Differences in Biomarkers of Inflammation Between Novel Subgroups of Recent-Onset Diabetes

Christian Herder,[1,2,3] Haifa Maalmi,[1,2] Klaus Strassburger,[2,4] Oana-Patricia Zaharia,[1,2] Jacqueline M. Ratter,[1,2] Yanislava Karusheva,[1,2] Mohamed A. Elhadad,[5,6,7] Kálmán Bódis,[1,2,3] Brenda W.C. Bongaerts,[2,4] Wolfgang Rathmann,[2,4] Sandra Trenkamp,[1,2] Melanie Waldenberger,[5,6,7,8] Volker Burkart,[1,2] Julia Szendroedi,[1,2,3] and Michael Roden,[1,2,3] for the GDS Group*

GENETICS/GENOMES/PROTEOMICS/METABOLOMICS

A novel clustering approach identified five subgroups of diabetes with distinct progression trajectories of complications. We hypothesized that these subgroups differ in multiple biomarkers of inflammation. Serum levels of 74 biomarkers of inflammation were measured in 414 individuals with recent adult-onset diabetes from the German Diabetes Study (GDS) allocated to five subgroups based on data-driven cluster analysis. Pairwise differences between subgroups for biomarkers were assessed with generalized linear mixed models before (model 1) and after (model 2) adjustment for the clustering variables. Participants were assigned to five subgroups: severe autoimmune diabetes (21%), severe insulin-deficient diabetes (SIDD) (3%), severe insulin-resistant diabetes (SIRD) (9%), mild obesity-related diabetes (32%), and mild age-related diabetes (35%). In model 1, 23 biomarkers showed one or more pairwise differences between subgroups (Bonferroni-corrected $P < 0.0007$). Biomarker levels were generally highest in SIRD and lowest in SIDD. All 23 biomarkers correlated with one or more of the clustering variables. In model 2, three biomarkers (CASP-8, EN-RAGE, IL-6) showed at least one pairwise difference between subgroups (e.g., lower CASP8, EN-RAGE, and IL-6 in SIDD vs. all other subgroups, all $P < 0.0007$). Thus, novel diabetes subgroups show multiple differences in biomarkers of

inflammation, underlining a prominent role of inflammatory pathways in particular in SIRD.

Diabetes is a multifactorial disease characterized by a complex combination of different but only partly understood etiologies (1). This heterogeneity is not adequately reflected by the current classification into the main forms of type 1 diabetes, type 2 diabetes, and gestational diabetes mellitus. In particular, the diagnosis of type 2 diabetes usually comprises forms of diabetes that cannot be assigned to any other specific diabetes types. This classic classification fails to consider possible differences in disease mechanisms, does not allow identification of people with different risk of developing complications, and precludes stratification of care and treatment regimens.

Recently, a data-driven cluster analysis of Scandinavian cohorts identified five diabetes subgroups (clusters) based on six variables: age at diagnosis, BMI, HbA_{1c}, HOMA-2 estimates of β-cell function and insulin resistance (HOMA2-B and HOMA2-IR), and GAD antibodies (GADA) (2). The five subgroups were validated in the German Diabetes Study (GDS) (3) and other cohort studies (4) and multinational trial populations (5). These studies demonstrated that the five diabetes subgroups have

[1]Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Düsseldorf, Germany

[2]German Center for Diabetes Research (DZD), Partner Düsseldorf, München-Neuherberg, Germany

[3]Division of Endocrinology and Diabetology, Medical Faculty and University Hospital Düsseldorf, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

[4]Institute for Biometrics and Epidemiology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Düsseldorf, Germany

[5]Research Unit of Molecular Epidemiology, Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg, Germany

[6]Institute of Epidemiology, Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg, Germany

[7]German Research Center for Cardiovascular Disease (DZHK), Partner site Munich Heart Alliance, Germany

[8]German Center for Diabetes Research (DZD), München-Neuherberg, Germany

Corresponding author: Christian Herder, christian.herder@ddz.de

Received 15 October 2020 and accepted 16 February 2021

This article contains supplementary material online at https://doi.org/10.2337/figshare.14043530.

