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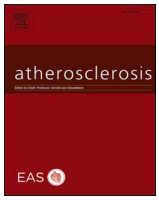

Sex-specific differences in cytokine signaling pathways in circulating monocytes of cardiovascular disease patients

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ABSTRACT

Background and aims: This study aims to identify sex-specific transcriptional differences and signaling pathways in circulating monocytes contributing to cardiovascular disease.
Methods and results: We generated sex-biased gene expression signatures by comparing male *versus* female monocytes of coronary artery disease (CAD) patients (n = 450) from the Center for Translational Molecular Medicine–Circulating Cells Cohort. Gene set enrichment analysis demonstrated that monocytes from female CAD patients carry stronger chemotaxis and migratory signature than those from males. We then inferred cytokine signaling activities based on CytoSig database of 51 cytokine and growth factor regulation profiles. Monocytes from females feature a higher activation level of EGF, IFN1, VEGF, GM-CSF, and CD40L pathways, whereas IL-4, INS, and HMGB1 signaling was seen to be more activated in males. These sex differences were not observed in healthy subjects, as shown for an independent monocyte cohort of healthy subjects (GSE56034, n = 485). More pronounced GM-CSF signaling in monocytes of female CAD patients was confirmed by the significant enrichment of GM–CSF–activated monocyte signature in females. As we show these effects were not due to increased plasma levels of the corresponding ligands, sex-intrinsic differences in monocyte signaling regulation are suggested. Consistently, regulatory network analysis revealed jun-B as a shared transcription factor activated in all female-specific pathways except IFN1 but suppressed in male-activated IL-4.
Conclusions: We observed overt CAD-specific sex differences in monocyte transcriptional profiles and cytokine- or growth factor-induced responses, which provide insights into underlying mechanisms of sex differences in CVD.

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1. Introduction

Although ischemic cardiovascular disease (CVD), including myocardial infarction (MI) and stroke, is the leading cause of death in both men and women [1], the sexual dimorphism in prevalence and presentation of the disease is well-known [2]. Ischemic heart disease (IHD) incidence in young women was reported to be lower than in men, however with increasing age, particularly after menopause, this risk profile is reversed [3]. Estrogen is thought to be a major contributor to these sex differences, by improving arterial function and lipoprotein profile, amongst others. Indeed, estrogen responses were enriched in atherosclerotic plaques of female coronary artery disease (CAD) patients, even beyond menopause [4].

Culminating evidence points out that chronic inflammation, affected by macrophages and their precursors, monocytes, plays an important causal role in IHD; it contributes to atherosclerotic plaque formation and ruptures [5]. Indeed, the CANTOS trial showed a significantly reduced secondary event rate after IL1 receptor intervention [6], although sex differences were not taken into account in this study. Macrophages and monocytes are major drivers of atherosclerosis [7,8] and IHD injury repair [9,10]. They are plastic cells that adapt to their environment [11], which may well differ between males and females. This has prompted several groups to study sex-specific differences in macrophages and monocytes [12–14], showing differences in monocyte number [15,16] or inflammatory response [17]. Macrophages in atherosclerotic lesions were also shown to differ between males and females, with interferon responses, besides estrogen responses, being enriched in females [4]. However, still little is known about sex differences in signaling and transcriptional profiles of circulating monocytes in female and male CAD patients.

This study has assessed a monocyte cohort from the Center for Translational Molecular Medicine (CTMM) – Circulating Cells Cohort presented to the Maastricht University Medical Center, The Netherlands, for sex-related differences in signaling and transcriptional makeup. We first generated the gene expression signatures (GES) for male and female CAD patient monocytes. Gene set enrichment analysis (GSEA) was then used for interpreting sex-specific differential genes, and cytokine and transcription factor (TF) activities were inferred based on Cytosig [18] and Dorothea [19] databases respectively. Finally, the CAD-specificity of pathways and TFs and sex-specific differences of plasma cytokines were validated in an independent healthy subject cohort and CAD plasma proteomics cohort, respectively.

2. Patients and methods

2.1. CTMM cohort

2.1.1. Human blood samples from the Center for Translational Molecular Medicine

A total of 460 CAD patients from the Center for Translational Molecular Medicine (CTMM) Circulating Cells Cohort were used to assess sex differences in CAD (see Fig. 1 for workflow and experimental design). CAD patients were defined as subjects diagnosed with stable angina, unstable angina, Non-ST Elevation Myocardial Infarction (NSTEMI), or silent ischemia. All participants provided written informed consent prior to participation. This study was approved by the Institutional Medical Ethical Review Board of the University Medical Center Utrecht, The Netherlands.

2.1.2. Cell isolation

When patients provided written informed consent, blood samples were obtained in ethylenediaminetetraacetic acid (EDTA) anticoagulated vacuum tubes and processed as previously described [20]. Briefly, blood was centrifuged at 156*g* for 15 min, plasma was withdrawn, and $CD14^{+}$ monocytes were isolated using Ficoll-Paque Plus (Sigma), magnetic bead isolation (BD Bioscience). Plasma and monocytes were stored at −80 °C until needed.

2.1.3. Flow cytometry

To study and quantify monocyte subsets, 50 μl of EDTA anticoagulated whole blood was analyzed by flow cytometry (Beckman Coulter FC 500), as previously described [20], using the antibody panel shown in Table 1. Lymphocytes and granulocytes were gated based on their scatter properties and confirmed using lymphocyte and granulocyte-specific antibodies. Monocytes were identified based on their scatter properties and positive CD14 staining. Expression of surface markers was quantified by relative marker expression and mean fluorescence intensity (MFI).

Table 1
Antibodies for flow cytometry.

