Circulation Research

ORIGINAL RESEARCH

Blood Monocyte Phenotype Is A Marker of Cardiovascular Risk in Type 2 Diabetes

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BACKGROUND: Diabetes is a major risk factor for atherosclerotic cardiovascular diseases with a 2-fold higher risk of cardiovascular events in people with diabetes compared with those without. Circulating monocytes are inflammatory effector cells involved in both type 2 diabetes (T2D) and atherogenesis.

METHODS: We investigated the relationship between circulating monocytes and cardiovascular risk progression in people with T2D, using phenotypic, transcriptomic, and metabolomic analyses. cardiovascular risk progression was estimated with coronary artery calcium score in a cohort of 672 people with T2D.

RESULTS: Coronary artery calcium score was positively correlated with blood monocyte count and frequency of the classical monocyte subtype. Unsupervised k-means clustering based on monocyte subtype profiles revealed 3 main endotypes of people with T2D at varying risk of cardiovascular events. These observations were confirmed in a validation cohort of 279 T2D participants. The predictive association between monocyte count and major adverse cardiovascular events was validated through an independent prospective cohort of 757 patients with T2D. Integration of monocyte transcriptome analyses and plasma metabolomes showed a disruption of mitochondrial pathways (tricarboxylic acid cycle, oxidative phosphorylation pathway) that underlined a proatherogenic phenotype.

CONCLUSIONS: In this study, we provide evidence that frequency and monocyte phenotypic profile are closely linked to cardiovascular risk in patients with T2D. The assessment of monocyte frequency and count is a valuable predictive marker for risk of cardiovascular events in patients with T2D.

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Responsible for 16% of globally recorded deaths, ischemic heart disease is the leading cause of death worldwide.[1] Ischemic heart diseases are linked to atherogenesis and its progression in coronary arteries. Atherosclerosis initiation and progression is mainly due to circulating monocytes being recruited to the subendothelial space of coronary arteries, where they differentiate into macrophages.[2] These macrophages become foam cells by capturing oxLDL (oxidized-low density lipoprotein) via scavenger receptors and produce inflammatory

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Novelty and Significance

What Is Known?

- Diabetes is a major risk factor for atherosclerotic cardiovascular diseases.
- However, there is cardiovascular risk heterogeneity among patients with type 2 diabetes (T2D) that cardiovascular risk scores fail to detect.
- None of these cardiovascular risk scores take into account proatherogenic immune effector, monocyte blood count.

What new information does this article contribute?

- Blood monocyte frequency can identify subgroup of T2D with higher cardiovascular risk
- Blood monocyte count is predictive of major adverse cardiovascular event among T2D population.
- Metabolic rewiring of monocytes is mediated by alteration of mitochondrial activity.

Diabetes is one of the major risk factors for atherosclerotic cardiovascular diseases with a 2-fold higher risk of cardiovascular events in patients with T2D, compared with those without. However, the cardiovascular risk heterogeneity within this population, is not captured by the diagnostic scores that perform poorly for this population. Noticeably, no cardiovascular risk scores include circulating immune cell population counts, despite atherosclerosis being an inflammatory disease. Monocytes, inflammatory effector cells both involved in T2D and atherosclerosis pathophysiology, could be a target to improve cardiovascular risk scores in patients with T2D. In a cross-sectional study population of 672 participants with T2D, we investigated the relationship between circulating monocytes and cardiovascular risk progression in people with T2D. We demonstrate that blood monocyte count is predictive of major adverse cardiovascular event among the T2D population and identified a proatherogenic phenotype of monocytes positively associated the risk of cardiovascular events. Altogether, our results provide evidence that the assessment of monocyte frequency and count is a valuable predictive marker for risk of cardiovascular events in patients with T2D.

Nonstandard Abbreviations and Acronyms

CAC	coronary artery calcium
MACE	major adverse cardiovascular event
OXPHOS	oxidative phosphorylation
PBMC	peripheral blood mononuclear cell
T2D	type 2 diabetes
TCA	tricarboxylic acid cycle
tWGCNA	transcriptomic weighted gene correlation network analysis

cytokines. Inflammatory cytokines and matrix metallopeptidases (enzymes capable of degrading extracellular matrix) produced by monocytes and macrophages further amplify endothelial activation.[3,4] This increases recruitment and adhesion of more immune cells, including monocytes, simultaneously enlarging and destabilizing the plaque.[3,4] Circulating monocytes are subdivided into 3 main subtypes: classical, intermediate, and nonclassical,[5] and an increase of specific subtypes has been associated with plaque vulnerability.[6] This indicates potentially different roles in atherogenesis between these subsets. Diabetes is one of the major risk factors for atherosclerotic cardiovascular diseases with a 2-fold higher risk of cardiovascular events in patients with type 2 diabetes (T2D), compared with those without.[7–9] Nevertheless, cardiovascular risk assessment in the T2D population, through cross-sectional studies based on coronary artery calcium (CAC) scoring, showed risk heterogeneity within this population.[10,11] This heterogeneity could be explained by differences in other concomitant clinical risk factors and their management (sex, age, dyslipidemia, high blood pressure, smoking, etc). Although algorithms have been developed to take into account multiple clinical variables, including those specific to T2D (diabetes duration, glycated hemoglobin), the cardiovascular risk scores they derive perform poorly.[12] This reflects the lack of effective diagnostic scores specifically tailored to individuals with T2D. Furthermore, many cardiovascular risk scores take into account the traditional risk factors, but only 1 score includes total white blood cell counts and none include circulating immune cell population counts, despite atherosclerosis being an inflammatory disease.[13–20] As inflammatory effector cells, specifically monocytes, are both involved in T2D and atherosclerosis pathophysiology,[21–24] they could be considered valuable target variables to improve the scores that evaluate cardiovascular risk in patients with T2D. This could also address previously uninterpretable heterogeneity among patients with T2D. In a cross-sectional study population of 672 participants with T2D, we demonstrated that the risk of cardiovascular events, evaluated by CAC score, is positively associated with blood leukocyte and monocyte counts. Then, using the 3 monocyte subtypes frequencies, we proposed 3 endotypes of participants with T2D differing in blood monocyte counts, classical monocyte frequency (CD45+ CD14++ CD16−), and the risk of cardiovascular events. The predictive association between monocyte count and major adverse cardiovascular events (MACEs) was validated through an independent prospective T2D cohort. Analyses of monocyte transcriptome, mitochondrial status, and blood metabolome suggested that amino acid metabolism could trigger the monocyte switch toward proatherogenic activity. Complementary analysis of the monocyte transcriptome also revealed deregulated genes involved in matrix remodeling and mitochondrial function. Altogether, our results provide evidence that a specific switch of monocyte phenotypic profile is closely linked to cardiovascular risk in patients with T2D.

METHODS

Data Availability

RNA sequencing (RNAseq) data are available from the NCBI Gene Expression Omnibus with GEO accession number GSE201087.

Patient Recruitment

We included patients from the AngioSafe-2 cohort named discovery cohort (ClinicalTrials.gov Identifier: NCT02671864), further detailed in the Supplemental Material.

We used data from the ongoing prospective GLUTADIAB cohort named validation cohort (ClinicalTrials.gov Identifier: NCT04353869), described in the Supplemental Material. We included 279 patients with type 2 diabetes, without cardiovascular disease, with both immunophenotyping through flow cytometry and CAC score available.

We used data from the prospective T2D (n=1042) monocenter SURDIAGENE cohort[25] detailed in Supplemental Material, to validate the link between monocytes and cardiovascular risk. The primary end point was a composite of cardiovascular death, nonfatal myocardial infarction, and nonfatal stroke (MACE)[26]; the assessment of each end point is further described in the Supplemental Methods.

