug or alcohol abuse, serious concomitant disease, serious recent infectious disease in the last 6 weeks or suspected elevated state of the immune system, and non-cooperativeness .The *Circulating Cells* study protocol was approved by the review board or ethics committee of each participating center[38]. Nine months after inclusion in the study, patients were contacted and any MACE that had occurred in the preceding months were recorded for outcome analysis. MACE is the primary endpoint of this study and is defined as death, myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting and cardiovascular accident. The cohort consists of 18 non-significant atherosclerosis, 438 stable angina, 53 unstable angina and 54 non-STEMI diagnosed patients. The non-significant atherosclerosis patients were removed for unstable vs stable CAD analysis. Thirty-three patients were lost to follow-up and were subsequently excluded from primary endpoint MACE analysis.

### Flow cytometry

Whole blood flow cytometry analysis was performed to identify and quantify T cell subsets. Cells were stained for the following markers: CD127, CD134, CD152, CD152, CD16, CD25, CD28, CD31, CD3, CD40, CD46, CD49b, CD4, CD56, CD62L, CD69, CD8, CXCR4, ICOS, IL12R, IL23R, NKG2c, TCRαβ and TCRγδ. See supplemental data for antibody information and staining combinations.

Blood was collected in a BD vacutainer EDTA blood collection tube and 50

µL whole blood was stained with the respective antibodies for 30 minutes on 4ºC. Red blood cells were lysed in 500 µL Versalyse solution (IM3648; Beckman Coulter) for 5 minutes at room temperature immediately afterwards cells were analyzed by flow cytometry. Each center was equipped with an identical flow cytometer (FC500, Beckman Coulter) and before each run cytometers were calibrated with fluorosphere setup kits (Beckman Coulter). Analysis was performed on Kaluza 1.3 flow cytometer analysis software (Beckman Coulter).

### Flow cytometry gating strategy

Figure 1A-G shows an example of the gating strategy that was used for the analysis of the flow cytometry data. T cell subsets were defined by the presence or absence of the expression of a cellular marker (e.g. $CD8^{+}$ and $CD8^{-}$). When there was a distinct population of cells that expressed the marker to a higher degree than other positive cells, that population is separately indicated as a high population (e.g. $CD8^{high}$) and the population of cells that are positive for a certain marker but expresses it to a lower degree is indicated with low (e.g. $CD8^{low}$). When a population of cells does not express a marker or only expresses it to a low degree this is indicated with a minus/low indicator (e.g. $CD8^{-/low}$). The Kaluza flow cytometry analysis software tree plot was used for generating the data for all possible combinations of cell markers (Figure 1H).

### Statistics

Statistical calculations were performed using R (version 3.0.2) for Windows. A P value lower than 0.05 was considered significant. Categorical data was compared using the chi-square test. For continuous data the nonparametric Mann-Whitney U test was used.

For logistic regression the patients were divided into 3 equally sized groups (tertiles) according to the measured values for each variable. Firth's penalized likelihood logistic regression was used to analyze stratified data. Two models were used, one simple model (Model 1) adjusted for age and an extended model (Model

2), which adjusted for age, hypertension, diabetes mellitus, current smoker, BMI and HDL-C level.

To identify variables that can classify patients into separate groups (e.g. stable vs. unstable CAD patients) we used the machine learning method Random Forest[39]. Taking advantage of combinations of predictor variables this algorithm classified subjects into different groups. The Random Forest method and the way it's applied in this project are further explained in the supplemental material.

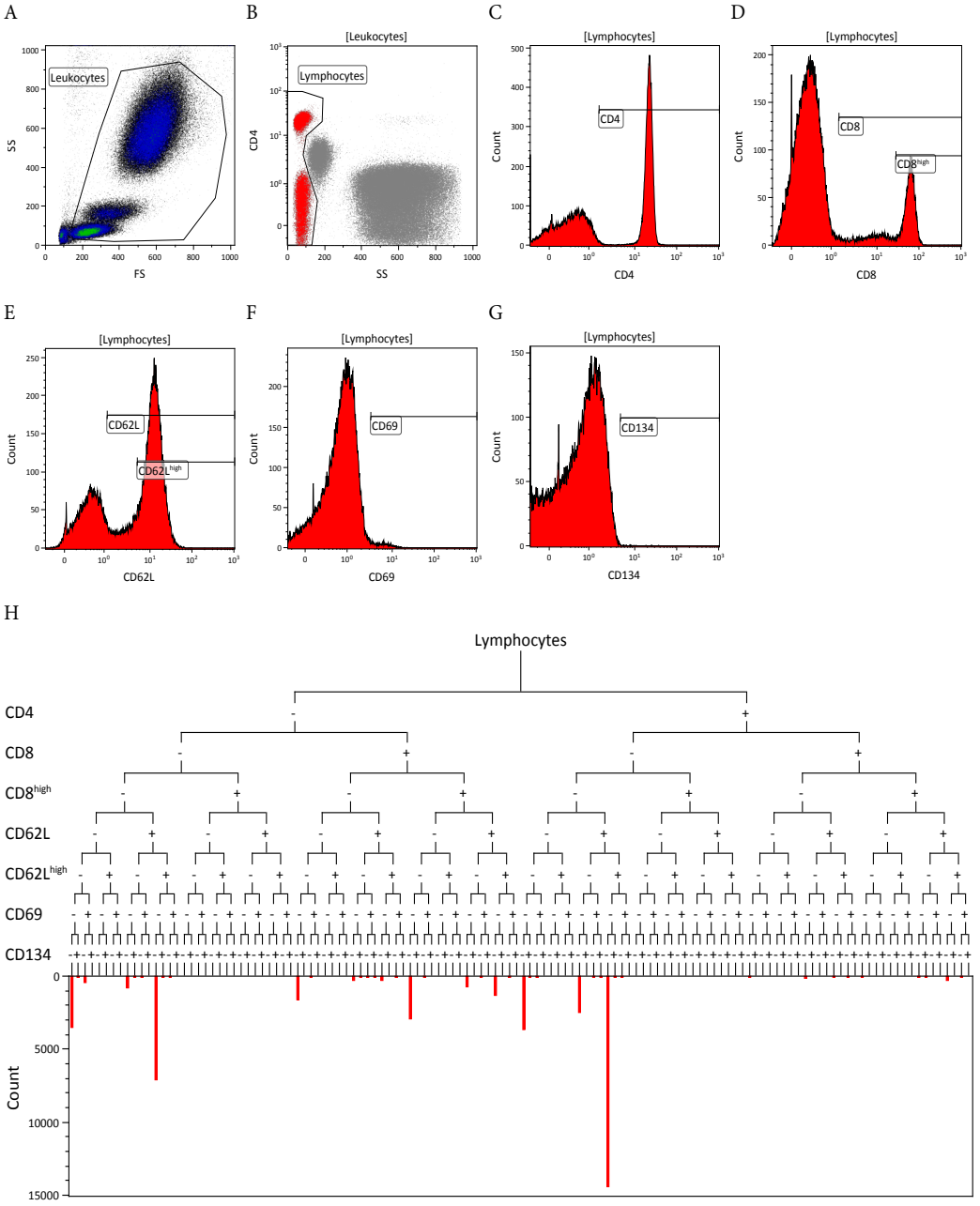


**Figure 1:** The T cell flow cytometry gating strategy. First cells were gated to exclude monocytes and granulocytes from the analysis by using the forward scatter, side scatter (A) and CD4 or CD3 staining characteristics (B). Cells were subsequently gated on expression of different T cell related markers, if present a separate low and high populations were also gated (C-G). Finally a tree diagram was generated to calculate the amount of cells in each possible combination of markers (H).