Antibody	Company	Dilution
CD14 PC7	BD Biosciences	1:10
CD16 PC5	BD Biosciences	1:10
CCR2 PE	BD Biosciences	1:5
CX3CR1 FITC	BD Biosciences	1:5

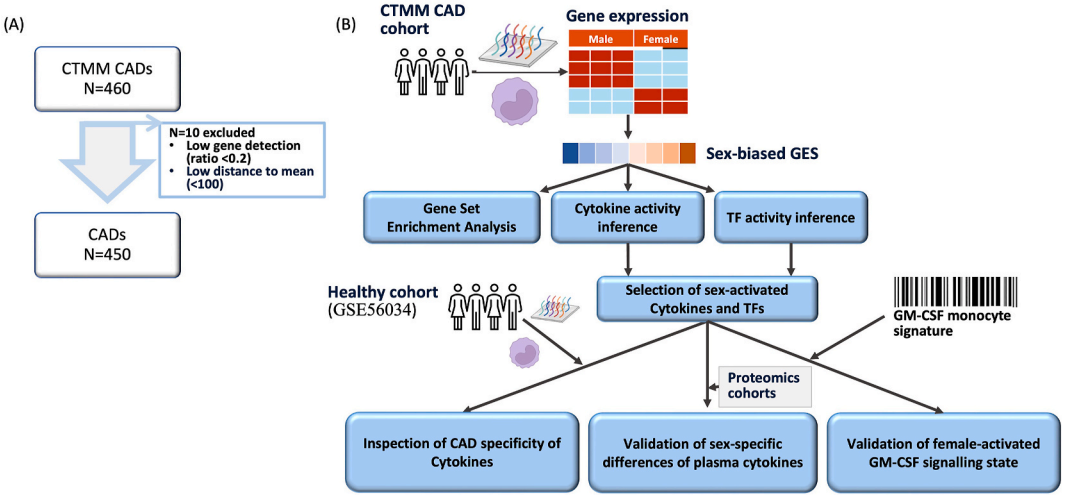

Fig. 1. CTMM cohort and analysis procedure.
(A) Schematic of cohort build-up; (B) schematic diagram of the analysis.

2.1.4. RNA isolation and micro-array analysis

RNA isolation and micro-array data generation of monocyte samples were completed by AROS (Denmark). In brief, RNA was isolated using Illumina TotalPrep RNA Amplification Kit (Illumina, San Diego, CA, USA) and cDNA was produced. Next, labeled cRNA was prepared and used on the array for hybridization. Hybridized chips were scanned by Illumina BeadStation (Illumina, Inc., San Diego, CA, U.S.A.). Raw image analysis and signal extraction were performed with Illumina Beadstudio Gene Expression software with default settings (no background subtraction) and no normalization.

2.1.5. Data preprocessing

The CTMM microarray data were exported to R v3.6.3 after quality control using GenomeStudio software. Ten of 460 profiles were excluded due to their low number of detected genes (<20% significantly detected genes) or large distance to the mean (>100) based on quality control files obtained from GenomeStudio. Log2 transformation and Robust Spline Normalization (RSN) were performed using lumi R package [21]. For the resulting cohort of 450 profiles, to reduce the effect of background noise, genes with low intensity (more than 90% of patients' log2 expressions lower than 7.5) were filtered out. To inspect the preprocessing results, batch correction was performed using the sva R package before visualization [22]. Eventually, 450 microarrays from 316 men and 134 women with CAD, including 9092 genes, were used for differential gene expression analysis. All probes have been converted to official gene symbols. The schematic diagram of the cohort build-up and workflow of the study is shown in Fig. 1A.

2.2. Validation cohorts

A monocyte cohort from Harvard Medical School, Boston, USA (GSE56034) was downloaded from GEO database for validation. $CD14^{+}CD16^{-}$ monocytes were sorted from peripheral blood mononuclear cells (PBMCs) of 485 healthy individuals (272 women and 213 men). mRNAs of these monocytes were then profiled on Affymetrix GeneChip Human Gene ST 1.0 microarrays. Log2 transformation and quantile normalization were used for data preprocessing. After filtering out genes with low intensity (more than 95% of patients' expressions lower than 100), 13,556 genes were left for differential gene expression analysis. The mean and standard deviation of age for healthy males and females are shown in Supplementary Table S1.

Secondly, proteomics data from a cohort including 187 CAD patients (32 female and 155 male) and 341 healthy subjects (182 female and 159 male) [23] were used to study whether the augmented signaling activities were reflecting elevated levels of the corresponding ligands in plasma. The demographics of this cohort are shown in Supplementary Table S2.

2.3. Differential gene expression

Sex-biased genes were obtained using limma package [24]. More specifically, for each gene g, a linear model was built between gene expression values of g and sexes of all patients. Significant levels were calculated based on the moderated t-statistics, which used empirical Bayes methods to obtain posterior variance estimators (for details see the limma tutorial). Ages and batches were added to the linear model as covariates for removing their effects. The male/female-biased genes in this study are genes that show up/down regulation by comparing males and females. The GES here is the log2 fold change (log2FC) of expression between male and female subjects. p-values were adjusted by false discovery rate (FDR).

2.4. Gene set enrichment analyses (GSEA)

GSEA of Gene Ontology (including Biological Process (BP), Molecular Function (MF), and Cellular Component (CC)), KEGG, WikiPathway, and Reactome for sex-related GES were performed using the ClusterProfiler R-package [25]. The p-values were adjusted for multiple comparisons by FDR, and the cut-off was set to 0.05 to avoid presenting false discovery significant terms. Gene ratio stands for the percentage of genes associated with the given GO/Pathway term to the total number of genes in that term.

2.5. Cytokine signaling activity inference

Cytokine signaling activities were calculated based on the database of target genes modulated by cytokines from Cytosig platform (cytosig.ccr.cancer.gov). The Cytosig database includes a total of 51 cytokines (and growth factors) and 6466 response genes. As suggested by Jiang et al. [18], considering the signature collinearity of cytokine profiles, we utilized ridge regression (linear ridge function in 'ridge' R-package), as predictive model of cytokine signaling activities. Only 4130 genes that overlapped in the Cytosig database and the sex-biased GES CTMM cohort were used in this model. Composite profiles of cytokine response were the explanatory variables, and the sex-biased GES was the response variable. The regression coefficients represent cytokine target activities. The ridge regression parameter is chosen automatically using 'ridge' R package proposed by Cule and De Iorio [26]. The p-values are computed using the significance test of Cule et al. [27]. p-values were adjusted using FDR.