C.H. and H.M. contributed equally to this study.

*A list of members of the GDS Group can be found in the APPENDIX.

distinct progression trajectories of diabetes-related complications (2,3,5), which have been related to differences in clinical, metabolic, and genetic characteristics (3).

Given the established role of inflammatory processes in the development of diabetes-related complications (6–10), potential differences in biomarkers of inflammation between diabetes subgroups might also contribute to differences in outcomes. However, biomarkers of inflammation investigated in this context have been limited to hs-CRP (3), so there is an obvious need to investigate in further detail whether differences in other biomarkers of inflammation exist between these subgroups. Therefore, this study aimed *1)* to comprehensively characterize differences in biomarkers of inflammation between the diabetes subgroups as described by Ahlqvist et al. and Zaharia et al. using a multimarker panel of serum protein biomarkers (6) and *2)* to investigate whether these differences are fully or partly independent of the aforementioned six clustering variables.

RESEARCH DESIGN AND METHODS

Study Population
This study is based on data from the GDS (11), an ongoing prospective observational cohort study investigating the natural course of metabolic alterations and the development of chronic diabetes complications (ClinicalTrials.gov, clinical trial reg. no. NCT01055093). The GDS was approved by the ethics committee of Heinrich Heine University Düsseldorf (ref. 4508). The study is performed in accordance with the Declaration of Helsinki. All participants provided written informed consent.

The GDS enrolls individuals with recent-onset diabetes (known diabetes duration ≤1 year) aged 18–69 years and glucose-tolerant individuals, who serve as control subjects. Diabetes was diagnosed in accordance with the guidelines of the American Diabetes Association (12). Study design and cohort profile of the GDS have previously been described in detail (11). Exclusion criteria are any secondary forms of diabetes; poor glycemic control (HbA$_{1c}$ >9.0% [75 mmol/mol]); current pregnancy; acute or severe chronic cardiac, hepatic, renal, or psychiatric diseases; active cancer; anemia; and acute infections, leukocytosis, immunosuppressive therapy, autoimmune diseases, and infection with HIV (11).

This cross-sectional analysis was based on the consecutive sampling of 504 participants with diabetes who entered the GDS cohort between September 2005 and December 2011, of whom 414 had available data for both biomarkers of inflammation and cluster assignment to one of the five diabetes subgroups as previously described (2,3). As shown in Supplementary Fig. 1, the sample represents a subsample of the GDS previously used in our analysis of diabetes subgroups and complications over a 5-year follow-up period, which additionally comprised study participants with baseline examinations until September 2018 (3).

Phenotyping and Laboratory Measurements
The study design included a structured interview, anthropometry, blood sampling, and measurement of metabolic variables and autoantibodies (GADA, islet cell autoantibodies [ICA]) as previously described (3,11). HOMA2-B and HOMA2-IR were calculated with the HOMA2 calculator from the University of Oxford based on fasting C-peptide and fasting glucose concentrations (13). Anti-inflammatory medication was paused a minimum of 7 days prior to blood sampling.

Biomarkers of inflammation were measured in serum of fasting participants at baseline with use of the inflammation panel from Olink Proteomics (Uppsala, Sweden) as previously described (14). This assay is based on proximity extension assay technology and allows the simultaneous measurement of 92 protein biomarkers covering pro- and anti-inflammatory cytokines, chemokines, growth factors, and factors involved in acute inflammatory and immune responses, angiogenesis, fibrosis, and endothelial activation (6). Therefore, "biomarkers of inflammation" in this article refers to the biomarkers from this panel, although some of them may also be considered metabolic biomarkers or biomarkers also reflecting other pathways.

The assay provides a relative quantification of protein concentrations that are given as normalized protein expression (NPX) values. These biomarker levels are comparable in their distribution with log$_2$-transformed protein concentrations. The normalization procedure is required to convert cycle threshold values from the quantitative PCR assay to relative protein concentrations.