CAC Score

We stratified the 672 T2D participants according to their cardiovascular risk assessed by CAC score, a widely used predictor of cardiovascular risk: very low with CAC score=0 (CV0 group), low with CAC score between 1 and 100 (CV1 group), intermediate with CAC score between 101 and 400 (CV2 group), and high with CAC score > 400 (CV3 group).

Extracellular Flux Measurements

Whole blood samples from random patients were obtained from the French Blood Establishment (Etablissement Français du Sang [EFS]). The specific steps for peripheral blood mononuclear cell (PBMC) preparation are detailed in the Supplemental Methods. Real-time extracellular acidification rate and oxygen consumption rate were measured on the PBMCs using Seahorse XFe96 extracellular flux analyzer (Agilent), further detailed in the Supplemental Methods.

RNA Sequencing

CD14+ immunoselection for subsequent RNAseq was detailed in the Supplemental Methods. We exclusively recruited male subjects for the RNAseq analysis to avoid hormonal biases and focus on gene expression modulation linked to cardiovascular risk.[27] Seventy-eight RNAseq samples were sequenced in 2 batches, with the specific sequencing protocol detailed in the Supplemental Methods. The processing steps detailed in Supplemental Methods were then identical for all batches. Normalization and differential gene expression analyses were performed with R package DESeq2 v1.32.0, including batch in the design formula, and Benjamini-Hochberg correction was applied with a threshold for significance set at adjusted $P<0.05$.

Bioinformatics Analysis

The weighted gene correlation network analysis pathway enrichment analysis and statistical analyses were all detailed in the Supplemental Methods.

RESULTS

Increased Classical Monocyte Frequency Identifies High Cardiovascular Risk Individuals among People With T2D

Our first aim was to evaluate the relationship between monocyte count and cardiovascular risk quantified by CAC score, a clinical test that reflects coronary calcification, in 672 participants with T2D from the Angiosafe-2 cohort.[28] In this cohort, 60% were males, the median age was 61 years, and body mass index 28.4 kg/m^2, with an average diabetes duration of 13 years and a mean glycated hemoglobin of 7.6% (clinical characteristics in Table S1 and Figure S1A and S1B). Correlative analyses revealed that CAC score distribution was positively correlated with monocyte and leukocyte counts (Spearman correlation coefficient rho=0.16, $P<0.001$), in addition to age and diabetes duration (Figure 1A; Figure S1C). Correlation between CAC score and monocyte counts was near significant when age was taken into account (Spearman coefficient partial correlation controlling $P=0.057$). Stratification of participants with T2D into 4 groups ranging from CV0 to CV3 (increasing cardiovascular risk) based on their CAC score (Table 1; Figure S1D) confirmed the gradual increase of both monocytes ($P=2.3\times10^{-3}$) and leukocytes ($P=1.0\times10^{-2}$) when CAC score increases (Figure 1B). To confirm this link between monocytes and cardiovascular risk among people with T2D, we compared the blood leukocyte and monocyte counts between 1041 participants with T2D

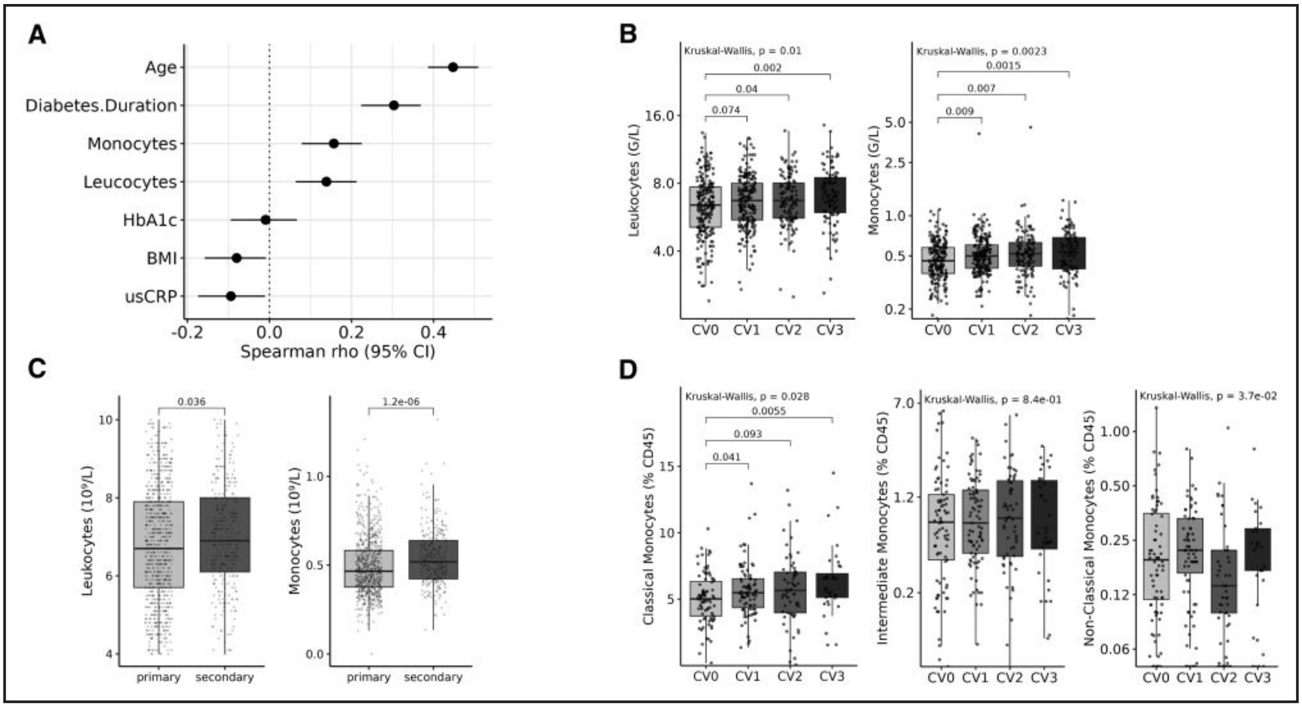

Figure 1. Monocyte subtypes frequencies determine groups with different cardiovascular risk.
A, Forest plot of Spearman correlation between coronary artery calcium (CAC) score and cardiovascular (CV) risk factors from 672 type 2 diabetes (T2D) participants (population 1). **B**, Box plots of circulating monocytes and leukocytes blood counts per CV risk group defined by participants' CAC score in 672 T2D participants (population 1), Kruskal-Wallis test with post hoc comparisons. **C**, Box plot of circulating monocytes and leukocytes blood counts of SURDIAGENE (Survival Diabetes and Genetics) T2D population (n=1041) subdivided in primary prevention (n=757) vs secondary prevention major adverse cardiovascular event groups (n=284). Mann-Whitney test. **D**, Box plots of monocyte subtypes flow cytometry percentages per CV risk group defined by participants' CAC score in 266 T2D participants (population 2), Kruskal-Wallis test with post hoc comparisons.

of the SURDIAGENE cohort (Table 2) in either primary or secondary cardiovascular prevention. Interestingly, we observed significantly higher counts of both circulating monocytes and leukocytes in the secondary cardiovascular prevention group, known to be at higher cardiovascular risk compared with the participants with T2D in the

Table 1. Clinical Cardiovascular Risk Factors of Individuals Included in the Correlation Analysis and Grouped According to Their CAC Score–Based Cardiovascular Risk Groups (With CV0: CAC Score=0, CV1: CAC Score [1–100], CV2: [101–400], and CV3: CAC Score >400)