2.6. TF activity inference

In this study, we predicted both activities of TFs that drive sex-biased GES and that drive cytokine-modulated gene profiles, based on the TF-target interaction network provided by DoRothEA [19]. Specifically, we first filtered out the TF–target interactions with the low levels of evidence (confidence score D and E), and then selected the intersection of TF target genes in DoRothEA database and 4130 genes we used for cytokine activity inference, to ensure consistency between the inferred Cytokine activities and TF activities. These steps result in 265 TF–target interactions and 1560 target genes being used for TF activity prediction. TF activities were calculated using the Weighted Connectivity Score (WTCS), a bi-directional version of the weighted Kolmogorov-Smirnov enrichment statistic (ES) described in Ref. [28]. For a given TF, assuming q_{pos} and q_{neg} are the activated and inhibited target sets of TF q in DoRothEA, its activity w on a cytokine-modulated profile (or sex-biased GES) s is as follows:

$$w_{q,s} = \begin{cases} \dfrac{NES_{pos} - NES_{neg}}{2}, \; if \; sgn\left(NES_{pos}\right) \neq sgn\left(NES_{pos}\right) \\ otherwise \end{cases} \quad (1)$$

where NES_{pos} is the normalized enrichment score of q_{pos} in s and NES_{neg} is the normalized enrichment score of q_{neg} in s. P-value is calculated based on the 10000-time gene-wise permutation test.

2.7. Association of sex-specific cytokine signaling activities with recurrent ischemic event incidence

The sex-specific cytokine activities were inferred for all CAD patients of the CTMM cohort using the run_wmean function in DecoupleR package [29]. Specifically, this function calculates a patient's signaling activity score by computing the weighted mean of this patient's gene expression profile, where weights reflect the gene's contribution to the gene expression signatures of this signaling pathway in the Cytosig database [18]. P-values were calculated from 1000 gene permutation test runs. Finally, the differences in cytokine activities of event-free CAD patients (males: n = 218; females: n = 89) *versus* CAD patients with recurrent angina (males: n = 36 in males; females: n = 24) were calculated for female and male subjects, respectively using Wilcoxon test.

2.8. Visualization

Cytoscape was applied to visualize the WTCS between cytokines and TFs. All other plots were generated using ggplot2 package in R.

2.9. Validation on GM–CSF–induced RNA-Seq dataset

Human CD14^{+} peripheral blood monocytes were stimulated for 24 h with GM-CSF (5 ng/ml; n = 3) and expression profiles were assessed by RNA-sequencing. Parental monocytes were used as controls. Differential gene expression analysis revealed 132 upregulated and 179 down-regulated genes in GM-CSF stimulated *vs*. unstimulated monocytes (the absolute value of log2FC > 0.5; $p_{adj} < 0.05$). This differentially expressed gene set (GSE210855) was used to perform GSEA to check if the up-regulated genes were enriched in the female-biased gene set from GES in the CTMM monocyte cohort.

2.10. Statistical analysis

Unpaired two-sided t-tests with non-equal variance were applied to compare gene expression from females and males in Fig. 5B and C and Supplementary Figs. S3B and C. Significant level is denoted by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in Fig. 4C. All statistical analyses were performed in R (v3.6.3).