**Table 1:** Baseline characteristics of stable and unstable CAD patients

| | Male | | Female | |
|---|---|---|---|---|
| | Stable CAD (n=313) | Unstable CAD (n=75) | Stable CAD (n=125) | Unstable CAD (n=37) |
| Age | 62(56-69) | 62(56-69) | 64(58-72) | 63(56-71) |
| BMI | 27(25-30) | 27(25-30) | 28(25-31) | 25(23-28)‡ |
| SBP (mm Hg) | 135(125-145) | 130(120-141) | 138(124-150) | 135(120-148) |
| DBP (mm Hg) | 80(70-85) | 79(70-86) | 76(70-85) | 78(70-81) |
| Glucose (mmol/L) | 6.00(5.40-6.70) | 6.38(5.70-7.60)* | 6.00(5.40-6.90) | 6.70(5.64-7.40) |
| Hemoglobin (mmol/L) | 8.90(8.50-9.30) | 9.00(8.60-9.60) | 8.20(7.73-8.60) | 8.30(7.75-8.60) |
| Cholesterol (mmol/L) | 3.90(3.40-4.65) | 4.45(3.90-5.29)‡ | 4.35(3.80-5.20) | 4.82(3.85-5.63) |
| LDL-C (mmol/L) | 2.22(1.73-2.84) | 2.72(2.12-3.49)‡ | 2.46(1.96-3.10) | 2.72(2.00-3.52) |
| HDL-C (mmol/L) | 0.99(0.88-1.19) | 0.96(0.79-1.15) | 1.18(1.00-1.47) | 1.14(0.97-1.37) |
| Triglycerides (mmol/L) | 1.30(0.96-1.87) | 1.67(1.20-2.45)‡ | 1.39(1.01-1.82) | 1.53(1.18-2.05) |
| hsCRP (ng/mL) | 2516(1685-5524) | 5594(2044-18154)‡ | 3626(1666-6976) | 3669(2136-7281) |
| WBC (X1000 cells/μL) | 6.80(5.78-8.00) | 8.10(6.90-9.80)‡ | 6.95(5.50-8.30) | 8.80(7.20-9.90)‡ |
| Current smoker | 20%(64) | 24%(18) | 17%(21) | 49%(18)‡ |
| Diabetes | 22%(70) | 19%(14) | 25%(31) | 22%(8) |
| Hypertension | 61%(191) | 60%(45) | 75%(94) | 59%(22) |
| Beta-blocker | 76%(237) | 60%(45)† | 83%(104) | 54%(20)† |
| Ca-antagonist | 32%(99) | 23%(17) | 31%(39) | 14%(5) |
| Aspirin | 83%(261) | 68%(51)† | 94%(117) | 62%(23)‡ |
| Vitamin K antagonist | 10%(32)* | 21%(16)* | 7%(9) | 16%(6) |
| ADP receptor blocker | 50%(155)* | 35%(26)* | 51%(64) | 46%(17) |
| ACE inhibitor | 37%(116) | 28%(21) | 31%(39) | 30%(11) |
| ATII receptor blocker | 17%(52) | 12%(9) | 31%(39) | 14%(5) |
| Diuretic | 19%(61) | 17%(13) | 28%(35) | 14%(5) |
| Statin | 84%(264) | 65%(49)‡ | 82%(103) | 49%(18)‡ |

Baseline characteristics table for the *Circulating Cells* cohort; values are median (interquartile range) or percentage (n). Stable CAD was compared to unstable CAD within each sex; categorical data was compared using the chi-square test and continuous data was compared with the Man-Whitney U test. * $p<0.05$, † $p<0.01$, ‡ $p<0.001$.

## Results

### Baseline cohort characteristics

Unstable angina, non-ST elevated myocardial infarction (non-STEMI) and stable angina are three distinct coronary syndromes. Stable angina often has a chronic character, unstable angina and non-STEMI are an acute form of a coronary syndrome. In this study non-STEMI and unstable angina patients were pooled and referred to as unstable CAD patients, this group was compared with stable angina patients (hereafter referred to as stable CAD patients).

Due to the chronic nature of stable CAD as compared to the more acute nature of unstable CAD, prescribed drug use was substantially higher in the stable CAD group both for males and females. Beta-blockers, aspirin, statins and in men also ADP receptor blockers are more commonly prescribed to stable CAD patients (Table 1). The only drug that did not follow this trend were Vitamin K antagonists, which were used more frequently in the unstable CAD group but only in males. Statin use was much higher in the stable CAD patients (males: 84%, females: 82%) compared to the unstable CAD patients (males: 65%, females:49%). The higher statin prescription in stable CAD patients might have contributed to the significantly lower total cholesterol, LDL-C and triglyceride levels in male stable CAD patients but in females the cholesterol levels did not differ between groups. In females smoking was significantly more common in the unstable CAD group. The inflammation marker hsCRP was significantly increased in male unstable CAD patients to more than twice the concentration in stable CAD patients, in contrast females did not show an increase in hsCRP. The increased level of inflammation was further confirmed by an increase in white blood cell (WBC) count in unstable CAD patients both in males and females.

### Sex-specific differences in CD3, CD4 and CD8 T cell distribution

Analysis of the flow cytometry data showed significant sex-specific differences in immune cell distribution. Lymphocyte count was significantly higher

**Table 2:** Comparison of T cell populations between males and females

| | Female (n=162) | Male (n=388) | P value |
|---|---|---|---|
| Lymphocytes (cells/μL) | 2151(1746-2715) | 1943(1529-2459) | p<0.001 |
| $CD3^+$ T cells (cells/μL) | 1450(1182-1889) | 1256(955-1666) | p<0.001 |
| $CD4^+$ T cells (cells/μL) | 1013(796-1328) | 808(603-1063) | p<0.001 |
| $CD8^+$ T cells (cells/μL) | 306(213-451) | 307(204-467) | N.S. |
| Naïve $CD4^+$ T cells (cells/μL) | 696(528-963) | 534(348-723) | p<0.001 |
| Naïve $CD8^+$ T cells (cells/μL) | 98(59-143) | 88(50-141) | N.S. |
| Activated $CD4^+$ T cells (cells/mL) | 3456(2333-6653) | 3191(1876-5136) | p<0.05 |
| Activated $CD8^+$ T cells (cells/mL) | 7187(4317-12097) | 7215(4368-11400) | N.S. |
| T regulatory cells (cells/μL) | 32(24-48) | 26(17-40) | p<0.001 |

Differences in T cell population between sexes; values are median (interquartile range). Treg cells, naïve and activated CD4 and CD8 cells were respectively identified by marker combinations $CD4^+CD25^+CD127^-$, $CD4^+CD62L^+CD69^-$, $CD8^+CD62L^+CD69^-$, $CD4^+CD62L^-CD69^+$ and $CD8^+CD62L^-CD69^+$.

by 13% in females as compared to males (Table 2). Examining the T cell distribution we observed that the difference in lymphocyte population was primarily caused by a higher $CD4^+$ and $CD3^+$ T cell count, which was approximately 20% higher in women as compared to men. Concurrently with the higher number of $CD4^+$ T cells, an increased number of naïve CD4 T cells ($CD4^+CD62L^+CD69^-$) and Treg cells ($CD4^+CD25^{high}CD172$-) was seen in women.

### Increased T cell numbers in female unstable CAD patients

Large differences in T cell distribution were observed between stable and unstable CAD patients within the female cohort; total lymphocyte counts as well as $CD3^+$ and $CD4^+$ T cell count were significantly increased by approximately 20% in female unstable CAD patients compared to female stable CAD patients. Surprisingly in the male cohort no significant differences were observed in lymphocyte, $CD3^+$, $CD4^+$ or $CD8^+$ T cell count between stable and unstable CAD patients (Figure 2A-F, Table S2).