Characteristic	CV0 N=219 (33%)	CV1 N=222 (33%)	CV2 N=128 (19%)	CV3 N=103 (15%)	*P* value
Sex					8.72×10^{-7}*
Female	113 (52%)	94 (42%)	36 (28%)	25 (24%)	
Male	106 (48%)	128 (58%)	92 (72%)	78 (76%)	
Age, y	56 (51–63)	61 (56–67)	66 (59–71)	67 (61–73)	1.45×10^{-27}*
BMI, kg/m²	28.9 (25.7–32.4)	28.2 (24.8–31.4)	28.0 (25.5–30.9)	27.7 (25.0–31.4)	2.21×10^{-1}
Dyslipidemia	112 (51%)	139 (63%)	91 (71%)	85 (83%)	2.19×10^{-7}*
Coronary calcium score	0 (0–0)	20 (5–52)	183 (129 270)	900 (600–1352)	4.25×10^{-137}*
HbA1c (%)	7.51 (6.80–8.60)	7.60 (6.80–8.40)	7.30 (6.80–8.35)	7.70 (7.00–8.30)	4.07×10^{-1}
Diabetes duration, y	10 (5–15)	14 (8–20)	16 (10–21)	17 (12–26)	3.75×10^{-14}*
Age at diagnosis, y	45 (39–52)	46 (40–53)	50 (41–56)	47 (40–57)	1.48×10^{-2}*
CRP, mg/L	1.91 (0.90–4.16)	1.37 (0.69–3.56)	1.27 (0.60–2.94)	1.53 (0.76–3.21)	5.60×10^{-2}
Leukocytes, /mL	6.40 (5.10–7.70)	6.80 (5.50–8.00)	6.70 (5.60–8.03)	7.10 (5.95–8.50)	9.95×10^{-3}*
Monocytes, /mL	0.46 (0.37–0.58)	0.50 (0.41–0.61)	0.52 (0.42–0.63)	0.54 (0.40–0.69)	2.60×10^{-3}*

n (%) with χ^2 test, or median (IQR) with Kruskal-Wallis test. BMI indicates body mass index; CAC, coronary artery calcium; CRP, C reactive protein; IQR, interquartile range; and HbA1c, glycated hemoglobin.
*Significant difference between groups.

Table 2. Clinical Characteristics of SURDIAGENE Population

	Primary cardiovascular prevention (n=757)	Secondary cardiovascular prevention (n=284)	*P* value
Men, n (%)	399 (53%)	206 (73%)	<0.0001*
Age, y	63+11	69+9	<0.0001*
BMI, kg/m²	32+6	30+5	<0.0001*
HbA1c (%)	7.8+1.7	7.6+1.3	0.0319*
Diabetes duration, y	13+9	16+10	<0.0001*
Leukocytes, 10%/L	6.8+1.4	7.0+1.3	0.0339*
Monocytes, 10%/L	0.49+0.16	0.54+0.16	<0.0001*

n (%) with χ^2 test, or mean (IQR) with Student test. BMI indicates body mass index; HbA1c glycated hemoglobin; and SURDIAGENE, Survival Diabetes and Genetics study.

primary prevention group (Figure 1C). Taken together, our observations suggest that monocyte count could be a cardiovascular risk marker in populations with T2D. Blood monocytes are composed of 3 subtypes based on the variable expression of surface markers: classical (CD45+ CD14++ CD16–), intermediate (CD45+ CD14++ CD16+), and nonclassical (CD45+ CD14+ CD16++). To evaluate the monocyte subtype distribution related to cardiovascular risk, we performed blood monocyte subtyping by flow cytometry in 266 participants with T2D of the AngioSafe-2 cohort (population 2, Figure S1A and S1B; Table S1). We found a significant increase (P=2.8×10^{-2}) of classical monocytes in higher cardiovascular risk groups (Figure 1D) compared with the lowest cardiovascular risk group (CV0). In contrast, we did not observe similar changes in the frequencies of intermediate (P=8.4×10^{-1}) or nonclassical (P=3.7×10^{-2}) monocytes, between cardiovascular risk groups (Figure 1D).

Blood Monocyte Count and Classical Monocyte Frequency Predict Cardiovascular Events in T2D

As we observed a difference in monocyte subtype distribution between groups at different cardiovascular risk categories, we performed unsupervised K-means clustering using the frequency of monocyte subpopulations obtained by flow cytometry from the 266 participants (Figure 2A). Our approach identified 3 clusters of participants (Figure 2A): 147 participants (55%) in the main cluster (clust.1), 87 participants (33%) in clust.2, characterized by a higher percentage of classical monocytes (Figure 2B), and 32 participants (12%) in clust.3, characterized by a higher percentage of intermediate and nonclassical monocytes (Figure 2B and clinical characteristics in Table 3). Our unsupervised analyses revealed that participants from cluster 2 are characterized by (1) a higher proportion of classical monocytes (Figure 2B), (2) higher counts of blood monocytes (median=0.6×10×10^9/mL, Kruskal-Wallis P<0.001, Figure 2C; Table 3), (3) a higher CAC score (Kruskal-Wallis P=0.004; Table 3), and (4) with a greater proportion of individuals in the CV3 risk category (Figure 2D). CAC score differences persisted after age and sex adjustments (P=0.048 after sex and age adjustments).

Importantly, these results were confirmed in an independent validation cohort of 279 patients with T2D for which we had fluorescence-activated cell sorting data and CAC score (GlutaDiab cohort). By projecting these patients onto the previous clustering, we confirmed higher cardiovascular risk in cluster 2 (Figure 2A through 2D), with a higher CAC score in cluster 2 compared with cluster 1 (median, 42 [interquartile range, 0–402] versus 7 [0–98]; P=0.019). Thus, we provide evidence, using complementary approaches, that blood monocyte count and frequency of classical monocytes are associated with the increased risk of cardiovascular events in a T2D population.

To assess the association between circulating monocyte count and MACE among individuals with T2D, 757 primary cardiovascular prevention participants with T2D of the SURDIAGENE cohort (Table 2) were divided into quartiles based on baseline monocyte counts (quartile 1 [0–0.4], to quartile 2 [0.4–0.5], to quartile 3 [0.5–0.6] and quartile 4 [0.6–1.2] per 10^9/L). We compared the cumulative incidence of both MACE (n=269, 3.55 per 100 person year) and cardiovascular death (n=196, 2.55 per 100 person year) between quartiles. Interestingly, participants with above-median monocyte count (0.5×10^9/L, that is, Q3 and Q4) suffered significantly more MACE (P log-rank <0.001) and cardiovascular deaths (P log-rank <0.001) than individuals below the median (ie, Q1 and Q2), as is shown in the Kaplan-Meier curves (Figure 2E). Furthermore, adjusted Cox analysis with cardiovascular risk factors (age, sex, NT-proBNP levels, estimated glomerular filtration rate [eGFR], urine albumin-creatinine ratio [uACR], and glycated hemoglobin) confirmed that a monocyte count increase was associated with a statistically significant 2.8-fold increase of MACE and a 5.1-fold increased risk of cardiovascular deaths (P=0.03 and P=0.003, respectively, Table 4). Of note, we observed no such association between leukocyte blood levels and MACE or cardiovascular death (p log-rank = 0.0112 and 0.166, respectively; Figure S2). Interestingly, in the cross-sectional study, 71% of participants belonging to cluster 2 have above-median monocyte counts (qHigh = Q3 and Q4), compared with only 40% of cluster 1 participants (Figure 2F). Applying quartile stratification in the Angiosafe-2 cohort (n=266, Population 2) revealed that people belonging to Q4 [0.6-1.2 per 10^9/L] had enriched classical monocytes compared with other quartiles while we did not observe statistically significant differences for nonclassical and intermediate monocytes (Figure 2G).