All biomarkers are listed in Supplementary Table 1 together with UniProt numbers, gene names, intra-assay coefficients of variation (CV) and interassay CVs. As previously described (14), the calculation of intra- and interassay CVs was based on three control sera measured in duplicates on each plate ($n = 7$). Due to technical issues with the assay for brain-derived neurotrophic factor, data for this biomarker were not reported. Further, 17 biomarkers were excluded because >25% of values for each were below the limit of detection. We had a priori defined a threshold level of 20% for intra- and interassay CVs as a criterion for exclusion of biomarkers, but as previously reported (14) intra- and interassay CVs ranged between 0.4 and 12.5% and between 0.9 and 11.6%, respectively, so no biomarkers needed to be excluded and 74 biomarkers remained for analysis. Two of these 74 biomarkers (IL-6, IL-18) had been measured before with ELISAs (15,16). Log$_2$-transformed absolute protein concentrations (ELISA) and NPX (proximity extension assay) were highly correlated for both IL-6 ($r = 0.89$, $P < 0.0001$) and IL-18 ($r = 0.74$, $P < 0.0001$) (Supplementary Fig. 2).

Statistical Analysis
The allocation to previously defined diabetes subgroups (clusters) was performed based on age at diagnosis, BMI, HbA$_{1c}$, HOMA2-B, HOMA2-IR, and GADA as previously described (2,3). The cluster assignment was performed in
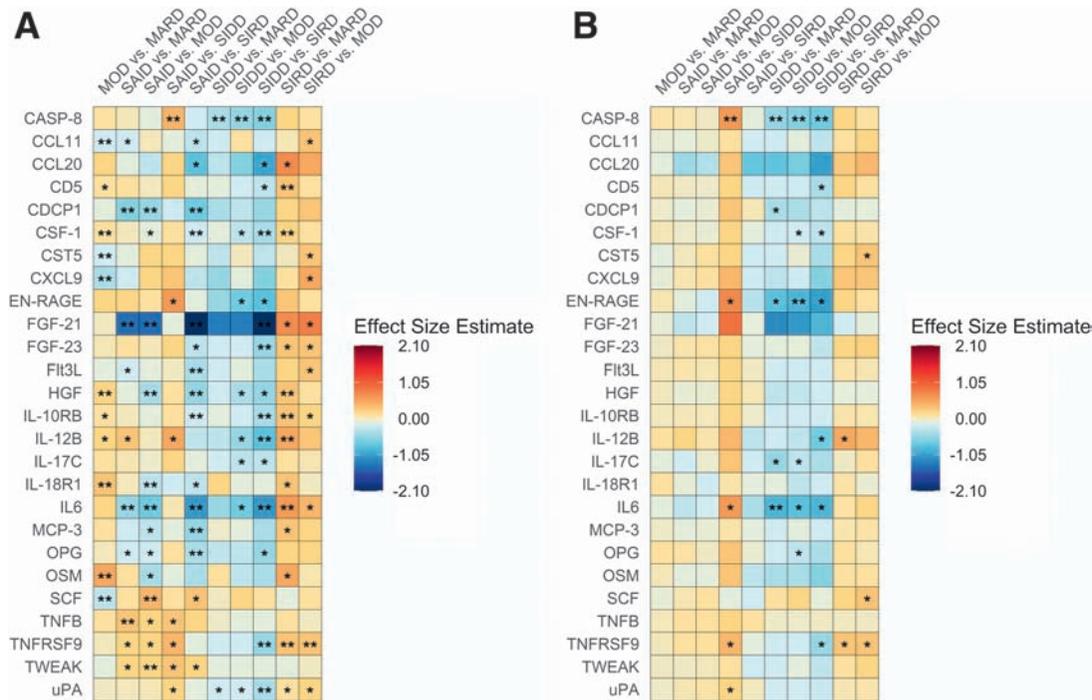
A