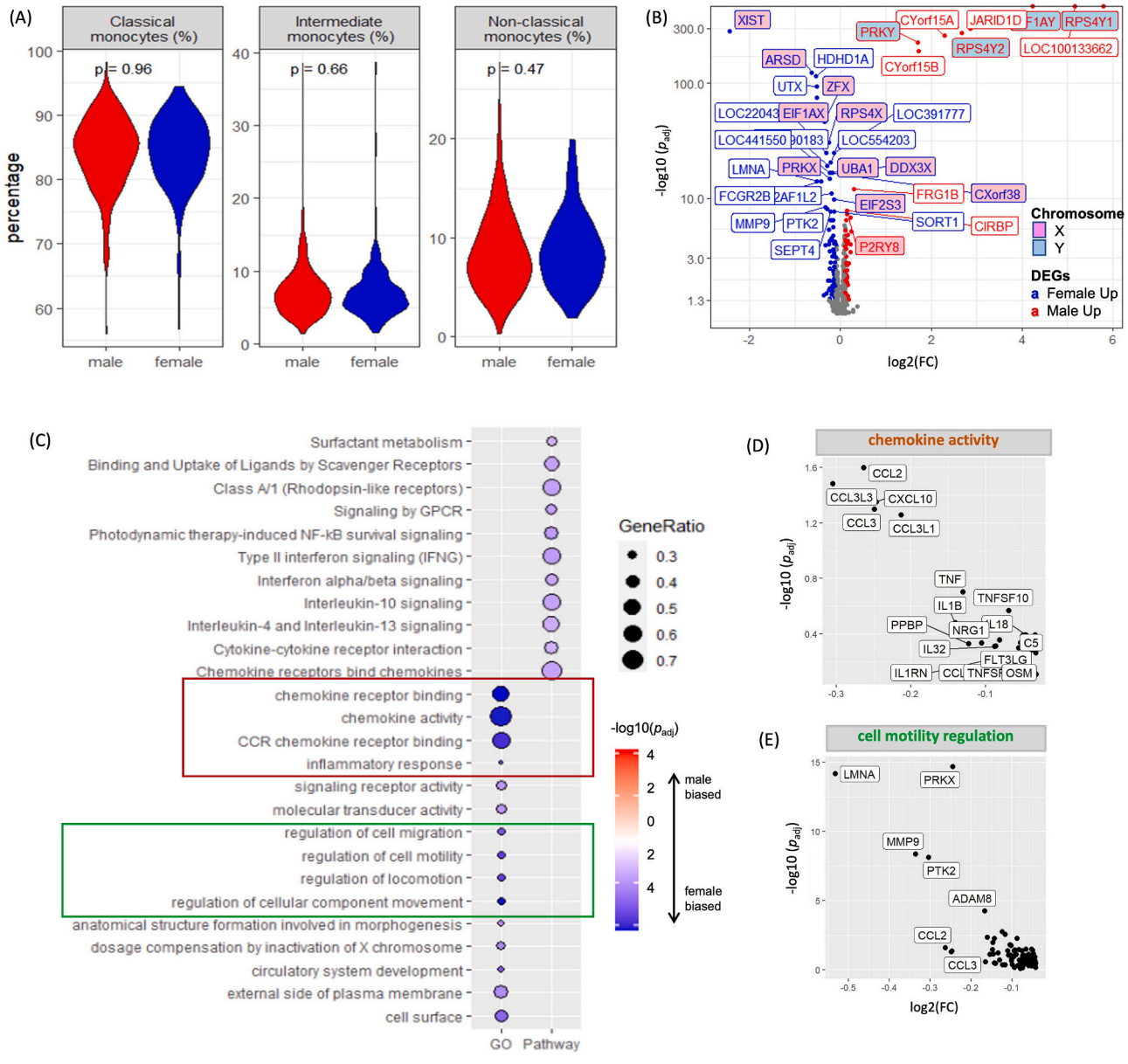

Fig. 2. Monocyte transcriptional signature of female CAD patients points to stronger migratory potential than that of men.
(A) Violin plots showing the percentages of the quantity of classical (CD14^{++}CD16^{-}), non-classical (CD14^{+}CD16^{+}), and intermediate (CD14^{++}CD16^{+}) monocytes from female and male patients. (B) Volcano plot showing sex-biased genes in the CTMM cohort. Significant female-biased (log2FC < −0.1 and $p_{adj} < 0.05$) and male-biased (log2FC > 0.1 and $p_{adj} < 0.05$) genes were color-coded with blue and red. (C) Dot plots visualized the significant levels of top enriched GO terms and pathways using GSEA. Male/female-biased categories are color-coded with red/blue. GO terms relating to cellular movement and chemokine- or interleukin signaling in the green and red boxes, respectively. Significant levels are shown by using log10-transformed adjusted *p*-values. (D and E). Log2FCs of genes in the enriched GO terms (chemokine activity and regulation of cell motility) have the top two highest significant levels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

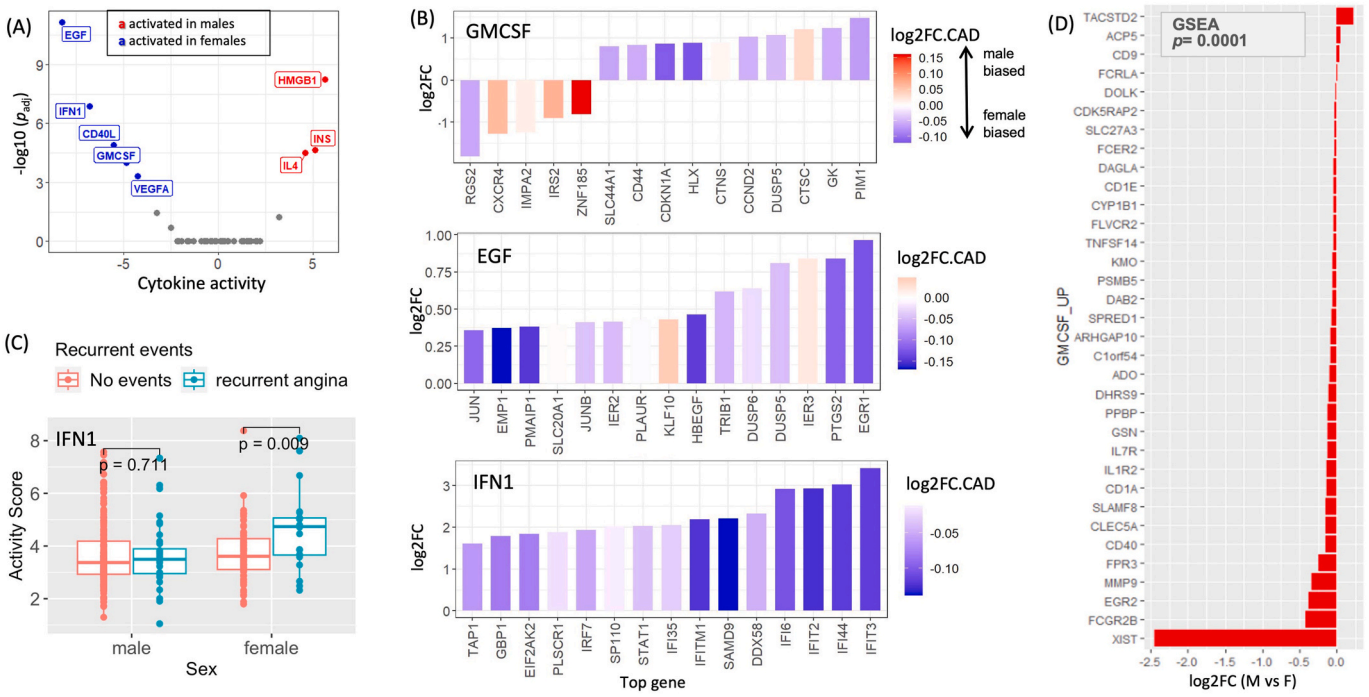

Fig. 3. Inference of male- and female-biased cytokines using Cytosig.
(A) Volcano plot visualizing the cytokine activities of CTMM CAD patients by comparing males *vs* females. By setting the cutoff of FDR-adjusted *p*-value as 0.05, female- and male-biased cytokines are highlighted in blue and red. (B) Bar plots showing the top 15 genes (ranked by absolute log2FC) modulated by IFN1, GMCSF, and EGF from Cytosig platform. Bar colors indicate the log 2-fold changes of these genes in the CTMM cohort, comparing male *vs* female patients. (C) Box plot compares IFN1 activities between male/female CAD patients with recurrent angina and without recurrent events. (D) Bar graph showing the enrichment of GMCSF-induced upregulated genes from the RNAseq dataset over the sex-biased GES from the CTMM cohort. y-axis shows the overlapped genes between GMCSF-induced upregulated genes from the RNAseq dataset and GES from the CTMM cohort. X-axis indicates the log 2-fold changes (male *vs* female) of these genes in the CTMM cohort. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Monocytes from female CAD patients show stronger chemotaxis and migratory gene signatures than males

To identify sex differences in monocyte transcriptomes of CAD patients, we compared microarray data from monocytes of male (n = 316) and female (n = 134) CAD patients within the CTMM cohort. The workflow of this study is shown in Fig. 1B. Clinical information of patients in this cohort is listed in Table 2. Except for higher HDL and lower creatinine in females, there were no significant differences in other clinical characteristics (e.g. age, BMI, blood pressure, glucose, LDL and triglyceride levels, smoking, and diabetes prevalence) between males and females. Similarly, flow cytometry analyses confirmed that the number of classical, non-classical, and intermediate monocytes was essentially similar between male and female CAD patients (Fig. 2A). We observed 48 male- and 107 female-biased genes (Fig. 2B; a full list of sex-biased genes is presented in the Supplemental excel file, including several notorious X or Y chromosome-linked genes, such as *PRKY*, *RPS4Y1*, *EIFAY*, and *XIST*.