To extend our analysis for T cell subsets that have a sex-specific effect on

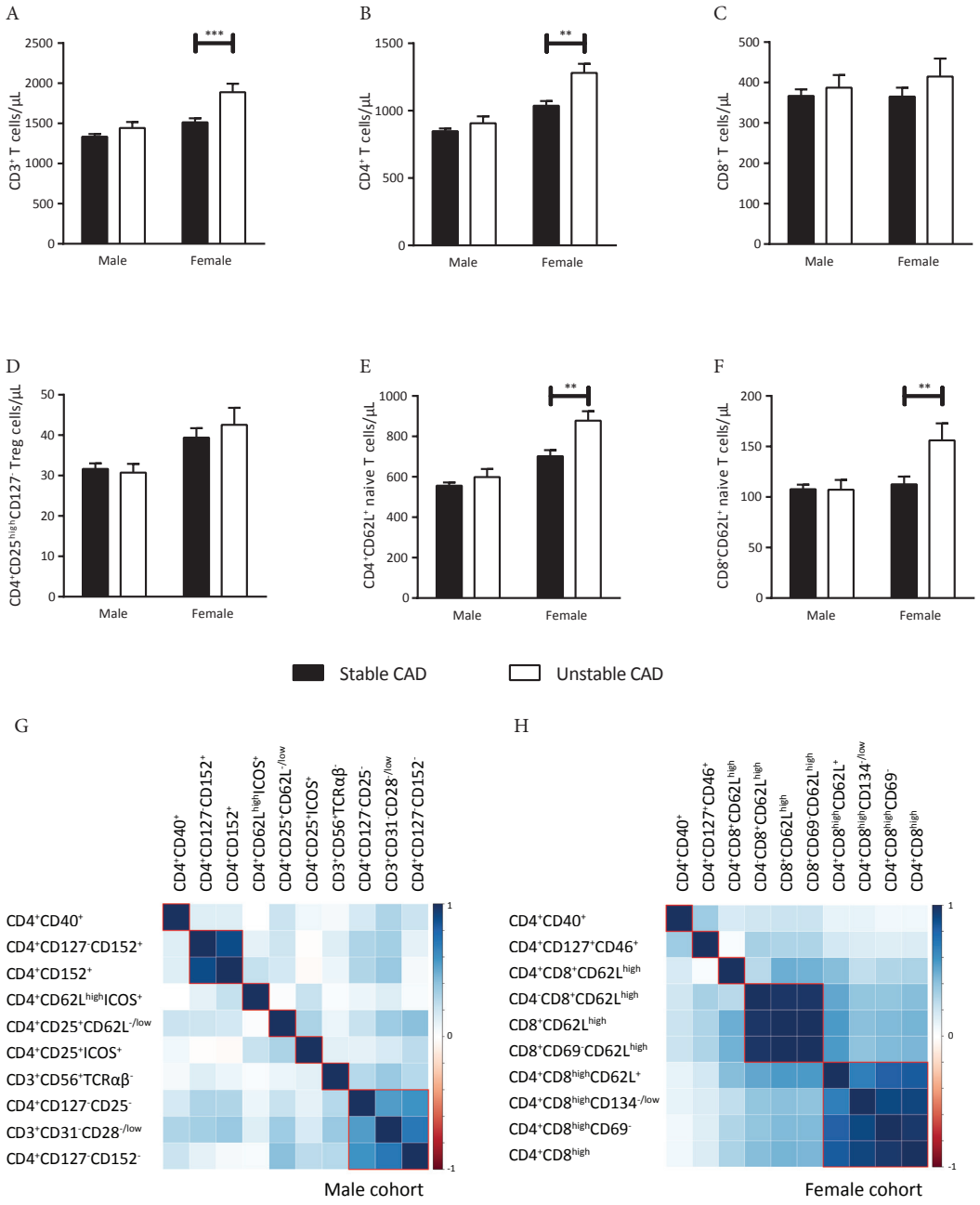


**Figure 2:** Sex-specific comparison of number of circulating $CD3^+$ (A), $CD4^+$ (B), $CD8^+$ (C), Treg (D), naïve $CD4^+$ (E) and naïve $CD8^+$ (F) cells between stable and unstable CAD patients. ** $p<0.01$, ***$p<0.001$. Correlation matrix of the 10 top ranked cell subsets determined by Random Forest modeling of stable and unstable CAD patients for the male (G) and female (H) cohort. The variables were clustered using hierarchical clustering. Blue indicates a positive correlation while red indicates a negative correlation, the intensity of the color indicates the size of the correlation.

CAD development we enlarged the flow cytometry panel with 24 markers known to be associated with specific T cell subsets (Table S1). The gating strategy we used was not focused on specific T cell subsets but included all possible combinations using the markers present in the panel, the panel can identify approximately 4500 cell subsets. Random Forest modeling was used to find T cell subsets that could discriminate between stable and unstable patients.

The ten T cell subsets best able to stratify patients into stable and unstable CAD groups were selected for further analysis for both the male and female cohort. A correlation matrix in combination with hierarchical clustering was used to identify overlapping cell subsets (Figure 2G, H). From each cluster the cell subset with the shortest marker combination and best able to stratify patients was chosen for further analysis. Using this strategy 7 cell subsets were selected for the male cohort and 5 cell subsets were selected for the female cohort (Table S3, S4).

In the male cohort, 3 T cell subsets showed a significant difference between stable and unstable patients: a higher number of circulating $CD3^+CD31^-CD28^-$, $CD3^+CD56^+TCR\alpha\beta^-$ and $CD4^+CD152^+$ T cells were observed in male unstable CAD compared to stable CAD patients (Table S3). The largest change was seen in the $CD3^+CD56^+TCR\alpha\beta^-$ T cell subset, which is a small cell subset with approximately 19 cells/μL blood (10-38 IQR) and we observed a 33% increase in the number of these cells in male unstable CAD compared to stable CAD patients.

The female cohort displayed larger changes in T cell subsets compared to the male cohort (Table S4). In the female cohort, $CD4^+CD127^+CD46^+$, $CD4^+CD40^+$, $CD4^+CD8^{high}$, $CD4^+CD8^+CD62L^{high}$ and $CD8^+CD62L^{high}$cells were significantly up regulated in female unstable CAD patients (Table S4). The largest changes were seen in the $CD4/CD8^-$double positive T cell subsets $CD4^+CD8^{high}$ and $CD4^+CD8^+CD62L^{high}$, which showed an almost 2-fold increase in unstable female CAD patients.

### Prognostic analysis of T cell subsets

When comparing patients that experienced MACE with control patients we observed in female MACE patients a significantly lower HDL-C concentration, this