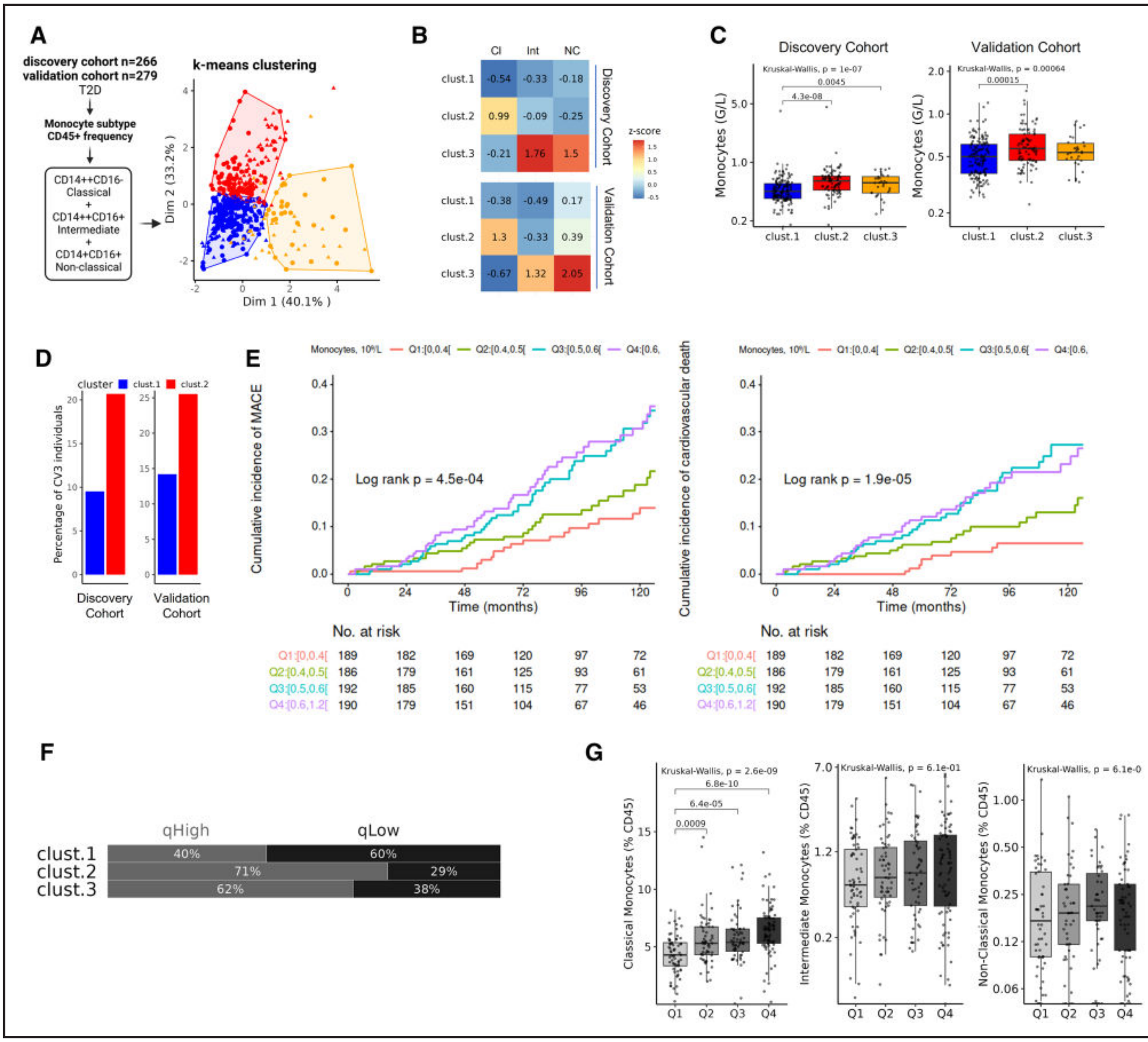

Figure 2. Clustering analysis reveals groups of type 2 diabetes (T2D) individuals with different monocyte subtype distribution and distinct cardiovascular (CV) risk.

A, Clustering workflow and principal component analysis of the T2D participants (n=266, population 2, discovery cohort) clustering based on circulating monocyte subpopulation percentages among CD45+ cells, with 3 k-means-defined clusters. Projection of 279 individuals from the validation cohort (n=279, triangles). **B**, Heatmap of the monocyte subtypes distribution among the T2D individuals in the discovery (n=266, population 2, top) and the validation (n=279, bottom) cohorts. **C**, Boxplot of the log2 values of monocyte blood levels among the T2D individuals (n=266, population 2) of each cluster (with n=78, 141, and 33, in cluster 1, 2, and 3, respectively), Kruskal-Wallis test. **Left**, discovery cohort. **Right**, validation cohort. **D**, Bar plot illustrating the percentage of T2D belonging to cluster 1 or cluster 2 individuals that have a coronary artery calcium (CAC) score >400 (CV3 group) in the discovery (left) and validation (right) cohorts. **E**, Kaplan-Meier curves of cumulative incidence of major adverse cardiovascular event (MACE) or CV death for each quartile of monocyte blood levels (10×10^{9}/L), in the SURDIAGENE cohort of 757 T2D individuals in primary prevention. **F**, Distribution in percentage of MACE-defined high (Q4+Q3) or low (Q1+Q2) CV risk quartiles of monocytes per cluster in 266 T2D participants (population 2). **G**, Box plot of monocyte subtypes flow cytometry percentages per MACE-defined monocyte quartile in 266 T2D participants (population 2), Kruskal-Wallis test with post hoc comparisons.

TCA Cycle and Mitochondrial Oxidative Pathways Are Deregulated in Classical Monocytes of Participants With T2D and High Cardiovascular Risk

To better characterize monocyte phenotype and identify pathways that may influence monocyte subset proportions linked with cardiovascular risk heterogeneity, we analyzed CD14+ monocyte transcriptomes by RNA-seq in a subset of 78 participants with T2D (Figure 3A and clinical characteristics in Table S1). First, we performed a weighted gene correlation network analysis (Figure 3B) to identify modules of coregulated genes associated with phenotypic clusters, monocyte count, monocyte subtype

Table 3. Clinical Cardiovascular Risk Factors Differences Between Individuals of Each Cluster

Characteristic	clust.1 N=147 (55%)	clust.2 N=87 (33%)	clust.3 N=32 (12%)	*P* value
Sex				0.001*
Female	59 (40%)	16 (18%)	7 (22%)	
Male	88 (60%)	71 (82%)	25 (78%)	
Age, y	60 (55–67)	65 (58–69)	61 (53–69)	0.013*
BMI, kg/m^2	28.3 (25.3–31.7)	26.9 (25.4–30.5)	29.6 (25.4–33.3)	0.26
Dyslipidemia	106 (72%)	61 (70%)	23 (72%)	0.95
Coronary calcium score	8 (0–131)	97 (3325)	52 (0–134)	0.004*
HbA1c, %	7.70 (7.00–8.60)	7.60 (6.75–8.30)	7.45 (6.97–8.33)	0.34
Diabetes duration, y	13 (8–20)	15 (9–20)	12 (7–18)	0.30
Age at diagnosis, y	45 (40–52)	46 (42–55)	45 (41–55)	0.45
CRP, mg/L	1.47 (0.78–3.44)	0.95 (0.54–2.49)	1.81 (0.64–2.86)	0.028*
Leukocytes, /mL	6.80 (5.60–7.80)	6.60 (5.40–7.80)	6.95 (5.20–8.03)	0.88
Monocytes, /mL	0.45 (0.37–0.56)	0.60 (0.46–0.69)	0.57 (0.43–0.68)	<0.001*