B

Effect Size Estimate
2.10
1.05
0.00
−1.05
−2.10

Figure 1—*A*: Unadjusted pairwise differences between diabetes subgroups (model 1). The heat map shows effect estimates and corresponding *P* values of pairwise comparisons of the 26 biomarkers with at least one significant pairwise difference after correction for pairwise and/or multiple comparisons (model 1). Extended names of biomarkers are given in Supplementary Table 1. Full results are given in Supplementary Table 3. Positive/negative effect estimates result from higher/lower biomarker levels in the diabetes subgroup named first in the respective comparison. *Tukey-Kramer corrected *P* < 0.05; **Bonferroni-corrected Tukey-Kramer *P* < 0.0007. Biomarkers are ordered alphabetically from top to bottom. *B*: Pairwise differences between diabetes subgroups with adjustment for clustering variables (model 2). The heat map shows effect estimates and corresponding *P* values of pairwise comparisons of the 26 biomarkers with at least one significant pairwise difference after correction for pairwise and/or multiple comparisons (model 2). Extended names of biomarkers are given in Supplementary Table 1. Full results are given in Supplementary Table 3. Positive/negative effect estimates result from higher/lower biomarker levels in the diabetes subgroup named first in the respective comparison. *Tukey-Kramer corrected *P* < 0.05; **Bonferroni-corrected Tukey-Kramer *P* < 0.0007. Biomarkers are ordered alphabetically from top to bottom.

the previously described sample from the GDS cohort (3) according to the sex-specific classification rules from Ahlqvist et al. (2) using the nearest centroid approach so that every individual was assigned to one of five predefined clusters, i.e., severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), severe insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD), and mild age-related diabetes (MARD). All individuals who tested positive for GADA were allocated to the SAID cluster. We did not develop novel or updated classification rules in the GDS, as our previous study primarily aimed at evaluation of specific features of the originally introduced subgroups (3), and because of the possible selection bias in recruitment, whereas the initial clustering algorithm was developed with a population-based sample (2). Differences in subgroup proportions between this sample and excluded individuals from the previously described sample from the GDS cohort (3) were analyzed with the χ^2 test.

Data are presented as median (25th percentile; 75th percentile) or percentages. Differences in the clinical characteristics of the study population according to diabetes subgroups were tested with Wilcoxon-Mann-Whitney test,

χ^2 test, and Fisher exact test. *P* values <0.05 were considered to indicate statistically significant differences.

Overall differences in biomarkers of inflammation between diabetes subgroups were analyzed with the Kruskal-Wallis test. Bonferroni-corrected *P* values <0.0007 (0.05/74) indicated significant differences.

Pairwise differences between means of biomarkers of inflammation across diabetes subgroups were estimated with use of generalized linear mixed models. The GLIMMIX procedure fits statistical models to unbalanced data and provides the capability of accounting for unequal residual variances between diabetes subgroups. Data were analyzed without (model 1) and with (model 2) adjustment for age at diagnosis, BMI, HbA$_{1c}$, HOMA2-B, HOMA2-IR, and GADA (all covariables entered the model as continuous variables). *P* values of pairwise mean differences were adjusted for pairwise multiple comparisons with the Tukey-Kramer procedure (based on 10 different combinations of subgroups) and additionally for the total number of biomarkers with the Bonferroni procedure. A Bonferroni-corrected Tukey-Kramer *P* value <0.0007 was considered statistically significant ($\alpha = 0.05/74 = 0.0007$).
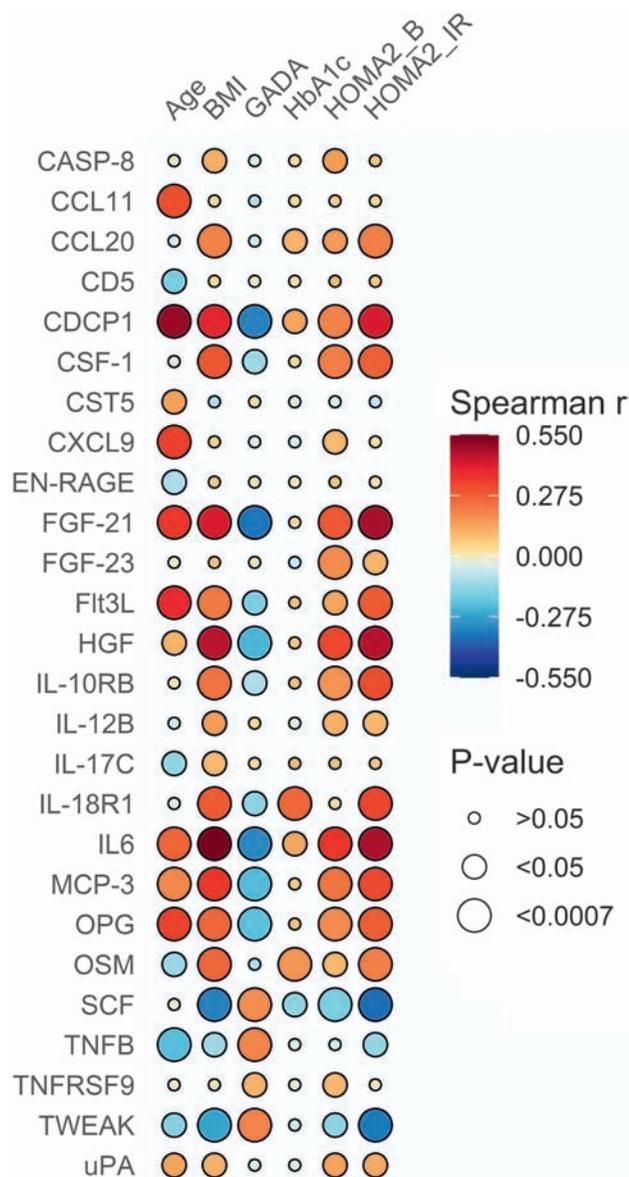
Figure 2—Correlations between biomarkers of inflammation and clustering variables in the total study sample. Correlations with the clustering variables were assessed for the 26 biomarkers of inflammation with pairwise differences between diabetes subgroups. Age, age at diagnosis. Extended names of biomarkers are given in Supplementary Table 1.