We then performed GSEA based on sex-biased signatures to obtain a more detailed view of the functions of sex-biased genes (Fig. 2C). While male-biased genes did not show clear, significantly enriched GO terms, female-biased genes were significantly enriched in pathways related to cellular movement and chemokine- or interleukin signaling. Analysis of the genes responsible for the observed enrichment of chemokine activity (Fig. 2D) term revealed that all GO term chemokine activity genes (e.g. *CCL2, CCL3, CXCL10*) were upregulated in female CAD patients compared to males. Similarly, *LMNA, PRKX, MMP9*, and *PTK2* are the top 4 differentially expressed 'regulation of cell motility' term members (Fig. 2E), next to *CCL2* and *CCL3*.

3.2. Identification of male- and female-biased cytokine signaling

Since chemokine/interleukin signaling and cellular motility-related pathways were significantly different between sexes, we inferred the cytokine, chemokine as well as growth factor pathway activities in male *vs*. female CAD based on the intersection of genes from our CTMM cohort and Cytosig database [18] (n = 4130 genes). EGF, IFN1, GMCSF, VEGFA, and CD40L signaling activities were significantly enriched in female patients, while INS, HMGB1, and IL4 pathways were enriched in male patients (Fig. 3A). Indeed, for the female-enriched pathways, the majority of cytokine- and growth factor-induced genes are upregulated in female compared to male CAD patients (indicated by the blue colored bars in Fig. 3B and Supplementary Fig. S1B), while genes suppressed by the female-biased ligands were enriched in males (red colors). Similarly, genes induced by IL4 and HMGB1 were mostly enriched in males (Supplementary Fig. S1C). Interestingly, although CVD event rates during 1-year follow-up in the CTMM cohort were limited (Table 2), female CVD patients with recurrent angina had significantly higher monocyte IFN1 signaling activity than female patients without recurrent events ($p = 0.009$), while IFN signaling did not differ for these endpoints in the male patient population (Fig. 3C, Supplementary Fig. S1D).

To determine whether the enhanced signaling activities were reflecting higher levels of the ligands in plasma, we studied a plasma proteomics dataset from the CAPIRE study cohort of CAD and healthy subjects [23]. In this plasma cohort (n = 155 for CAD males; n = 32 for CAD females, n = 159 for healthy males, and n = 182 for healthy females), we could only demonstrate a minor, yet significant, increase in VEGF levels in female plasma compared to males, but none of the other ligands showed even a trend towards significant differences between male and female CAD (Supplementary Fig. S1E), suggesting enhanced sensitivity for these ligands and/or intrinsic signaling differences within

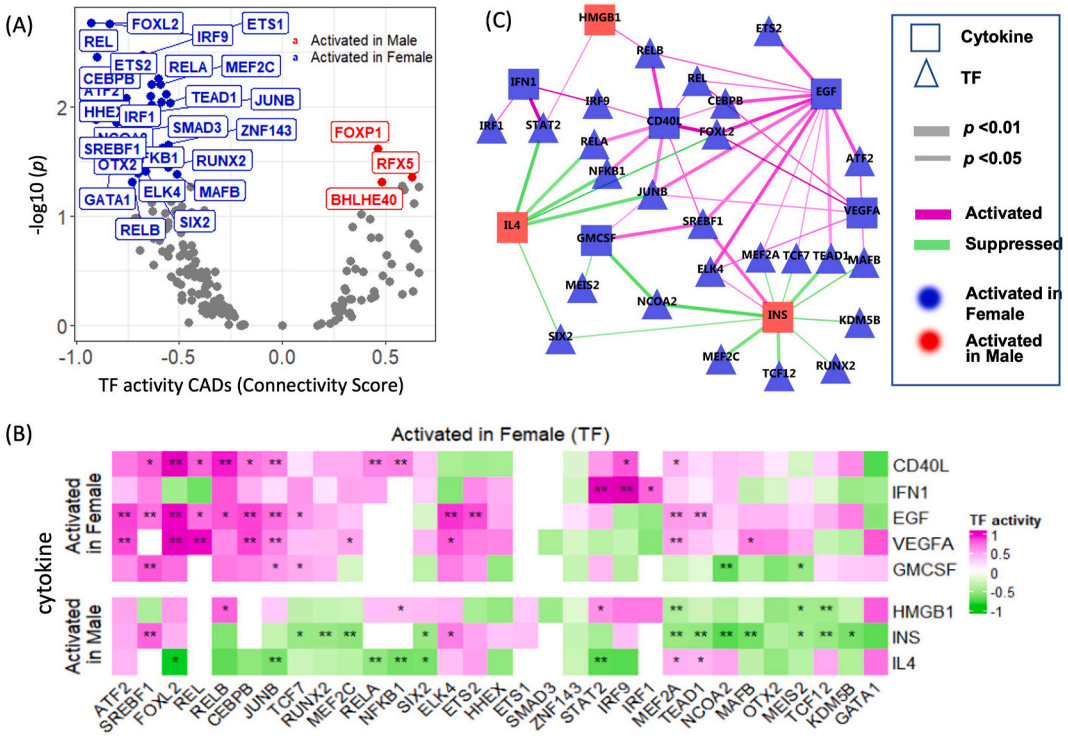

Fig. 4. Associations between sex-biased enriched cytokines and TFs.
(A) Volcano plot of inferred TF activities on CAD cohort by comparing male and female subjects. Nodes are color-coded based on the WTCS. TF was defined as a female/male-activated TF if its absolute WTCS is higher than 0.4 and $p < 0.05$. (B) Heatmap visualizing the activities of TFs that drive female-biased TFs and sex-biased cytokine in CAD. (C) Bipartite graph showing the connections between TFs that drive female-biased TFs and sex-biased cytokine in CAD. Only TFs that are significantly enriched in a cytokine-modulated gene profile are visualized ($p < 0.05$ and absolute value of connectivity score >0.5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

monocytes. In line with the enrichment of chemokine activity term members in female monocytes (Fig. 2D), we did find significantly increased plasma levels of CCL3 in female CAD patients (Supplementary Fig. S1E). Next, we performed GSEA of signature gene expression profiles of human $CD14^{+}$ peripheral blood monocytes stimulated or not with GM-CSF (24h; 193 genes with log2FC > 0.5, $p_{adj} < 0.05$). The intersection of genes between CTMM GES and up-regulated genes induced by GM-CSF was visualized in Fig. 3D. GM–CSF–induced signature indeed was significantly enriched in females compared to male CAD patient monocytes (GSEA, $p = 0.0001$). Most GM–CSF–induced genes, including *MMP9*, *LMNA*, and *CCL2* (all enriched GO term members, Fig. 2D and E) were indeed seen to be significantly increased in female CAD patients. Interestingly, the gene transcript with the strongest upregulation in females (see Fig. 2B), i.e. the X-linked non-coding RNA *XIST*, also appeared to be induced by GM-CSF (log2FC = 0.53, $p_{adj} = 3.54*10^{-10}$), next to Fc-gamma receptor 2B (*FCGR2B*), Early growth response gene-2 (*EGR-2*) and the motility transducer Formyl-peptide receptor 3 (*FPR3*). Moreover, we also found CD40, the receptor mediating CD40L signaling (activated in females) to be significantly induced by GM-CSF and enriched in female over male CAD patients.