The statistical differences between each cluster were calculated by Kruskal-Wallis test and are considered statistically significant when the $P<0.05$. n (%) with χ^2 test, or median (IQR) with Kruskal-Wallis test. BMI indicates body mass index; CRP, C reactive protein; IQR, interquartile range; and HbA1c, glycated hemoglobin.

frequencies, CAC score, and cardiovascular risk groups. Our approach revealed 12 coexpressed gene modules (Figure S3A). Gene expression of each transcriptome weighted gene correlation network analysis module (tWGCNA) was summarized by a surrogate eigengene for all genes belonging to the module for each participant (Figure S3A). Two main modules (tWGCNA-1 with 751 coexpressed genes and tWGCNA-2 with 295 coexpressed genes) were significantly associated with cluster type (P=0.001 and 0.01, respectively, Figure 3B). Interestingly, tWGCNA-1 was also significantly associated with classical monocyte frequencies (P=0.05) and CV3 or CV0 risk group (P=0.01, Figure 3B). Functional over-representation analysis revealed that the gene network belonging to this tWGCNA-1 module is involved in controlling mitochondrial respiration (tricarboxylic acid cycle [TCA] cycle $P<1\times10^{-6}$; oxidative phosphorylation $P<1\times10^{-6}$; Figure 3B; Figure S3B). To associate a specific monocyte transcriptome signature to the high cardiovascular risk in participants from clust.2, we compared their transcriptomes to those derived from clust.1 (lowest median CAC score), with matching for sex and age (clinical characteristics in clinical Table S2). This analysis revealed 2529 differentially expressed genes (adjusted $P<0.05$), with 1343 upregulated and 1186 downregulated genes in clust.2 (Figure 3C). Pathway enrichment analysis of the differentially expressed genes displayed significant enrichment of genes involved in epigenetic pathways such as histone modifications (adjusted $P=1.4\times10^{-12}$), and Chromatin Organization (adjusted $P<1.0\times10^{-12}$, details of the over-representation analysis in Figure S3C). Noticeably, both the TCA cycle and oxidative phosphorylation (OXPHOS) pathways were also enriched (gene set enrichment analysis; adjusted $P=2.06\times10^{-14}$ and 8.37×10^{-11}, respectively; Figure S3C), and mainly composed of downregulated genes in cluster 2 (Figure 3C; Figure S3C). To identify genes specifically linked to increased coronary artery calcification, we performed differential gene expression analysis between groups with the lowest (CV0) and the highest (CV3) CAC score. By comparing CV0 (n=21) and CV3 (n=16) groups (clinical characteristics in Table S3), 315 genes were significantly differentially expressed (adjusted $P<0.05$; Figure 3D), with 245 upregulated and 70 downregulated genes in the CV3 group. It should be noted that although participants belonging to cluster 2 have a higher CAC score compared with the other clusters, only 21% are also part of CV3, thus the individuals from the clustering and the CAC score analysis are not identical. Plasma membrane organization, glycerolipid metabolism, and glycerophospholipid metabolism were the top over-represented pathways (Figure 3E; Figure S3D). The enrichment of these lipid-associated pathways was independent of the presence of dyslipidemia (glycerolipid P=3.6e-04 and glycerophospholipid P=3.1e-03 after adjustment). In addition, a decreased expression of genes involved in the OXPHOS pathway, glutathione metabolism, TCA cycle, and fatty acid metabolism was observed in the highest cardiovascular risk group (CV3) according to CAC score (Figure 3E). Of note, age adjustment in our observations (Figure S3E) and detailed over-representation analysis results in Figure S3F. Altogether, our transcriptomic analyses revealed that deregulation of cellular metabolism, including unsaturated fatty acids, glutathione, TCA cycle, and OXPHOS pathways, contributes to the phenotypic reprogramming of blood monocytes that is associated with cardiovascular risk (Figure 3F and 3G). Furthermore, downregulation of these mitochondrial pathways is observed when CAC score increases as well as between clusters (Figure 3G). This implicates 4 out of 5 of the mitochondrial complexes and impacts a subset of genes that regulate the TCA cycle (Figure 3H and 3I). To confirm these transcriptomic results, we analyzed the metabolic flux in PBMCs of 34 participants with T2D from the CV0 or CV3 groups. There was no statistically significant difference in the levels of nonmonocyte cell types including lymphocytes and neutrophils between CV0 and CV3 groups (Figure S4A). We found that individuals from the CV3 group (n=26) had a significantly lower oxygen consumption rate compared with those from the CV0 group (n=8; Figure 4A). Levels of basal and maximal mitochondrial respiration as well as ATP production are reduced in the PBMCs of participants

Table 4. Cox Analysis of Monocyte Association With MACE and CV Death in SURDIAGNE T2D Cohort

	Univariate			Age-sex			Multivariate*		
	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
MACE (158 events)									
Monocytes (+1%)	1.1	(1.0:1.2)	8.7×10^{-4}*	1.0	(1.0–1.1)	0.296	1.0	(0.9–1.1)	0.931
Monocytes (%)			0.004*			0.535			0.626
Ref. Q1: (0–5.9)									
Q2: (5.9–7.0)	1.7	(1.0–2.8)		1.3	(0.8–2.1)		1.4	(0.8–2.3)	
Q3: (7.0–8.5)	1.6	(1.0–2.6)		1.3	(0.8–2.1)		1.1	(0.7–1.9)	
Q4: (8.5–18.1)	2.4	(1.5–3.7)		1.4	(0.9–2.4)		1.3	(0.8–2.2)	
Monocytes (+10%/L)	6.8	(3.0–15.7)	6.4×10^{-6}*	3.7	(1.5–9.1)	0.004*	2.8	(1.1–7.1)	0.030*
Monocytes (10%/L)									
Ref. Q1: (0–0.4)			0.001*			0.067			0.136
Q3: (0.5–0.6)	2.1	(13–3.4)		1.8	(1.1–2.9)		1.8	(1.0–3.0)	
Q4: (0.6–1.2)	2.6	(1.6–4.1)		1.9	(1.1:3.0)		1.8	(1.1–3.0)	
CV death (109 events)									
Monocytes (+1%)	1.2	(1.1–13)	4.5×10^{-5}*	1.1	(1.0–1.2)	0.033*	1.0	(1.0–1.2)	0.331
Monocytes (%)									
Ref. Q1: (0–5.9)			0.001*			0.214			0.541
Q2: (5.9–7.0)	2.0	(1.1–3.8)		1.4	(0.7–2.7)		1.5	(0.7–2.8)	
Q3: (7.0–8.5)	1.9	(1.0–3.5)		1.5	(0.8–2.9)		1.2	(0.6–2.4)	
Q4: (8.5–18.1)	3.2	(1.8–5.7)		1.9	(1.0–3.6)		1.5	(0.8–3.0)	
Monocytes (+10//L)	12.6	(4.9–32.9)	2×10^{-7}*	7.6	(2.7–21.6)	1.4×10^{-4}*	5.1	(1.7–15.2)	0.003*
Monocytes (10%/L)									
Ref. Q1: (0–0.4)			8.4×10^{-5}*			0.005*			0.014*
Q2: (0.4–0.5)	2.5	(1.2–53)		2.3	(1.1–4.7)		3.1	(1.4–7.0)	
Q3: (0.5–0.6)	4.1	(2.0–8.2)		3.3	(1.6–6.6)		3.5	(1.6–7.8)	
Q4: (0.6–1.2)	4.6	(2.3–9.2)		3.3	(1.6–6.7)		3.4	(1.5–7.5)	

CV indicates cardiovascular; HbA1c, glycated hemoglobin; MACE, major adverse cardiovascular event; NTpro-BNP, natriuretic peptide; SURDIAGNE, Survival Diabetes and Genetics; and T2D, type 2 diabetes.