Correlations between inflammation-related biomarkers showing differences between subgroups in model 1 and the six aforementioned clustering variables were estimated with use of Spearman rank correlation coefficients and corresponding P values.

The aforementioned statistical analyses were conducted with SAS, version 9.4 (SAS Institute, Cary, NC).

We performed exploratory principal components analysis (PCA) in RStudio (version 4.0.2) using the factoextra package to compare the separation of the diabetes

subgroups 1) based on clustering variables and 2) based on biomarkers of inflammation. As GADA is the sole criterion for the allocation to SAID, we focused on the remaining clustering variables to visualize clustering of the SIDD, SIRD, MOD, and MARD subgroups.

In a causal inference analysis, we applied the two-sample Mendelian randomization (MR) method to evaluate the bidirectional causal relationship between biomarkers, which showed significant differences between diabetes subgroups, and type 1 diabetes, type 2 diabetes, and their related complications. This analysis was performed with the MR-Base web interface (17). Instrumental variables (IVs) were extracted from a published genome-wide association study (GWAS) of SOMAscan-measured proteins ($n = 3,301$) (18). For diabetes and diabetes-related complications (angiopathy, retinopathy, neuropathy, renal complications), we used the latest GWAS for type 1 and type 2 diabetes of the MRC IEU OpenGWAS Project with 12,375 case and 82,665 control subjects of the FinnGen biobank (19). All data sets include observations in men and women of European ancestry. All IVs passed the threshold of $P < 5 \times 10e-8$. To select IVs, we first removed palindromic single nucleotide polymorphisms (SNPs) (defined as SNPs with A/T or G/C) with minor allele frequencies close to 0.5 as the effect allele will be ambiguous. Then, we replaced these SNPs with proxies with a minimum linkage disequilibrium R^2 value of 0.8 when available and allowed palindromic SNPs with a minor allele frequency threshold up to 0.3. Finally, to identify the independent signals among correlated SNPs, we clumped the SNPs by removing SNPs in linkage disequilibrium with the lead SNP using the R^2 cutoff 0.001. A Bonferroni-corrected P value <0.005 was considered statistically significant ($\alpha = 0.05/10 = 0.005$).

Data and Resource Availability
The data are subject to national data protection laws. Therefore, data cannot be made freely available in a public repository. However, data can be requested through an individual project agreement with the Steering Committee of the GDS (speaker: Michael Roden, michael.roden@ddz.de).