3.3. Associations between sex-biased enriched cytokines and TFs

Our validation experiments point to cell-intrinsic differences in sex-specific signaling pathways. Therefore, we investigated whether common regulatory mechanisms underlie the sex-specific pathway activation. Hereto we first calculated TFs activities driving these sex-biased genes in CAD based on a TF-target interaction network from DoRothEA [19]. In line with our previous findings, we identified more activated TFs in women (n = 31) than in men (n = 3) (Fig. 4A). We then inferred the activities of these sex-biased TFs to identify which TFs are linked to the 8 ligand-induced gene profiles from the Cytosig database (Fig. 4B) and visualized the TFs that are significantly activated or suppressed by the ligands in a bipartite graph (Fig. 4C). CD40L, EGF, and VEGF were found to be linked to several of the TFs enriched in female CAD patients. As expected, we found STAT2, IRF1, and IRF9 to be significantly associated with IFN1, while NFKB1 and RELA were associated with CD40L. Both pathways were significantly enriched in females, while the male-enriched IL-4 cytokine activity was linked to suppressed STAT2, NFkB and RELA signaling. Most notably, JUNB was associated with 4 out 5 female-enriched pathways, i.e. CD40L, EGF, VEGF and GM-CSF, and 1 of 2 male-enriched pathways (IL-4).

To identify the key target genes of TFs associated with these female-activated ligand pathways, we visualized the differential gene expressions between male/female CAD patients for selected TF's target genes and their dysregulation (log2FC) induced by the respective cytokine/growth factors (Supplementary Fig. S2). Even though both the ligand- and TF pathways were highly significantly enriched in females, their target genes' expression showed only modest dysregulation in females compared to males. Interestingly, both GM-CSF and JUNB specifically induce the expression of *LMNA*, which is one of the genes with the strongest upregulation in female CAD patients compared to males (Fig. 2A and Supplementary Fig. S1A), and a dominant gene involved in the female enriched GO term 'cell motility' (Fig. 2D).

3.4. Validation on monocytes from healthy cohort

To validate if the sex differences we observed in the CTMM cohort are specific for CAD, we calculated pathway activities for the 5 ligands that are enriched in female CAD patients (i.e. CD40L, EGF, GMCSF, IFN1, VEGFA) and the 3 ligands that are enriched in male CAD patients (i.e. IL4, HMGB1, INS) in a monocyte cohort from 485 healthy males and females (GSE56034). Fig. 5A shows that all these ligands did not show any significant activation or suppression scores in healthy men *versus*

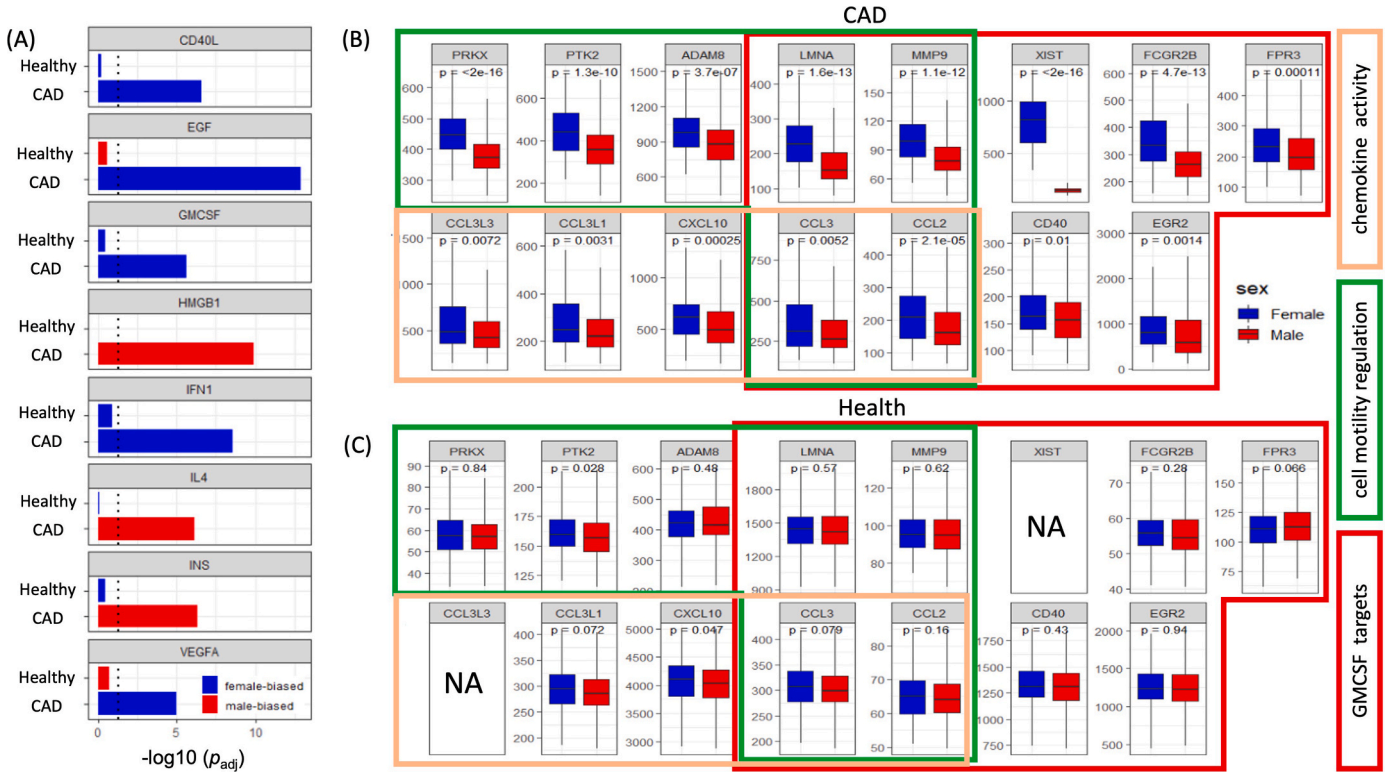

Fig. 5. Female-/male-biased genes and cytokines inferred from CTMM CAD cohort were validated on a healthy cohort (GSE56034).
(A) Activities of 8 CAD-related sex-biased cytokines on CAD and healthy (HE) cohort. Dashed lines indicate the significant threshold 0.05. (B) Expression levels in the CTMM cohort for 15 differentially expressed genes (male *vs* female) that are involved in chemokine activity (yellow box), regulation of cell motility (green box) and GMCSF-driven target genes (red box). (C) Expression levels of the same 15 sex-biased genes as in panel B in a healthy cohort (GSE56034). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Demographics of females and males who participated in the CTMM cohort expressed as mean ± SD or frequencies (%).