*Adjusted for age, sex, NTpro-BNP, HbA1c, estimated glomerular filtration rate, and urine albumin-to-creatinine ratio.

from the CV3 group indicating decreased mitochondrial respiration (Figure 4A). Quantification of extracellular acidification rate revealed that the glycolytic capacity (and reserve) is also significantly reduced in PBMCs of participants with T2D from the CV3 group (Figure 4B). Integration of basal and maximal oxygen consumption rate and extracellular acidification rate levels revealed an overall suppression of respiration in PBMCs from participants of the CV3 group compared with the CV0 group (Figure 4C). Additionally, we measured the mitochondrial mass in the blood monocytes using mitotracker assay in the same population. We found a decrease in the mitochondrial mass of CD14+ monocytes in participants with T2D belonging to the CV3 high cardiovascular risk group (Figure 4D). We also observed a significant decrease of mitochondrial mass and activity in both total blood monocytes and classical monocytes of high cardiovascular risk participants (high level of monocytes, high CAC score, and belonging to clust.2 compared with clust.1 with low cardiovascular risk, low level of blood monocytes, and low CAC score (Figure 4E). This decrease in mitochondrial mass and activity was only observed in classical monocytes (whose RNAseq data were analyzed) and not in CD16+ cells including intermediate and nonclassical monocytes (Figure S4B).

Interestingly, plasma metabolomes of participants with T2D (clinical characteristics in Table S1) from high cardiovascular risk were characterized by an accumulation of specific metabolites that indicate the TCA cycle may be perturbed, as well as amino acid metabolism, for example, accumulation of tryptophan, alanine, aspartate, and glutamate; and this compared with participants with low cardiovascular risk (Figure 4F; Figure S4C through S4E). These results may reflect a contribution of amino acid metabolism in the monocyte phenotype associated with cardiovascular risk increase. Hence, the monocyte transcriptome and more specifically the mitochondrial metabolism genes downregulated in the high cardiovascular risk group (CV3) could be a consequence of alterations in plasma metabolome (Figure 4G). To test this hypothesis, we incubated PBMCs of a healthy donor with serum of either high cardiovascular risk (high level

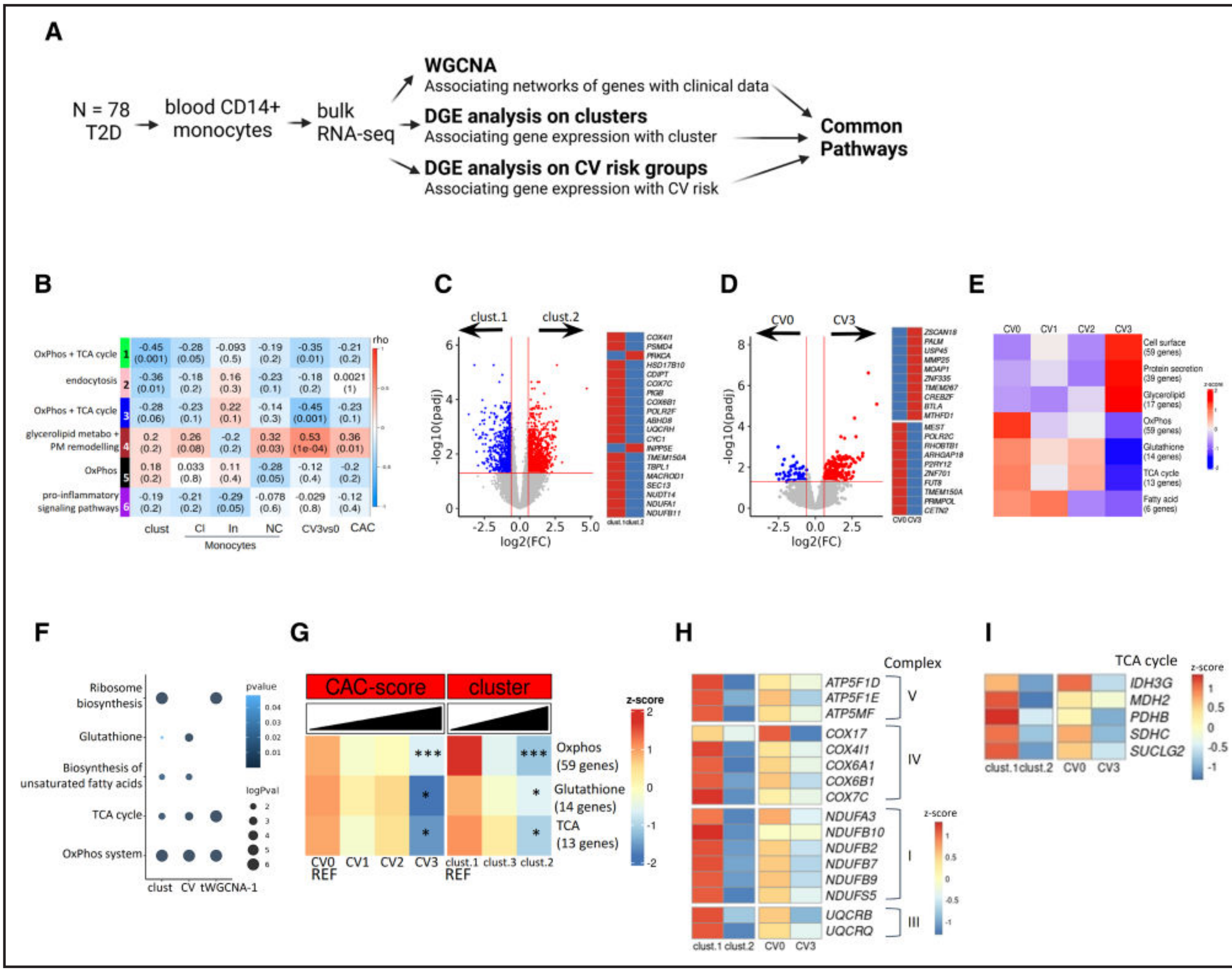

Figure 3. Monocyte transcriptome is linked to clusters and cardiovascular (CV) risk in patients with type 2 diabetes (T2D), and integration reveals possible mitochondrial dysfunction.