RESULTS

Study Population
As shown in Table 1, each of the 414 study participants was assigned to one of the five subgroups based on the clustering approach previously described (2,3): SAID, 21% ($n = 87$); SIDD, 3% ($n = 12$); SIRD, 9% ($n = 39$); MOD, 32% ($n = 133$); and MARD, 35% ($n = 143$). The proportions of subgroups in our sample did not differ from those in the previously described larger sample from the GDS cohort (SAID, 22%; SIDD, 3%; SIRD, 11%; MOD, 29%; and MARD, 35%; $P = 0.377$ for the difference between included and excluded individuals) (3). Participants in these subgroups differed not only in the clustering variables but also in all other variables tested except total

Table 1—Clinical characteristics of the study population according to diabetes subgroup allocation

	SAID	SIDD	SIRD	MOD	MARD	P
N (% of study sample)	87 (21)	12 (3)	39 (9)	133 (32)	143 (35)	
Age, years	34.5 (25.9; 46.2)	46.3 (33.4; 52.9)	56.4 (50.7; 64.8)	43.9 (35.3; 53.1)	59.8 (53.8; 64.5)	**<0.0001**
Sex (men/women), %	59.7/40.3	91.7/8.3	64.1/35.9	57.1/42.9	71.3/28.7	**0.02**
BMI, kg/m²	24.0 (21.9; 26.5)	27.1 (24.5; 29.0)	34.9 (31.1; 37.4)	33.7 (30.0; 38.5)	27.3 (25.4; 29.9)	**<0.0001**
Waist circumference, cm	86.0 (76.5; 96.0)	94.0 (82.2; 106.0)	114.0 (106.0; 122.0)	109.5 (98.2; 119.0)	96.1 (90.0; 104.0)	**<0.0001**
HOMA2-B	47.8 (34.9; 69.3)	35.8 (24.1; 45.2)	165.9 (136.5; 187.6)	91.2 (70.4; 119.9)	81.6 (63.3; 105.9)	**<0.0001**
HOMA2-IR	1.0 (0.7; 1.3)	1.4 (1.0; 2.7)	4.4 (3.8; 5.5)	2.7 (1.9; 3.4)	1.8 (1.4; 2.3)	**<0.0001**
Total cholesterol, mg/dL	185.0 (163.5; 213.5)	196.5 (190.5; 200.0)	203.5 (173.5; 228.5)	198.5 (167.5; 231.5)	197.5 (173.5; 226.5)	0.13
Triglycerides, mg/dL	74.3 (53.4; 109.0)	100.1 (66.1; 191.6)	176.6 (110.5; 243.6)	141.3 (91.5; 206.0)	109.6 (83.0; 152.2)	**<0.0001**
HbA$_{1c}$, %	6.4 (6.0; 7.1)	8.8 (8.0; 10.2)	6.0 (5.8; 6.3)	6.4 (5.8; 7.0)	6.1 (5.8; 6.5)	**<0.0001**
HbA$_{1c}$, mmol/mol	46.4 (42.1; 54.1)	72.7 (63.4; 87.4)	42.1 (39.9; 45.3)	46.4 (39.9; 53.0)	43.1 (39.9; 47.5)	**<0.0001**
eGFR (mL/min per 1.73 m²)	101.0 (86.7; 110.3)	102.6 (92.1; 114.8)	78.6 (68.7; 88.8)	95.4 (85.3; 108.3)	87.6 (75.9; 97.7)	**<0.0001**
CKD, %	0	0	7.9	3.0	6.4	0.05
Known diabetes duration, days	162 (123; 255)	113.0 (49.5; 192.0)	197 (124; 279)	179 (122; 176)	194 (132; 266)	0.52
Hypertension, %	25.6	41.7	78.9	61.0	75.7	**<0.0001**
Antihypertensive medication, %	10.3	16.7	64.1	45.1	51.0	**<0.0001**
Lipid-lowering medication, %	2.3	8.3	30.7	9.8	25.2	**<0.0001**
Glucose-lowering medication (insulin/metformin/none/other), %	77/7/11/5	50/25/8/17	0/67/25/8	19/33/38/10	10/35/49/6	**<0.0001**
GADA >0.9 units/mL, %	100	0	0	0	0	**<0.0001**
ICA >20 JDF units, %	83.9	8.3	2.6	6.0	2.8	**<0.00001**

Data are presented as median (25th percentile; 75th percentile) unless otherwise indicated. The frequency of missing data are <2% for each variable. CKD was defined by eGFR <60 mL/min/1.73 m² (calculated using the CKD-EPI equation based on creatinine and cystatin C). Boldface type indicates significant P values <0.05. CKD, chronic kidney disease; JDF, Juvenile Diabetes Foundation.

cholesterol levels, known diabetes duration, and prevalence of chronic kidney disease (Table 1).