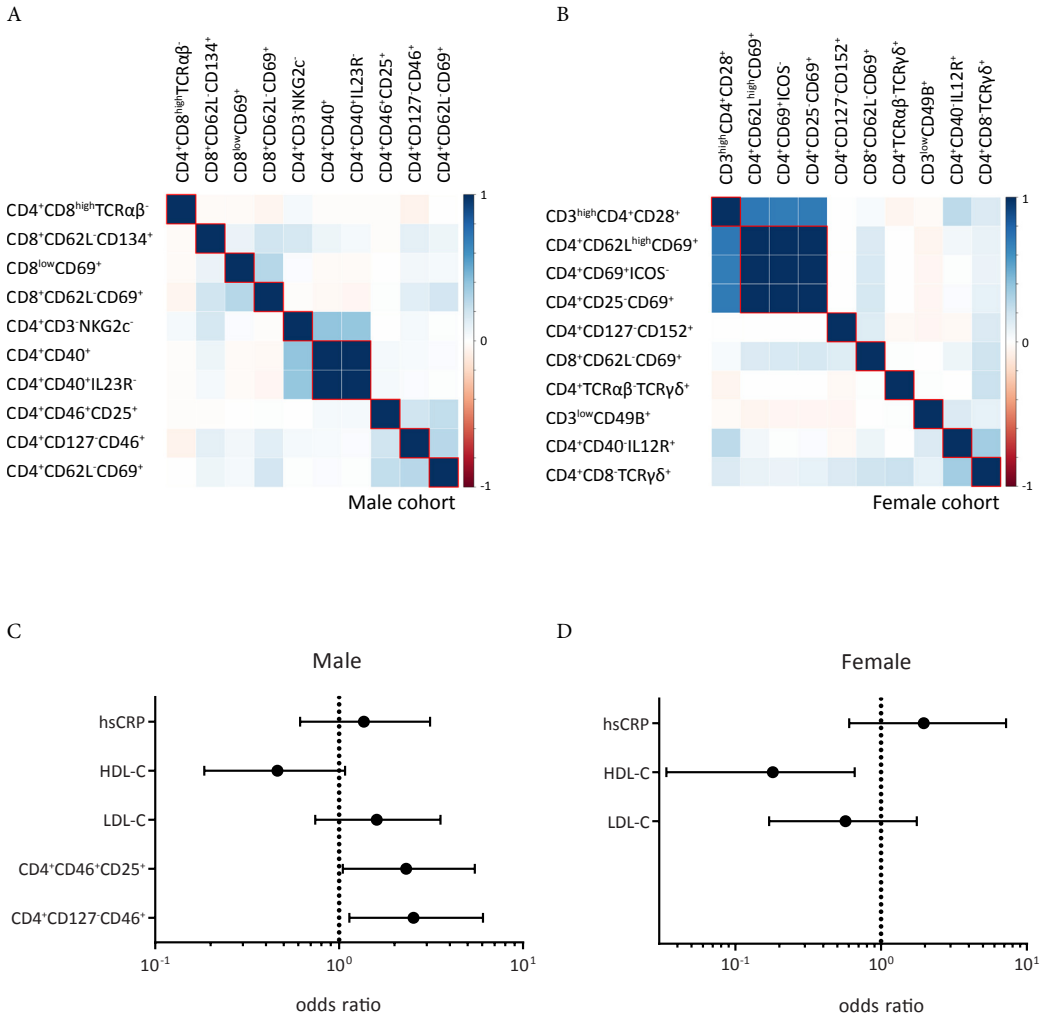


**Figure 3:** Correlation matrix and odds ratios of potential prognostic T cells subsets. Correlation matrix of the 10 top ranked cell subsets determined by Random Forest modeling of MACE and control patients for the male (A) and female (B) cohort. The variables were clustered using hierarchical clustering. Blue indicates a positive correlation while red indicates a negative correlation, the intensity of the color indicates the size of the correlation. Forest plots of ORs from granulocyte cell subsets and major blood based biomarkers such as hsCRP, LDL-C and HDL-C in the male (C) and female (D) cohort. Median value with corresponding 95% confidence interval (CI) for each variable, dotted vertical line indicates no effect.

difference was not significant in the male cohort (Table S5).

As with stable and unstable CAD patients, a model was generated using Random Forest for discriminating MACE patients from controls. The ten top-ranked T cell subsets from the Random Forest model were used to generate a correlation matrix for the male and female cohort (Figure 3A, B). Overlapping cell subsets were identified using hierarchical clustering of the correlation matrix and one representative subset was selected from each cluster.

Logistic regression was subsequently used to determine the odds ratio (OR) to evaluate the biomarker potential of each T cell subset (Table S6 and S7). The patients were distributed into tertiles based on their respective cell subset measurements, odds of MACE in the first tertile (reference tertile) were compared with the second and third tertile to determine the respective ORs. Using a simple model that adjusts for age (Model 1) we found that 2 subsets in the male cohort had significant ORs (first compared to third tertile). The $CD4^+CD127^-CD46^+$ and $CD4^+CD46^+CD25^{high}$ have an OR of 2.54 (1.14-6.06 95%IC, p=0.023) and 2.32 (1.05-5.47 95%IC, p=0.037) respectively and are associated with an increased risk of MACE. The subsets represent small T cell subsets with circulating cell numbers for $CD4^+CD127^-CD46^+$ of 14 cells/μL(9-23 IQR) and for $CD4^+CD46^+CD25^{high}$ 7 cells/μL (3-13 IQR) accounting for approximately 1% of CD4 T cells. When this model was extended to adjust for hypertension, diabetes mellitus, current smoker, BMI and HDL-C in addition to age (Model 2) the ORs for both cell subsets were no longer significant (Table S6). In contrast, in the female cohort none of the T cell subsets showed a significant association with MACE (Table S7).

In the male cohort traditional blood-based biomarkers such as LDL-C, HDL-C and hsCRP performed poorly as predictive biomarkers for MACE. The T cell subsets $CD4^+CD127^-CD46^+$ and $CD4^+CD46^+CD25^{high}$ performed better than the traditional biomarkers (Figure 3C). In contrast, in females HDL-C had a significant OR (first compared to third tertile) and an increased concentration of HDL-C was associated with a decreased chance of MACE, while none of the T cell subsets showed a significant OR (Figure 3D).

## Discussion

The concept that both the innate and the adaptive immune system are of central importance in the initiation and progression of atherosclerosis is widely accepted. Modulation of specific T cell subsets in mice has been shown to affect the progression of atherosclerosis and as such T cells have been shown to play a central role in atherosclerotic disease progression. The translation of experimental research performed in mouse models into human pathology has been complicated by the inherent limitations in human research. In this study we investigated a broad spectrum of T cells in circulation to determine whether a T cell subset could be identified that associated with severity of coronary syndrome or with the chance to develop adverse cardiac events.

One of the prominent findings of this study is the significant sex based difference in the association of the immune system and lymphocyte subsets with cardiovascular disease. It is well known that sex based differences in the manifestation of cardiovascular disease exist. Women tend to develop CAD later in life and present with angina pectoris or atypical symptoms more often than men, whereas men more often present with acute myocardial infarction or sudden death[36,40,41]. Morphologically, the plaque disruption is also different between sexes as women (primarily pre-menopausal women) more often show plaque erosion, while men more often show plaque rupture when presenting with acute coronary syndrome[42,43]. In the Circulation Cells cohort, we found a 20% higher number of $CD3^+$ and $CD4^+$ T cells in women compared to men, this in contrast to a lower number of monocytes observed in women. These numbers indicate that it might be advised in future observational studies to separate men and women when analyzing an immune mediated disease in a gender mixed cohort.

Unstable CAD is associated with an increased systemic inflammation[44]. Here we found significantly higher numbers of circulating CD3 and CD4 positive T cells in female unstable CAD patients compared to stable female CAD patients, however a corresponding effect was not observed in the male cohort. While the differences for most T cell subsets in males were relatively small, one exception was

$CD3^+CD56^+TCR\alpha\beta^-$ cells which was increased in male unstable CAD patients. The $CD3^+CD56^+TCR\alpha\beta^-$ cell subset partially corresponds to natural killer T cells, most evidence points toward a pro-atherogenic role of NKT cells[45–47]. In contrast to our results a previous study has found a decreased numbers of circulating NKT cells in patients with CAD[48]. The discrepancy in result could be due to a difference in study setup as they compared healthy subjects to CAD patients and we compare stable CAD to unstable CAD patients.

Females show particularly large changes in CD4/CD8 double positive T cell subsets where the number of circulating $CD4^+CD8^{high}$ and $CD4^+CD8^+CD62L^{high}$ cells almost doubled in female unstable CAD patients. Equivalent changes in these subsets were not found in the male cohort. Double positive cells are most commonly described in the thymus as immature T cells but multiple studies have shown the presence of a small subset in blood, less than 5% of the circulating T cells in the blood that are positive for both CD4 and CD8[49–51]. Several studies have found an increase in this double positive cell subset in cases of viral infection, cancer and autoimmune disease but very little is known on the function of this cell subset[52]. We now show for the first time a direct correlation between CD4/CD8 double positive T cells and coronary syndrome. Additional research will have to reveal whether the CD4/CD8 double positive T cells have a functional role in the development of atherosclerosis in females.

To our knowledge only two prospective studies have been performed on circulating T cells in relation to major adverse coronary events, one focused on Th2 cells and the second on Tregs and their association with cardiovascular events. Engelbertsen *et al.* showed a strong negative association between Th2 count in circulation and the incidence of coronary events in women but not in men. In the panel that was used in this study it was impossible to identify Th2 cells, but it is interesting to note that Engelbertsen *et al.* found a strong sex-specific effect, which we also observed in different T cell subsets[32]. Wigren *et al.* found that increased Treg counts associate with a decreased incidence of myocardial infarction and interestingly in males we find two T cell subsets that are very similar to Treg cells,

$CD4^+CD127^-CD46^+$ and $CD4^+CD46^+CD25^{high}$, which are predictive for MACE[33]. These subsets should partially overlap with the standard Treg $CD4^+CD25^{high}CD127^-$ cell subsets but in contrast to Wigren *et al*. we found an increased chance of MACE with an increase in these cell subsets. The study by Wigren *et al*. has a significantly longer follow-up time of 15 years compared to the 9 months follow-up in the current study and in their study blood samples had been stored for many years before analysis, whereas in comparison we used fresh blood for flow cytometry analysis. These differences in the setup of the study could have contributed to the different findings in comparison to Wigren *et al*.