A, Schematic of the different transcriptomic analysis performed in 78 male T2D individuals (population 3). B, Weighted gene coexpression network analysis (WGCNA) on the RNA sequencing (RNA-seq) data of 78 male participants with T2D. Heat map representation of the correlation between modules of genes with similar expression patterns across individuals and clinical traits (first line rho values, second line *P* value). **C**, Volcano plot of the differential gene expression analysis between 19 participants belonging to cluster 1 and 27 participants from cluster 2 (population 3). The top 20 most significant upregulated (in red) or downregulated (blue) genes of the tricarboxylic acid cycle (TCA)-oxidative phosphorylation (OXPHOS) pathways in clust.2 are represented on the heat map. **D**, **Left**, Volcano plot of the differentially expressed genes (DEG) between the 16 CV3 (CAC score >400) and the 21 CV0 (CAC score=0) male participants with T2D. Downregulated genes in CV3 compared with CV0 in blue and upregulated genes in red. **Right**, Heat map of the standardized mean expression of the top 10 CV3 genes, and the top 10 CV0 genes. **E**, Heat map of the mean expression per CV risk group of the DEG contributing to the over-represented pathways significantly enriched among the differentially expressed genes between CV3 vs CV0. **F**, Bubble plot of the *P* value of the over-represented and common pathways of the 3 transcriptomic analyses. The size is proportional to the −log10 transformation of the pathway enrichment *P* value, and the color reflects the *P* value. **G**, Heat map of the mean expression per CV risk group and cluster of the leading-edge genes contributing to the enrichment of the significant pathways in the Gene Set Enrichment Analysis (GSEA) for both cluster 2 vs 1 and CV3 vs CV0. *q-value ≤0.05, ***: q-value ≤0.001. **H**, Heat map of the 16 coregulated genes among the transcriptomic weighted gene correlation network analysis (tWGCNA)-1 network (**B** module 1 green), which are part of the oxidative phosphorylation mitochondrial pathway, and that are in common between the differential gene expression (DGE) analyses: cluster 2 vs cluster 1 and CV3 vs CV0 adjusted to age, classified by mitochondrial complex. **I**, Heat map of the 5 TCA cycle genes, among the tWGCNA-1 network (module 1 green, **B**), and that are in common between the DGE analyses: cluster 2 vs cluster 1 and CV3 vs CV0 adjusted to age.

of monocytes, high CAC score [CV3 group] and belonging to cluster 2, n=6) or low cardiovascular risk (low level of blood monocytes, low CAC score [CV0 group] and belonging to cluster 1, n=6) participants with T2D for 6 hours and then measured the frequency of classical monocytes and mitochondrial mass and activity of these circulating monocytes. In line with other results, we found that after incubation in the serum of high cardiovascular risk individuals, the percentage of classical monocytes significantly increased compared with the monocytes incubated with the serum of low cardiovascular risk participants (Figure 4H). Interestingly, this increase in

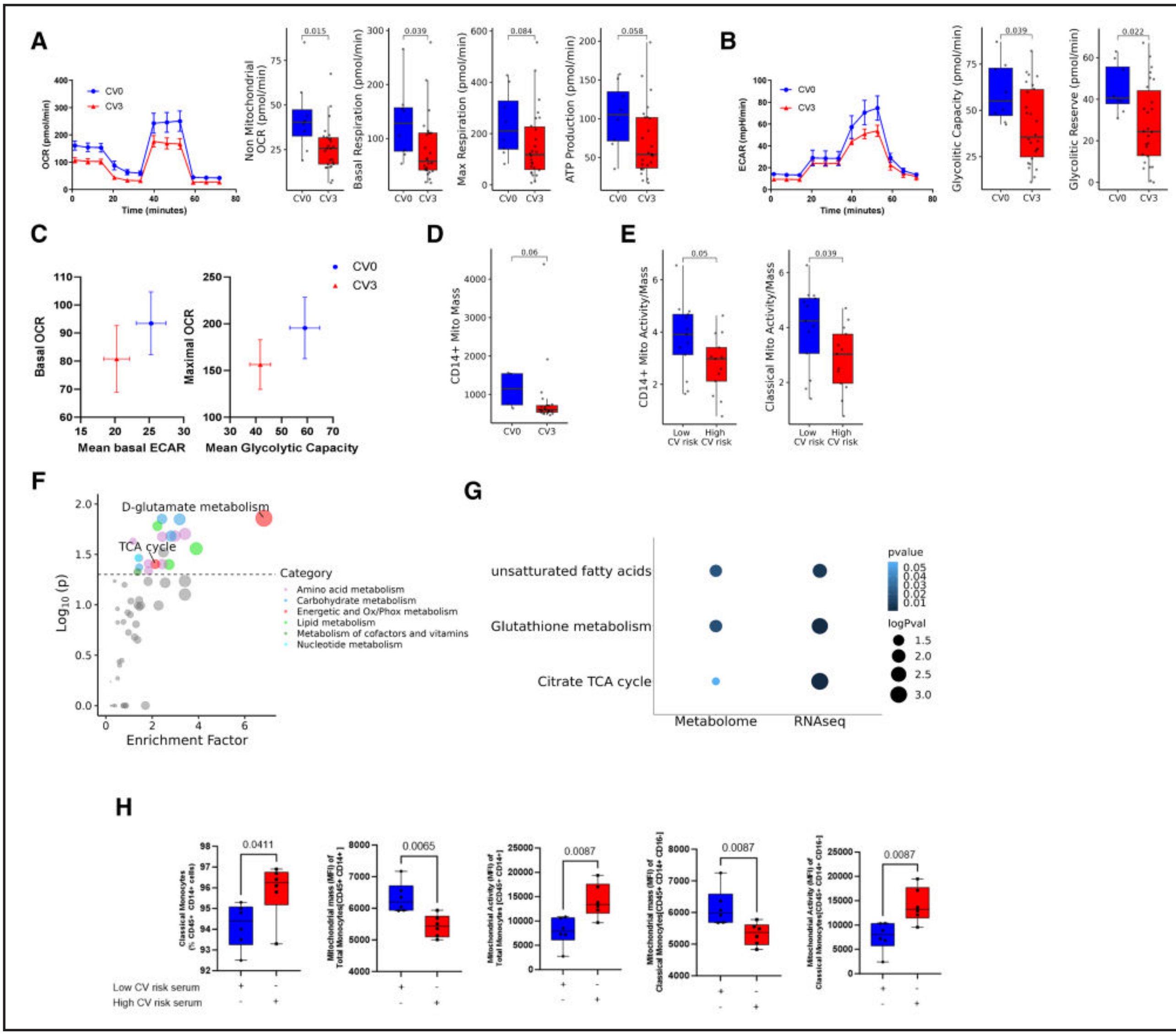

Figure 4. Metabolomic analysis reveals possible link between blood metabolome and cardiovascular (CV) risk-associated mitochondrial dysfunction.

A, **Left**, oxygen consumption rate (OCR) Seahorse profiles for the peripheral blood mononuclear cells (PBMCs) of 26 high (CV3) vs 8 low CV (CV0) risk patients with type 2 diabetes (T2D). **Right**, Boxplots of nonmitochondrial oxygen consumption, basal respiration, maximal respiration, and ATP production in the PBMCs of 26 high vs 8 low CV risk patients with T2D. **B**, **Left**, extracellular acidification (ECAR) Seahorse profiles for the PBMCs of 26 high vs 8 low CV risk patients with T2D. **Right**, Boxplots of the glycolytic capacity and the glycolytic reserve in the PBMCs of 26 high vs 8 low CV risk patients with T2D. **C**, Energetic plots representing basal energy consumption (**left**) and maximal energy consumption (**right**), in the PBMCs of 26 high vs 8 low CV risk patients with T2D. **D**, Mitochondrial mass of total blood monocytes in 4 low CV risk (CV0), and 22 high CV risk (CV3) individuals with T2D. **E**, Boxplot of the mitochondrial activity/mass in the circulating total monocytes or the classical monocytes of 13 high CV risk patients vs 13 low CV risk patients. **F**, Pathway enrichment analysis of the plasma metabolome analysis, comparing the blood metabolite footprints between low CV risk (n=27) vs high CV risk (n=21) of individuals with T2D. Dot plot of the metabolic enriched pathways among the differentially expressed metabolites. **G**, Bubble plot of the *P* value of the over-represented pathways in both the transcriptomic (performed in Figure 3F) and metabolomic analyses on CV risk. The size is proportional to the −log10 transformation of the pathway enrichment *P* value, and the color reflects the *P* value. **H**, Bar plots of classical monocyte frequency, total and classical monocyte mitochondrial mass and activity from healthy donors after incubation in the serum of 6 high CV risk, or 6 low CV risk T2D patients. Kruskal-Wallis comparisons $^{*}P<0.05$, $^{**}P<0.01$.

monocyte frequency is associated with a decrease in mitochondrial mass, as observed in monocytes of individuals with T2D at high cardiovascular risk (Figure 4H). However, the mitochondrial activity increased, which might be an initial transient compensation for compromised mitochondrial function. Altogether, functional characterization of monocytes from participants with T2D at high cardiovascular risk revealed a decrease in mitochondrial mass and activity which might be provoked by blood metabolites.