Unadjusted Differences in Biomarkers of Inflammation Between Diabetes Subgroups

The overall comparison of biomarkers between diabetes subgroups showed differences in the serum levels of 27 of 74 biomarkers of inflammation ($P < 0.0007$ after accounting for multiple testing) (Supplementary Table 2). Biomarker levels were generally highest in individuals from the SIRD subgroup, lowest in individuals from the SIDD subgroup, and intermediate in the SAID, MOD, and MARD subgroups (Supplementary Table 2).

The pairwise comparison of biomarkers between diabetes subgroups without any adjustment for covariables (model 1) showed significant differences in 26 of 74 biomarkers of inflammation after correction for pairwise multiple comparisons (based on 10 different combinations of subgroups) with use of the Tukey-Kramer test ($P < 0.05$). Among these 26 biomarkers, 23 showed at least one pairwise significant difference after additional Bonferroni correction ($P < 0.0007$). Figure 1A graphically summarizes the effect estimates and corresponding P values of between-means pairwise comparisons of the 26 biomarkers, while Supplementary Table 3 reports detailed results for all 74 biomarkers.

After Bonferroni correction, the largest number of pairwise differences between subgroups (at least four) were observed for caspase-8 (CASP-8), macrophage colony-stimulating factor 1 (CSF-1), fibroblast growth factor-21 (FGF-21), hepatocyte growth factor (HGF), and interleukin-6 (IL-6). With respect to the number of biomarkers, the largest number of differences after Bonferroni correction were observed for the comparison of SIRD with MARD, SAID, and SIDD, with higher serum levels of seven, eight, and nine biomarkers, respectively, in SIRD (all $P < 0.0007$). MARD, MOD, and SAID differed in four to eight biomarker levels but with different directions. Only one biomarker (CASP-8) was different in the comparisons of SIDD versus SAID, MARD, and MOD.

Differences in Biomarkers of Inflammation Between Diabetes Subgroups After Adjustment for Clustering Variables

The 26 biomarkers of inflammation with pairwise differences between diabetes subgroups were also correlated with at least one of the six clustering variables. Figure 2 provides an overview of correlation coefficients and P values (see Supplementary Table 4 for detailed results). Approximately two-thirds of these biomarkers (between 17 and 19) correlated with age, BMI, HOMA2-B, and HOMA2-IR, with mostly positive correlation coefficients between 0.1 and 0.5. Correlations between biomarker levels and presence of GADA were positive for 4 and inverse for 10 biomarkers. Only six biomarkers correlated with HbA_{1c}.

When we assessed differences in biomarker levels between subgroups with additional adjustment for these clustering variables (model 2), there were significant differences between subgroups for 13 biomarkers (Tukey-Kramer $P < 0.05$). The largest number of pairwise differences between subgroups ($n = 4$) were observed for CASP-8, S100 calcium-binding protein A12 (EN-RAGE), IL-6, and tumor necrosis factor receptor superfamily member 9 (TNFRSF9/CD137). Regarding the number of biomarkers, most differences were found for the comparison of SIRD versus SIDD, with higher serum levels of seven biomarkers in SIRD.

After additional adjustment for multiple testing, three biomarkers (CASP-8, EN-RAGE, IL-6) showed at least one pairwise difference between subgroups (Fig. 1B and Supplementary Table 3). Figure 3 shows the unadjusted serum levels of these three biomarkers in each diabetes subgroup. CASP-8 levels were lower in SIDD than in SAID, SIRD, MARD, and MOD; EN-RAGE levels were lower in SIDD than in MOD; and IL-6 levels were lower in SIDD than in MARD (all $P < 0.0007$). None of the biomarkers differed between SAID, MOD, and MARD after full adjustment.