Classic blood based biomarkers such as LDL-C, HDL-C and hsCRP performed poorly in this study as a predictive biomarker for the development of MACE. These markers have shown to work well for primary prediction but possibly in secondary event prediction their use is limited. This shows the need for novel biomarkers for the accurate risk assessment of this population of patients. The biomarker T cell subsets that were identified here could possibly fill this role and be a used for prediction of secondary cardiovascular events in male patients.

It should be noted that the current study does have a few limitations. Primarily due to the short follow-up time of 9 months the number of MACE events is limited, especially in the female cohort. The current findings need to be validated in a cohort study specifically designed to find sex-specific differences of T cell distribution in CAD patients.

Our analysis in a CAD based cohort showed sex-specific effects on the T cell distribution in patients. Female unstable CAD patients have a significantly higher circulating CD4 and CD8 T cell numbers compared to female stable CAD patients, in contrast in males no difference was found between stable and unstable CAD patients. In females we find a large increase in the number of circulating CD4/CD8 double positive T cells in unstable CAD patients compared stable CAD patients but no difference is found in males CAD patients. Prognostic analysis shows that classic blood based biomarkers LDL-C, HDL-C and hsCRP perform poorly as predictive biomarkers in males while in females HDL does show prognostic potential for

predicting secondary CAD events. Only in males do T cell subsets show prognostic potential for secondary CAD events, T regulatory cell subsets in particular could be of use as predictive CAD biomarkers.

## References


1. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol 2011; 12(3): 204–212. doi:10.1038/ni.2001.
2. Daugherty A et al. The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E-/- mice. J Clin Invest 1997; 100(6): 1575–1580. doi:10.1172/JCI119681.
3. Zhou X et al. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. Circulation 2000; 102(24): 2919–2922. doi:10.1161/01.CIR.102.24.2919.
4. Whitman SC et al. IFN-gamma deficiency exerts gender-specific effects on atherogenesis in apolipoprotein E-/- mice. J Interferon Cytokine Res 2002; 22(6): 661–670. doi:10.1089/10799900260100141.
5. Gupta S et al. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. J Clin Invest 1997; 99(11): 2752–2761. doi:10.1172/JCI119465.
6. Buono C et al. T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. Proc Natl Acad Sci U S A 2005; 102(5): 1596–1601. doi:10.1073/pnas.0409015102.
7. Lee TS et al. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 1999; 19(3): 734–742. doi:10.1161/01.ATV.19.3.734.
8. Hauer AD et al. Blockade of interleukin-12 function by protein vaccination attenuates atherosclerosis. Circulation 2005; 112(7): 1054–1062. doi:10.1161/CIRCULATIONAHA.104.533463.
9. Frostegard J et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis 1999; 145(1): 33–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10428293.
10. Zhou X et al. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. J Clin Invest 1998; 101(8): 1717–1725. doi:10.1172/JCI1216.
11. Davenport P, Tipping PG. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. Am J Pathol 2003; 163(3): 1117–1125. doi:10.1016/S0002-9440(10)63471-2.
12. King VL et al. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. Arterioscler Thromb Vasc Biol 2002; 22(3): 456–461. doi:10.1161/hq0302.104905.
13. Huber SA et al. T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. Circulation 2001; 103(21): 2610–2616. doi:10.1161/01.CIR.103.21.2610.
14. King VL et al. Interleukin-4 does not influence development of hypercholesterolemia or angiotensin II-induced atherosclerotic lesions in mice. Am J Pathol 2007; 171(6): 2040–2047. doi:10.2353/ajpath.2007.060857.
15. Taleb S et al. Interleukin-17: friend or foe in atherosclerosis? Curr Opin Lipidol 2010; 21(5): 404–408. doi:10.1097/MOL.0b013e32833dc7f9.
16. Mallat Z et al. Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. Circulation 2003; 108(10): 1232–1237. doi:10.1161/01.CIR.0000089083.61317.A1.
17. Van Puijvelde GHM et al. Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. Circulation 2006; 114(18): 1968–1976. doi:10.1161/CIRCULATIONAHA.106.615609.
18. Ait-Oufella H et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nat

Med 2006; 12(2): 178–180. doi:10.1038/nm1343.

19. Gotsman I et al. Impaired regulatory T-cell response and enhanced atherosclerosis in the absence of inducible costimulatory molecule. Circulation 2006; 114(19): 2047–2055. doi:10.1161/CIRCULATIONAHA.106.633263.
20. Van Es T et al. Vaccination against Foxp3(+) regulatory T cells aggravates atherosclerosis. Atherosclerosis 2010; 209(1): 74–80. doi:10.1016/j.atherosclerosis.2009.08.041.
21. Kolbus D et al. CD8+ T cell activation predominate early immune responses to hypercholesterolemia in Apoe-/- mice. BMC Immunol 2010; 11(1): 58. doi:10.1186/1471-2172-11-58.
22. Kyaw T et al. Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. Circulation 2013; 127(9): 1028–1039. doi:10.1161/CIRCULATIONAHA.112.001347.
23. Liuzzo G et al. Monoclonal T-cell proliferation and plaque instability in acute coronary syndromes. Circulation 2000; 101(25): 2883–2888. doi:10.1161/01.CIR.101.25.2883.
24. Methe H et al. Enhanced T-helper-1 lymphocyte activation patterns in acute coronary syndromes. J Am Coll Cardiol 2005; 45(12): 1939–1945. doi:10.1016/j.jacc.2005.03.040.
25. Mor A et al. Altered status of CD4(+)CD25(+) regulatory T cells in patients with acute coronary syndromes. Eur Heart J 2006; 27(21): 2530–2537. doi:10.1093/eurheartj/ehl222.
26. Sardella G et al. Frequency of naturally-occurring regulatory T cells is reduced in patients with ST-segment elevation myocardial infarction. Thromb Res 2007; 120(4): 631–634. doi:10.1016/j.thromres.2006.12.005.
27. Ji Q et al. Downregulation of T helper cell type 3 in patients with acute coronary syndrome. Arch Med Res 2009; 40(4): 285–293. doi:10.1016/j.arcmed.2009.04.002.
28. Ammirati E et al. Circulating CD4+CD25hiCD127lo regulatory T-cell levels do not reflect the extent or severity of carotid and coronary atherosclerosis. Arterioscler Thromb Vasc Biol 2010; 30(9): 1832–1841. doi:10.1161/ATVBAHA.110.206813.
29. Zhao Z et al. Activation of Th17/Th1 and Th1, but not Th17, is associated with the acute cardiac event in patients with acute coronary syndrome. Atherosclerosis 2011; 217(2): 518–524. doi:10.1016/j.atherosclerosis.2011.03.043.
30. Potekhina A V et al. CD4(+)CD25(high)CD127(low) regulatory T cells in patients with stable angina and their dynamics after intracoronary sirolimus-eluting stent implantation. Hum Immunol 2011; 72(7): 553–557. doi:10.1016/j.humimm.2011.03.015.
31. Zhang WC et al. Impaired thymic export and increased apoptosis account for regulatory T cell defects in patients with non-ST segment elevation acute coronary syndrome. J Biol Chem 2012; 287(41): 34157–34166. doi:10.1074/jbc.M112.382978.
32. Engelbertsen D et al. T-helper 2 immunity is associated with reduced risk of myocardial infarction and stroke. Arterioscler Thromb Vasc Biol 2013; 33(3): 637–644. doi:10.1161/ATVBAHA.112.300871.
33. Wigren M et al. Low levels of circulating CD4+FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke. Arterioscler Thromb Vasc Biol 2012; 32(8): 2000–2004. doi:10.1161/ATVBAHA.112.251579.
34. Markle JG, Fish EN. SeXX matters in immunity. Trends Immunol 2014; 35(3): 97–104. doi:10.1016/j.it.2013.10.006.
35. Mosca L et al. AHA/ACC scientific statement: consensus panel statement. Guide to preventive cardiology for women. American Heart Association/American College of Cardiology. J Am Coll Cardiol 1999;

33(6): 1751–1755.