DISCUSSION

T2D is a major cardiovascular risk factor; however, this cardiovascular risk is heterogeneous among people with T2D and can be time-sensitive within the same patient. Better understanding of this heterogeneity could improve preventive management. In the first part of our article, we demonstrate that monocyte count, routinely assessed through a complete blood panel, is a strong predictive marker for 10-year cardiovascular risk in individuals with T2D. Thus, even among people considered to have a normal monocyte count, those with T2D and a monocyte count $>0.5\times10^9$/L have a higher risk of cardiovascular events over the next decade compared with those with a count $<0.5\times10^9$/L. These data support findings previously published in populations of people with T2D,[29] or nondiabetic individuals but at high cardiovascular risk due to their age,[30] renal status,[31,32] or even their indication for scheduled coronary angiography.[33] It is noteworthy that this increased risk associated with monocyte counts $>0.5\times10^9$/L is independent of age, sex, and renal status. It may be linked to other differences, such as smoking habits, blood pressure, or LDL cholesterol levels. Indeed, it has been previously demonstrated that dyslipidemia,[34] high blood pressure,[35] and smoking[36] were also associated with higher monocyte counts. The predictive value of the 0.5×109/L monocyte threshold should be compared with that of well-established risk factors and standard cardiovascular assessments to demonstrate its complementarity and added value.

To further understand the link between cardiovascular risk and blood monocytes, we investigated whether a specific subtype of monocytes or a particular distribution of the 3 monocyte subtypes was responsible, or preferentially associated, with increased cardiovascular risk. We found that it was the percentage of classical monocytes (absolute or relative to the other 2 subtypes) that was positively associated with cardiovascular risk in our population. Of note, this finding is based on cross-sectional data (AngioSafe-2 and Glutadiab cohorts), as the monocyte subtypes were not studied in the longitudinal SURDIAGENE study (Survival Diabetes and Genetics). The increase in the proportion of classical monocytes compared with intermediate and nonclassical monocytes in patients at high cardiovascular risk is controversial.[37–40] However, knowing on one hand that diabetes[41] and dyslipidemia[42] are known to increase monocytopoiesis, and on the other hand, that it is primarily the CD16+ monocytes that adhere to the endothelium and penetrate the intima during the process of atherosclerosis,[43–45] we can speculate that the observed increase in the proportion of classical monocytes at the expense of intermediate and nonclassical monocytes during the progression of cardiovascular risk is the result of these 2 simultaneous phenomena: a global increase in monocyte production by the bone marrow (as it releases monocytes in the classical form,[46,47] and an increase in the adhesion to the arterial endothelium of intermediate and nonclassical monocytes.

We next analyzed the transcriptome of monocytes (CD14+ cells) from patients with T2D classified as low or high cardiovascular risk, either based on their CAC score or according to the distribution of monocyte subtypes (clustering). In both scenarios, we observed a gene expression signature that indicates dysregulation of the TCA cycle and mitochondrial OXPHOS in patients at high cardiovascular risk. These findings were functionally validated by metabolic flux analysis on PBMCs, and MitoTracker assays on monocytes from these patients. These highlighted impaired oxidative respiration and a shift to quiescence in the energetic profile of those with T2D at high cardiovascular risk. Interestingly, the comparison of the blood metabolome between our patients with T2D at low and high cardiovascular risk also revealed differences in TCA cycle metabolites. Furthermore, treating monocytes from a healthy donor with serum from a high cardiovascular risk patient's ex vivo caused them to phenotypically transition to monocytes resembling those from high cardiovascular risk patients (high CAC score, high monocyte blood count, and belonging to cluster 2). Thus, we highlight that the progression of cardiovascular risk in patients with T2D is marked by monocyte mitochondrial dysfunction, the exact causes of which remain to be established.

Similarly, mitochondria in adaptive immune cells have already been identified as a key player in other chronic inflammatory diseases.[48,49] A possible mechanism, detailed by Hulsmans et al,[50] is the deleterious effect of reactive oxygen species (that can be initially produced in response to hyperglycemia[51]) on the mitochondria, leading to a vicious cycle during which, in response to reactive oxygen species aggression, the mitochondria themselves produce more reactive oxygen species, further exacerbating their dysfunction. This accumulation of reactive oxygen species likely plays a prominent role in the development of atherosclerosis, as demonstrated by numerous studies.[52–57] Another phenomenon could have an impact, which is the release by activated monocytes of free mitochondria and microvesicles containing mitochondrial elements, as demonstrated by Puhm et al.[58] The development of therapeutic interventions to limit this mitochondrial dysfunction and its impact on cardiovascular risk could be contemplated in patients with T2D, as is already the case in psychiatric[59] or neurological diseases.[60] Nevertheless, our study puts monocytes at the core of the equation for the heightened risk in patients with T2D and advocates for further research on antiatherosclerotic treatments targeting monocytes and mitochondrial dysfunction. A limitation of this section could be the exclusion of CD14+ CD16++ monocytes during CD14+ immunoselection, despite the CD16 monocytes being considered highly activated. However, the CD16+

monocyte levels are not correlated with cardiovascular risk estimated by CAC score, thereby leaving our overall message unchanged.

Study Limitations

Our study has several limitations. First, the main design is cross-sectional and from the AngioSafe-2 and Glutadiab cohorts. Monocyte subtypes were not investigated longitudinally in the SURDIAGENE study that was also used here. Finally, we must acknowledge the limitation of excluding CD14+CD16++ monocytes during CD14+ immunoselection, despite the potential significance of CD16 as a marker for highly activated monocytes. It is worth noting that CD16+ monocyte levels do not exhibit correlations with cardiovascular risk estimated by CAC score, thus maintaining the overall message of our study intact.

CONCLUSIONS

Our data underline an association between routine white blood cell–derived monocyte counts and cardiovascular risk among people with T2D. By combining monocyte transcriptomic, immunophenotypic, and plasma metabolomic analyses, this study reveals an OXPHOS and mitochondrial modulation in a specific monocyte subtype, linked to an endotype of participants with T2D that are at higher cardiovascular risk. We show an association of 2 phenomena: an increase in the monocyte pool, leading to more ready infiltration of atherosclerotic plaques, promoting progression. Mitochondrial dysfunction was apparent in these monocytes, providing a potential explanation for the increased cardiovascular risk associated with T2D. Our study presents monocytes as good candidates for the prediction of cardiovascular complication development among people with T2D and highlights the relevance of phenotyping circulating monocytes to assess cardiovascular risk in T2D.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

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