Risk factor	Male (n = 316)	Female (n = 134)	*p*
Age (years)	62.48 ± 10.02	62.92 ± 10.66	0.69
BMI (kg/m^2)	27.4 ± 3.89	27.7 ± 5.28	0.56
Triglyceride (mmol/L)	1.57 ± 0.93	1.54 ± 0.87	0.76
LDL (mmol/L)	2.47 ± 0.98	2.71 ± 1.12	0.08
HDL (mmol/L)	1.06 ± 0.26	1.23 ± 0.36	**9.48E-05***
Heart rate (bpm)	67.49 ± 13.53	69.42 ± 12.44	0.14
Systolic Blood Pressure (mmHg)	134.81 ± 18.92	137.89 ± 22.83	0.17
Diastolic Blood Pressure (mmHg)	78.84 ± 11.15	76.46 ± 12.32	0.06
Hypertension (# of patients)	192 (60.76%)	93 (69.4%)	0.08
Smoking (pack years)	25.41 ± 22.28	26.85 ± 24.33	0.56
Current smoker (# of patients)	66 (20.89%)	33 (24.63%)	0.38
Glucose (mmol/L)	6.51 ± 2.18	6.76 ± 2.09	0.32
Diabetes mellitus (# of patients)	64 (20.25%)	34 (25.37%)	0.26
Creatinine (mmol/L)	91.86 ± 53.76	74.99 ± 19.84	**2.24E-06***
Renal failure (# of patients)	6 (1.9%)	3 (2.24%)	0.81
Recurrent angina (# of patients)	36 (11.4%)	24 (17.9%)	0.09
No recurrent events (# of patients)	218 (69.0%)	89 (66.4%)	

p-values were calculated by Student's t-test (for continuous data) or Pearson's chi-squared test (for categorical data, analyzing sex differences in recurrence of angina, excluding "possible events").

women, confirming the specificity of these sex differences for CAD. As the average age of the healthy cohort (i.e. 32.9 ± 10.9 years for male; 28.5 ± 9.1 years for females) is much lower than that of the CTMM cohort, we also analyzed a subcohort of all subjects above 45 years old (n = 43 males, age = 48.8 ± 2.1; n = 29 females; age = 48.9 ± 2.549) (Supplementary Table S1). As shown in Supplementary Fig. S3, also in this subcohort, GMCSF, CD40L, IFN1, IL-4, and HMGB1 signaling was not showing any differences in activation status in healthy individuals. Surprisingly, EGF, VEGF, and Insulin activities were even found to be enriched in the opposite sexes in healthy subjects compared to CAD patients, pointing to sex and disease-specific differences in aging of these pathways.

We then visualized the expression of female-upregulated genes responsible for the female-biased GO term enrichment as well as the GM-CSF signature genes, i.e. *XIST, FCGR2B, EGR2, MMP9, FPR3*, and *CD40*. Fig. 5 B and C show that these genes were significantly upregulated in the monocytes of female compared to male CAD patients, but not in the healthy cohort. Only *LMNA* was already slightly elevated in females vs males, but only in the healthy >45 year sub-cohort suggestive of an age-dependent upregulation in women (Supplementary Fig. S3B). Of note,

chemokine CCL2 expression was significantly higher in healthy middle-aged men vs women, whereas in CAD it was elevated in women. Altogether, these data strongly indicate the observed sex-biased signaling activities are specifically found in CAD and not in healthy subjects.

4. Discussion

In this study, we examined sex-related differences in signaling pathways and their regulation in circulating monocytes of CAD patients. We found monocytes of female CAD patients to have significantly activated CD40L, GM-CSF, EGF, VEGF, and IFN1 signaling compared to men, whereas male CAD patients' monocytes displayed higher IL-4, Insulin, and HMGB1 signaling activities compared to females. These sex effects were not observed in healthy subjects of different age categories. The corresponding cytokine and growth factors activating these pathways did not differ between men and women in CAD, pointing to downstream differences in pathway regulation. Indeed, several of these pathways appeared to share a common transcriptional regulation, with JUNB central in 4 out of 5 female and 1 of 2 male signaling pathways as the most prominent sex-specific TF.

Although several studies have shown increased plasma levels of the X-chromosome-linked CD40L [30,31], we could not confirm this in the plasma proteomics dataset of the CAPIRE study [23]. Except for significantly higher VEGF levels in female CAD patients, we did not observe any differences in plasma levels of female- or male-biased ligands. Type I IFN responses, mediated by the X-chromosome linked Toll-like receptor 7 (*TLR7*), are known to be enhanced in females [4,32], yet we did not find differences in IFN1 plasma levels between male and female CAD patients. Likewise, levels of HMGB1 reported to be higher in men than in women [33], were similar in men and women in the CAPIRE study. Therefore, we assume that sex-biased differences in signaling activity are mainly pathway intrinsic and reflect sex differences in the monocyte's capacity to respond to a particular cytokine in CAD. These findings are in line with the observation of Gupta et al. that female neutrophils displayed increased responsiveness to type I IFNs, independent of serum IFN1 levels [8].

We could confirm that the monocyte GM-CSF signaling signature was enriched in the monocytes of female CAD patients. Moreover, several female-enriched GO term members, such as lamin A (*LMNA*), were induced by GM-CSF, which concurs with GM-CSF's reported role in myeloid cell recruitment in inflammatory and pathological conditions [34].

While some studies reported a role for the nuclear membrane integrity stabilizer *LMNA* in NFkB-dependent inflammation [35], differentiation and migration of monocyte/macrophages [36,37], sex differences in *LMNA* expression and the induction by GM-CSF are to our knowledge hitherto unknown.