36. Lerner DJ, Kannel WB. Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. Am Heart J 1986; 111(2): 383–390. doi:10.1016/0002-8703(86)90155-9.
37. Adams MR et al. Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys. Lack of an effect of added progesterone. Arteriosclerosis 1990; 10(6): 1051–1057. doi:10.1161/01.ATV.10.6.1051.
38. Hoefer IE et al. Circulating cells as predictors of secondary manifestations of cardiovascular disease: design of the CIRCULATING CELLS study. Clin Res Cardiol 2013; 102(11): 847–856. doi:10.1007/s00392-013-0607-9.
39. Breiman L. Random Forests. Mach Learn 2001; 45(1): 5–32. doi:10.1023/A:1010933404324.
40. Heer T et al. Gender differences in acute myocardial infarction in the era of reperfusion (the MITRA registry). Am J Cardiol 2002; 89(5): 511–517. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11867033.
41. Hochman JS et al. Sex, Clinical Presentation, and Outcome in Patients with Acute Coronary Syndromes. N Engl J Med 1999; 341(4): 226–232. doi:10.1056/NEJM199907223410402.
42. Farb A et al. Coronary Plaque Erosion Without Rupture Into a Lipid Core: A Frequent Cause of Coronary Thrombosis in Sudden Coronary Death . Circ 1996; 93 (7 ): 1354–1363. doi:10.1161/01.CIR.93.7.1354.
43. Kramer MC a et al. Relationship of thrombus healing to underlying plaque morphology in sudden coronary death. J Am Coll Cardiol 2010; 55(2): 122–132. doi:10.1016/j.jacc.2009.09.007.
44. Buffon A et al. Widespread coronary inflammation in unstable angina. N Engl J Med 2002; 347(1): 5–12. doi:10.1056/NEJMoa012295.
45. Nakai Y et al. Natural killer T cells accelerate atherogenesis in mice. Blood 2004; 104(7): 2051–2059. doi:10.1182/blood-2003-10-3485.
46. Tupin E et al. CD1d-dependent activation of NKT cells aggravates atherosclerosis. J Exp Med 2004; 199(3): 417–422. doi:10.1084/jem.20030997.
47. Major AS et al. Quantitative and qualitative differences in proatherogenic NKT cells in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2004; 24(12): 2351–2357. doi:10.1161/01.ATV.0000147112.84168.87.
48. Kyriakakis E et al. Invariant natural killer T cells: linking inflammation and neovascularization in human atherosclerosis. Eur J Immunol 2010; 40(11): 3268–3279. doi:10.1002/eji.201040619.
49. Blue ML et al. Coexpression of T4 and T8 on peripheral blood T cells demonstrated by two-color fluorescence flow cytometry. J Immunol 1985; 134 (4 ): 2281–2286. Available at: http://www.jimmunol.org/content/134/4/2281.abstract.
50. Ortolani C et al. Cytofluorimetric identification of two populations of double positive (CD4+,CD8+) T lymphocytes in human peripheral blood. Biochem Biophys Res Commun 1993; 191(2): 601–609. doi:http://dx.doi.org/10.1006/bbrc.1993.1260.
51. Ghia P et al. Age-dependent accumulation of monoclonal CD4+CD8+ double positive T lymphocytes in the peripheral blood of the elderly. Br J Haematol 2007; 139(5): 780–790. doi:10.1111/j.1365-2141.2007.06867.x.
52. Parel Y, Chizzolini C. CD4+ CD8+ double positive (DP) T cells in health and disease. Autoimmun Rev 2004; 3(3): 215–220. doi:10.1016/j.autrev.2003.09.001.

## Supplementary material

### Supplementary Methods

#### Flow cytometry

For T cell analysis cells were stained in 9 combinations (Table S1) with the following antibodies: CD127-FITC (11-1278-42; eBioscience, San Diego, USA), CD134-ECD (IM99512; Beckman Coulter, Fort Lauderdale, USA), CD152-PC7 (IM99512; Beckman Coulter), CD152-PE (IM2282; Beckman Coulter), CD16-FITC (IM0814U; Beckman Coulter), CD25-ECD (6607112; Beckman Coulter), CD28-ECD (6607111; Beckman Coulter), CD31-FITC (IM1431U; Beckman Coulter), CD3-ECD (A07748; Beckman Coulter), CD3-PC5 (A07749; Beckman Coulter), CD3-PC7 (737657; Beckman Coulter), CD40-PC5 (35-0409; eBioscience), CD46-PE (12-0469-42; eBioscience), CD49b-FITC (IM1425; Beckman Coulter), CD4-ECD (6604727; Beckman Coulter), CD4-PC7 (737660; Beckman Coulter), CD56-PC5 (A07789; Beckman Coulter), CD62L-PE (IM2214U; Beckman Coulter), CD69-PC5 (IM2656; Beckman Coulter), CD8-FITC (A07756; Beckman Coulter), CXCR4-PE (A07409; Beckman Coulter), ICOS-FITC (11-9948-42; eBioscience ), IL12R-PE (FAB839P; R and D Systems, Minneapolis, Minn., USA), IL23R-FITC (FAB14001F; R and D Systems), NKG2c-PE (FAB138P; R and D Systems), TCRαβ-PE (A39499; Beckman Coulter) and TCRγδ-PC5 (IM2662; Beckman Coulter).

#### Random Forest

Random forest is an ensemble learning method that generates models of the provided data and then uses all models combined to get better predictive performance. Random forest generates many decision trees based on bootstrap samples from the original data and for each split in a decision tree a number of variables are randomly selected and tested for classification performance. From these variables the best performing variable is selected for the split. This randomization in sampling and variable selection makes the model very robust and unbiased; it has

been shown to give good results in analyzing multivariate flow cytometry data[1,2].

As only part of the data is used to generate each individual decision tree, the remainder of the data can be used to test the performance of the decision tree and determine the importance of each variable. The importance of variables in the random forest is estimated by permuting one predictor variable while all else is kept unchanged and looking at how much prediction accuracy decreases. This is done per constructed decision tree and gives a mean decrease of accuracy per variable.

Because the dataset contains many hundreds of variables (all possible marker combinations) we first reduced the number of variables. Using random forest to estimate the importance of each variable and discarding variables with an importance of less than zero, we then generate a new random forest again discarding the variables with a negative importance score. This procedure is repeated until for a set amount of repetitions no variables with negative importance values are found. This technique is based on a similar technique implemented in random jungle[3].

The random forest can determine similarity between patients by looking at how many times two subjects fall into the same terminal node over all the decision trees, as it is assumed that two subjects that are similar to each other will frequently end up in the same terminal node. This attribute of random forest was used to impute missing predictor data in the database, as similar subjects were used to estimate the missing data.

## References

1. Eliot M et al. Tree-Based Methods for Discovery of Association between Flow Cytometry Data and Clinical Endpoints. Adv Bioinformatics 2009; 2009: 235320. doi:10.1155/2009/235320.
2. Normolle DP et al. Statistical classification of multivariate flow cytometry data analyzed by manual gating: stem, progenitor, and epithelial marker expression in nonsmall cell lung cancer and normal lung. Cytometry A 2013; 83(1): 150–160. doi:10.1002/cyto.a.22240.
3. Schwarz DF et al. On safari to Random Jungle: a fast implementation of Random Forests for high-dimensional data. Bioinformatics 2010; 26(14): 1752–1758. doi:10.1093/bioinformatics/btq257.

## Supplementary tables

**Table S1:** Flow cytometry staining panel

| FITC | PE | ECD | PE-Cy5 | PE-Cy7 |
|---|---|---|---|---|
| CD49b | TCRαβ | N.A. | CD56 | CD3 |
| CD16 | NKG2c | CD3 | CD244 | CD4 |
| CD31 | CXCR4 | CD28 | CD3 | CD4 |
| ICOS | CD62L | CD25 | CD69 | CD4 |
| IL-23R | IL-12R | CD134 | CD40 | CD4 |
| CD127 | CD46 | CD25 | N.A. | CD4 |
| CD127 | CD152 | CD4 | N.A. | (CD152) |
| CD8 | CD62L | CD134 | CD69 | CD4 |
| CD8 | TCRαβ | N.A. | TCRγδ | CD4 |

Twenty-four marker panel used for staining T cells in whole blood.

**Table S2:**Overview of T cell populations in stable and unstable CAD

| | Male | | Female | |
|---|---|---|---|---|
| | Stable CAD (n=313) | Unstable CAD (n=75) | Stable CAD (n=125) | Unstable CAD (n=37) |
| Lymphocytes (cells/μL) | 1943 (1518-2413) | 1961 (1591-2639) | 2111 (1719-2620) | 2643 (2003-3035)† |
| $CD3^+$ T cells (cells/μL) | 1256 (956-1659) | 1262 (953-1850) | 1407 (1152-1783) | 1761 (1438-2267)‡ |
| $CD4^+$ T cells (cells/μL) | 809 (584-1063) | 793 (631-1115) | 966 (753-1287) | 1175 (1003-1527)† |
| $CD8^+$ T cells (cells/μL) | 307 (202-468) | 299 (220-464) | 294 (200-448) | 361 (264-537) |
| Naïve $CD4^+$ T cells (cells/μL) | 540 (344-707) | 496 (363-813) | 642 (476-924) | 871 (673-1105)‡ |
| Naïve $CD8^+$ T cells (cells/μL) | 87 (49-139) | 88 (51-140) | 91 (55-136) | 126 (95-197)† |
| Activated $CD4^+$ T cells (cells/μL) | 3 (2-5) | 3 (2-5) | 4 (2-7) | 3 (2-6) |
| Activated $CD8^+$ T cells (cells/μL) | 7 (5-11) | 6 (4-12) | 7 (4-13) | 7 (5-11) |
| T regulatory cells (cells/μL) | 27 (16-40) | 26 (18-40) | 33 (26-48) | 36 (25-51) |

Difference in immune cell populations between patients with stable coronary artery disease and unstable coronary artery disease for the male and female cohort, values are median (interquartile range). † $p < 0.01$, ‡ $p < 0.001$

**Table S3:** Comparison of T cells selected by random forest model in male patients

| | Stable CAD (n=313) | Unstable CAD (n=75) | P value |
|---|---|---|---|
| $CD3^+CD31^-CD28^-$ (cells/μL) | 656 (483-880) | 714 (564-941) | p<0.05 |
| $CD3^+CD56^+TCR\alpha\beta^-$ (cells/μL) | 19 (10-37) | 28 (15-42) | p<0.05 |
| $CD4^+CD152^+$ (cells/μL) | 98 (60-157) | 129 (75-206) | p<0.05 |
| $CD4^+CD25^+CD62L^{-/low}$ (cells/μL) | 11 (6-18) | 9 (4-15) | N.S. |
| $CD4^+CD25^+ICOS^+$ (cells/μL) | 1 (1-3) | 2 (1-3) | N.S. |
| $CD4^+CD40^+$ (cells/μL) | 7 (5-11) | 8 (6-11) | N.S. |
| $CD4^+CD62L^{high}ICOS^+$ (cells/μL) | 4 (2-6) | 3 (2-7) | N.S. |

Circulating cell numbers for cell subsets selected by Random Forest modeling of stable and unstable CAD patients for the male cohort ,values are median (interquartile range).

**Table S4:** Comparison of T cells selected by random forest model in female patients

| | Stable CAD (n=125) | Unstable CAD (n=37) | P value |
|---|---|---|---|
| $CD4^+CD127^+CD46^+$ (cells/μL) | 274 (199-382) | 367 (269-483) | p<0.01 |
| $CD4^+CD40^+$ (cells/μL) | 8 (6-14) | 12 (9-16) | p<0.01 |
| $CD4^+CD8^{high}$ (cells/μL) | 2 (1-4) | 5 (3-7) | p<0.001 |
| $CD4^+CD8^+CD62L^{high}$ (cells/μL) | 13 (9-19) | 25 (14-39) | p<0.0001 |
| $CD8^+CD62L^{high}$ (cells/μL) | 149 (95-205) | 213 (167-272) | p<0.0001 |

Circulating cell numbers for cell subsets selected by Random Forest modeling of stable and unstable CAD patients for the female and female cohort ,values are median (interquartile range).

**Table S5:** Baseline characteristics of MACE and control patients

| | Male | | Female | |
|---|---|---|---|---|
| | Control (n=309) | MACE (n=43) | Control (n=146) | MACE (n=19) |
| Age | 62 (56-68) | 64 (55-72) | 64 (57-71) | 62 (56-69) |
| BMI | 27 (25-29) | 28 (26-31) | 27 (24-30) | 27 (23-29) |
| SBP (mm Hg) | 135 (124-145) | 130 (120-140) | 135 (120-150) | 137 (129-149) |
| DBP (mm Hg) | 80 (70-85) | 76 (70-88) | 75 (68-82) | 75 (70-85) |
| Glucose (mmol/L) | 6.00 (5.48-6.82) | 5.81 (5.30-6.62) | 6.15 (5.40-7.02) | 5.60 (5.44-8.60) |
| Hemoglobin (mmol/L) | 8.90 (8.58-9.40) | 9.00 (8.40-9.40) | 8.30 (7.75-8.60) | 8.30 (7.93-8.58) |
| Cholesterol (mmol/L) | 4.00 (3.40-4.74) | 4.00 (3.58-4.94) | 4.59 (3.84-5.30) | 4.15 (3.60-4.73) |
| LDL-C (mmol/L) | 2.30 (1.74-2.88) | 2.40 (1.91-3.04) | 2.60 (1.99-3.30) | 2.65 (2.00-2.85) |
| HDL-C (mmol/L) | 1.02 (0.86-1.21) | 0.96 (0.85-1.04) | 1.22 (1.01-1.47) | 1.01 (0.79-1.23)† |
| Triglycerides (mmol/L) | 1.30 (0.98-1.95) | 1.30 (0.98-1.75) | 1.40 (1.02-1.88) | 1.42 (1.00-1.72) |
| hsCRP (ng/mL) | 2720 (1688-6518) | 3510 (1785-7683) | 3626 (1889-7281) | 4678 (2151-7132) |
| WBC (X1000 cells/μL) | 7.00 (5.80-8.20) | 7.10 (6.05-8.50) | 7.30 (5.70-9.00) | 7.40 (5.90-8.60) |
| Current smoker | 21% (66) | 14% (6) | 25% (36) | 21% (4) |
| Diabetes | 20% (61) | 21% (9) | 23% (33) | 32% (6) |
| Hypertension | 61% (187) | 77% (33) | 70% (102) | 79% (15) |
| Unstable CAD | 17% (54) | 33% (15)* | 22% (34) | 16% (3) |
| Beta-blocker | 72% (222) | 79% (34) | 74% (108) | 84% (16) |
| Ca-antagonist | 30% (92) | 33% (14) | 23% (34) | 47% (9) |
| Aspirin | 82% (254) | 72% (31) | 83% (121) | 95% (18) |
| Vitamin K antagonist | 10% (31) | 19% (8) | 10% (14) | 11% (2) |
| ADP receptor blocker | 45% (140) | 47% (20) | 45% (66) | 58% (11) |
| ACE inhibitor | 35% (107) | 44% (19) | 29% (42) | 37% (7) |
| ATII receptor blocker | 15% (47) | 23% (10) | 26% (38) | 32% (6) |
| Diuretic | 17% (52) | 30% (13) | 26% (38) | 16% (3) |
| Statin | 82% (252) | 70% (30) | 71% (104) | 84% (16) |

Baseline characteristics of male patients that experienced major adverse cardiovascular event (MACE) versus control patients that did not experience MACE, values are median (interquartile range) or percentage (n). † $p < 0.01$