Next to *LMNA*, GMCSF also induced *CD40* which, at least partly, may explain the increased CD40L signaling activity in female monocytes. CD40-CD40L are co-stimulatory molecules well known for their role in inflammatory responses and atherosclerosis [38]. Interestingly, elevated expression of CD40L has been associated with cardiovascular diseases and risk for acute cardiovascular symptoms, also in women [39]. While GM-CSF is mainly thought to act pro-inflammatory, it also can dampen excessive inflammation. For instance, GM-CSF induced *XIST*, an X-chromosome linked long non-coding RNA and in fact, the RNA with the highest enrichment in females, was recently shown to accelerate wound healing in the skin by promoting macrophage polarization towards an anti-inflammatory M2 phenotype [40]. Similar anti-inflammatory effects were reported for the GM–CSF–induced female-enriched genes early-growth-response gene 2 (*EGR-2*) [41] and Fc-gamma receptor 2B (*FCGR2B*) [42]. Although it remains to be elucidated how monocyte pathway activities relate to sex differences in CAD development or manifestation, it is tempting to speculate that the abovementioned inflammation-dampening and wound-healing responses may account for the more stable, fibrocalcific plaque phenotype observed in females [43].

At premenopausal stages, most sex differences in CAD responses have been attributed to sex hormones. For instance, estrogen has been found to have anti-inflammatory effects on macrophages, by dampening NFκB signaling [17,44,45], to reduce oxidative stress response in healthy murine peritoneal macrophages to bias macrophages toward the M2 phenotype [46] and to attenuate their migratory capacity [14]. However, sex-specific disease mechanisms in post-menopausal women are less well studied, although CVD risk profiles of women increase profoundly even beyond that of men after menopause and enhanced estrogen responsiveness was observed even in post-menopausal women [4], suggesting that epigenetic mechanisms are responsible for these long-term effects. Although for CVD conclusive experimental proof is lacking, estrogens have indeed been shown to effect persistent epigenetic gene regulation by cooperating with several DNA (de)methylation and histone modification enzymes as well as chromatin remodeling complexes [47]. Considering monocyte's short half-life, such a mechanism should occur at the level of more long-lived myeloid precursor cells or even at hematopoietic stem cell level. Although we observed no differences in CVD risk factors between sexes in our cohort, except for creatinine and HDL levels, it is well known that the relative risk for CVD conferred by such risk factors is greater in females than in males [1,48]. This may also match the seemingly paradoxical bias towards proinflammatory signaling in women with CAD in the CTMM cohort.

Our study has some limitations. While the CTMM contains a reasonable amount of 316 male and 134 female CAD patients, the cohort of the CAPIRE study is more limited and contains an imbalance in the number of male and female CAD patients (Supplementary Table S2, 32 female CAD, and 155 male CAD). Therefore, the Welch *t*-test was used to compare the protein levels between males and females, to reduce type I error rates.

CVD risk factor profiles differed between cohorts. In fact, creatinine levels (both cohorts), smoking (CAPIRE cohort), and HDL-C levels (CTMM cohort), were different between male and female CAD patients. As HDL has been shown to impact monocyte inflammatory responses [49], this may have confounded our findings. However, we believe that an HDL-C and creatinine-associated bias of gender-specific signaling is unlikely for the following two reasons. Firstly, while HDL-C and creatinine levels in men and women are known to differ in health as well (as also seen in the CAPIRE study), the identified sex-specific pathways in CAD did not differ between sexes in healthy subject monocytes. Secondly, except for a very weak (yet significant) correlation of CD40L to smoking ($\rho = -0{,}12, p = 0.01$), we did not observe any correlation of the sex-biased signaling pathway with these risk factors.

The average age of the healthy cohort subjects was considerably lower than that of the CAD cohorts (i.e. the CTMM patient cohort and the CAPIRE study cohort). While >50% of women in the latter cohorts were post-menopausal, this did not hold for the healthy cohort (average age men: 32.93 ± 10.96 years, women: 28.49 ± 9.11 years). However, also using this subcohort the main findings on sex-biased pathways remain valid, giving confidence in our findings. Finally, the healthy control dataset included expression profiles obtained from sorted $CD14^{+}CD16^{-}$ (classical) monocytes, while the CTMM study cohort of CAD patients involved CD14 bead purified monocyte samples. However, the majority of monocytes in human blood (>85%) consisted of classical monocytes, which is in line with previous reports in healthy individuals [50]. Subset numbers did not differ between males and females in our CVD cohort, nor did we observe differences in subset distribution in the healthy cohort as inferred by deconvolution (data not shown).

In conclusion, our study shows sex differences in the signaling capacities of circulating monocytes in CAD patients. While male monocytes display enhanced signaling activities induced by HMGB1, insulin, and IL-4, female monocytes are enriched in EGF, IFN1, CD40L, GM-CSF, and VEGF signaling pathways. Although we did observe increases in plasma VEGF levels in female CAD patients compared to males, these enhanced signaling responses mostly represent increased intrinsic

signaling capacities. We showed increased GM-CSF signaling in female monocytes enhanced expression of various genes related to the female-enriched GO terms of chemokine activity and regulation of cell motility, as well as the expression of *CD40*, which may contribute to increased CD40L responsiveness.

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Data availability

The data that support the findings of this study are openly available in the Gene Expression Omnibus (GEO), reference number GSE56034.

CRediT authorship contribution statement

Chang Lu: Methodology, Data curation, Validation, Software, Writing – original draft, Writing – review & editing. **Marjo Donners:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Joël Karel:** Conceptualization, Supervision, Methodology, Writing – review & editing. **Hetty de Boer:** Investigation, Methodology, Writing – review & editing. **Anton Jan van Zonneveld:** Resources, Writing – review & editing. **Hester den Ruijter:** Resources, Writing – review & editing. **J. Wouter Jukema:** Resources, Writing – review & editing. **Adriaan Kraaijeveld:** Resources, Writing – review & editing. **Johan Kuiper:** Resources, Writing – review & editing. **Gerard Pasterkamp:** Resources, Writing – review & editing. **Rachel Cavill:** Conceptualization, Supervision, Writing – review & editing. **Javier Perales-Patón:** Conceptualization, Supervision, Writing – review & editing. **Ele Ferrannini:** Resources, Writing – review & editing. **Pieter Goossens:** Conceptualization, Supervision, Writing – review & editing. **Erik A.L. Biessen:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2023.04.005.

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