**Table S6:** Simple logistic regression for major adverse cardiovascular events in males

| Lymphocyte subset | Tertile | n | events | Model 1: | | Model 2: | |
|---|---|---|---|---|---|---|---|
| | | | | OR(95% CI) | P value | OR(95% CI) | P value |
| $CD4^+CD127^-CD46^+$ | First | 109 | 9 | 1 (reference) | | 1 (reference) | |
| | Second | 109 | 14 | 1.54 (0.66-3.79) | 0.322 | 1.22 (0.50-3.08) | 0.662 |
| | Third | 112 | 20 | 2.54 (1.14-6.06) | 0.023 | 2.14 (0.93-5.22) | 0.074 |
| $CD4^+CD3^-NKG2c^-$ | First | 112 | 11 | 1 (reference) | | 1 (reference) | |
| | Second | 112 | 8 | 0.71 (0.27-1.78) | 0.465 | 1.04 (0.38-2.81) | 0.934 |
| | Third | 115 | 21 | 2.05 (0.96-4.56) | 0.062 | 2.96 (1.29-7.21) | 0.010 |
| $CD4^+CD40^+$ | First | 117 | 13 | 1 (reference) | | 1 (reference) | |
| | Second | 116 | 15 | 1.25 (0.57-2.76) | 0.582 | 1.43 (0.62-3.37) | 0.396 |
| | Third | 121 | 18 | 1.55 (0.72-3.44) | 0.262 | 1.61 (0.70-3.78) | 0.262 |
| $CD4^+CD46^+CD25^+$ | First | 113 | 9 | 1 (reference) | | 1 (reference) | |
| | Second | 113 | 15 | 1.72 (0.74-4.18) | 0.206 | 1.45 (0.62-3.56) | 0.397 |
| | Third | 117 | 20 | 2.32 (1.05-5.47) | 0.037 | 1.49 (0.63-3.66) | 0.368 |
| $CD4^+CD62L^-CD69^+$ | First | 114 | 10 | 1 (reference) | | 1 (reference) | |
| | Second | 113 | 16 | 1.75 (0.78-4.12) | 0.177 | 1.72 (0.75-4.05) | 0.199 |
| | Third | 117 | 17 | 1.85 (0.82-4.33) | 0.137 | 1.31 (0.55-3.21) | 0.540 |
| $CD4^+CD8^{high}TCR\alpha\beta^-$ | First | 114 | 15 | 1 (reference) | | 1 (reference) | |
| | Second | 114 | 18 | 1.24 (0.60-2.59) | 0.569 | 1.00 (0.46-2.14) | 0.991 |
| | Third | 118 | 9 | 0.57 (0.23-1.32) | 0.188 | 0.53 (0.21-1.28) | 0.162 |
| $CD8^+CD62L^-CD134^+$ | First | 80 | 11 | 1 (reference) | | 1 (reference) | |
| | Second | 80 | 14 | 1.30 (0.55-3.11) | 0.545 | 1.63 (0.65-4.23) | 0.300 |
| | Third | 83 | 5 | 0.41 (0.13-1.20) | 0.105 | 0.58 (0.17-1.79) | 0.348 |

| Lymphocyte subset | Tertile | n | events | Model 1: | | Model 2: | |
|---|---|---|---|---|---|---|---|
| | | | | OR(95% CI) | P value | OR(95% CI) | P value |
| $CD8^+CD62L^-CD69^+$ | First | 113 | 12 | 1 (reference) | | 1 (reference) | |
| | Second | 112 | 19 | 1.74 (0.82-3.83) | 0.151 | 1.66 (0.75-3.79) | 0.212 |
| | Third | 116 | 11 | 0.86 (0.37-2.03) | 0.737 | 0.68 (0.28-1.65) | 0.397 |
| $CD8^{low}CD69^+$ | First | 113 | 10 | 1 (reference) | | 1 (reference) | |
| | Second | 113 | 23 | 2.57 (1.21-5.83) | 0.014 | 2.30 (1.03-5.39) | 0.041 |
| | Third | 116 | 9 | 0.86 (0.34-2.17) | 0.752 | 0.68 (0.26-1.77) | 0.430 |

Simple logistic regression on T cell subsets selected from the male cohort. Events refer to the number of major adverse cardiac events (MACE) in the respective group of patients (tertile). The odds ratio (OR) is calculated versus the first tertile for each respective tertile. Model 1 is adjusted for age; model 2 is furthermore adjusted for hypertension, diabetes mellitus, current smoker, BMI and HDL-C.

**Table S7:** Simple logistic regression for major adverse cardiovascular events in females

| Lymphocyte subset | Tertile | n | events(n) | Model 1: | | Model 2: | |
|---|---|---|---|---|---|---|---|
| | | | | OR(95% CI) | P value | OR(95% CI) | P value |
| $CD3^+CD49B^+CD3^{-/low}$ | First | 50 | 4 | 1 (reference) | | 1 (reference) | |
| | Second | 50 | 4 | 1.00 (0.24-4.14) | 0.997 | 1.18 (0.28-5.00) | 0.814 |
| | Third | 52 | 10 | 2.54 (0.81-9.22) | 0.112 | 2.17 (0.67-8.07) | 0.201 |
| $CD4^+CD127^-CD152^+$ | First | 50 | 4 | 1 (reference) | | 1 (reference) | |
| | Second | 50 | 3 | 0.80 (0.17-3.45) | 0.757 | 0.85 (0.18-3.83) | 0.830 |
| | Third | 51 | 7 | 1.73 (0.51-6.50) | 0.377 | 1.78 (0.50-7.19) | 0.378 |
| $CD4^+CD3^{high}CD28^+$ | First | 54 | 3 | 1 (reference) | | 1 (reference) | |
| | Second | 53 | 7 | 2.36 (0.66-10.18) | 0.190 | 1.87 (0.48-8.51) | 0.372 |
| | Third | 55 | 7 | 2.26 (0.60-10.13) | 0.231 | 1.78 (0.45-8.37) | 0.419 |
| $CD4^+CD40^-IL12R+$ | First | 53 | 4 | 1 (reference) | | 1 (reference) | |
| | Second | 52 | 5 | 1.28 (0.34-5.13) | 0.710 | 1.47 (0.36-6.28) | 0.587 |
| | Third | 54 | 9 | 2.31 (0.72-8.45) | 0.160 | 2.04 (0.62-7.70) | 0.246 |
| $CD4^+CD8^-TCR\gamma\delta^+$ | First | 53 | 5 | 1 (reference) | | 1 (reference) | |
| | Second | 52 | 11 | 2.38 (0.82-7.68) | 0.110 | 1.99 (0.65-6.70) | 0.231 |
| | Third | 54 | 2 | 0.39 (0.07-1.74) | 0.220 | 0.38 (0.06-1.77) | 0.220 |
| $CD4^+TCR\alpha\beta^-TCR\gamma\delta^+$ | First | 53 | 3 | 1 (reference) | | 1 (reference) | |
| | Second | 52 | 7 | 2.42 (0.66-10.59) | 0.184 | 2.83 (0.73-13.23) | 0.134 |
| | Third | 55 | 8 | 2.60 (0.74-11.21) | 0.139 | 2.04 (0.56-9.07) | 0.289 |
| $CD8^+CD62L^-CD69^+$ | First | 53 | 5 | 1 (reference) | | 1 (reference) | |
| | Second | 52 | 9 | 2.01 (0.66-6.73) | 0.220 | 1.29 (0.38-4.64) | 0.682 |
| | Third | 55 | 4 | 0.79 (0.20-2.96) | 0.729 | 0.55 (0.13-2.20) | 0.396 |

| Lymphocyte subset | Tertile | n | events(n) | Model 1: | | Model 2: | |
|---|---|---|---|---|---|---|---|
| | | | | OR(95% CI) | P value | OR(95% CI) | P value |
| $CD4^{+}CD69^{+}ICOS^{-}$ | First | 53 | 9 | 1 (reference) | | 1 (reference) | |
| | Second | 52 | 5 | 0.51 (0.15-1.57) | 0.245 | 0.42 (0.12-1.37) | 0.152 |
| | Third | 55 | 3 | 0.30 (0.07-1.02) | 0.053 | 0.27 (0.06-0.98) | 0.046 |

Simple logistic regression on T cell subsets selected from the female cohort. Events refer to the number of major adverse cardiac events (MACE) in the respective group of patients (tertile). The odds ratio (OR) is calculated versus the first tertile for each respective tertile. Model 1 is adjusted for age; model 2 is furthermore adjusted for hypertension, diabetes mellitus, current smoker, BMI and HDL-C..