PCA Using the Clustering Variables and Biomarkers of Inflammation

The first PCA using the clustering variables (except for GADA) indicated the largest difference between the SIRD and SIDD subgroups and an overlap between MOD and MARD (Supplementary Fig. 3A). The first two principal components (PC1, PC2) explained 64.7% of the variance. With use of all 74 biomarkers of inflammation for the PCA, the separation was less pronounced, but again SIRD and SIDD were the subgroups with the best separation (Supplementary Fig. 3B). In the second analysis, PC1 and PC2 explained 29.1% of the variance (Supplementary Fig. 4).

MR Analysis

We assessed the associations between genetically predicted levels of biomarkers that showed significant differences between diabetes subgroups (CASP-8, EN-RAGE, IL-6) and type 1 and type 2 diabetes and their related complications. No IVs were available for CASP-8 in the MR-Base platform. For EN-RAGE, a nominally significant association with renal complications in people with type 2 diabetes was observed (inverse variance–weighted β = 0.279, $P = 0.033$) (Supplementary Table 5), which did not remain significant after adjustment for multiple testing. No associations of genetically predicted EN-RAGE levels were detected with type 2 diabetes or the other tested complications in type 2 diabetes. There were also no associations of EN-RAGE with type 1 diabetes or any of the complications in people with type 1 diabetes (Supplementary Table 5). For IL-6, there was no evidence for causal effects on any of the aforementioned outcomes (all $P \geq 0.087$) (Supplementary Table 6).

We also performed MR analyses to examine whether type 2 diabetes, type 1 diabetes, or any of their complications had causal effects on EN-RAGE or IL-6 levels, but we found no evidence for this (all $P \geq 0.119$) (Supplementary Tables 7 and 8).

DISCUSSION

Diabetes subgroups identified with use of the clustering approach described by Ahlqvist et al. (2,20) and validated by Zaharia et al. (3) showed multiple differences in biomarkers of inflammation. Biomarker levels were highest in the SIRD subgroup, which is characterized by pronounced insulin resistance; lowest in the SIDD subgroup, which is characterized by severe insulin deficiency; and intermediate for the MOD, MARD, and SAID subgroups. Although adjustment for clustering variables attenuated these differences, we were still able to identify three biomarkers (CASP-8, EN-RAGE, IL-6) that showed at least one pairwise difference between the subgroups in the adjusted model.

This study represents a novel comprehensive investigation of the relationship between biomarkers of inflammation and the five novel diabetes subgroups (clusters). Our previous analysis focused on metabolic features and comorbidities of these subgroups but already reported that hs-CRP was higher in SIRD and MOD compared with the other subgroups (3). The identification of multiple differences in inflammation-related biomarkers validates the empirically derived classification based on age at diagnosis, BMI, metabolic parameters, and autoimmunity. As all of these clustering variables correlate with subclinical inflammation, it is important to emphasize that the observed differences were at least partly independent of the clustering variables. Of note, our study included only individuals with recent-onset diabetes, which is more appropriate for drawing pathophysiological conclusions than investigating individuals with long-standing diabetes whose inflammatory profiles are substantially affected by diabetes-related complications and comorbidities.

Overall, the highest levels of biomarkers of inflammation were observed in SIRD, i.e., the diabetes subgroup characterized by the most pronounced insulin resistance. SIRD represents one of the smaller subgroups, with 9% in our cohort and 10–17% in other European cohorts (2). However, this rather small subgroup is clinically important not only because of the high degree of insulin resistance but also because of higher prevalence and increased risk for diabetes-related comorbidities such as nephropathy (2,3) or hepatic steatosis and early fibrosis (3). A close relationship between inflammation and insulin resistance has previously been established in multiple studies, mostly in the context of obesity and dysfunctional adipose tissue (21). Inflammation-related processes in adipose tissue and spillover of inflammatory biomarkers into the circulation have been recognized as important mechanisms possibly initiating abnormal hepatic glucose
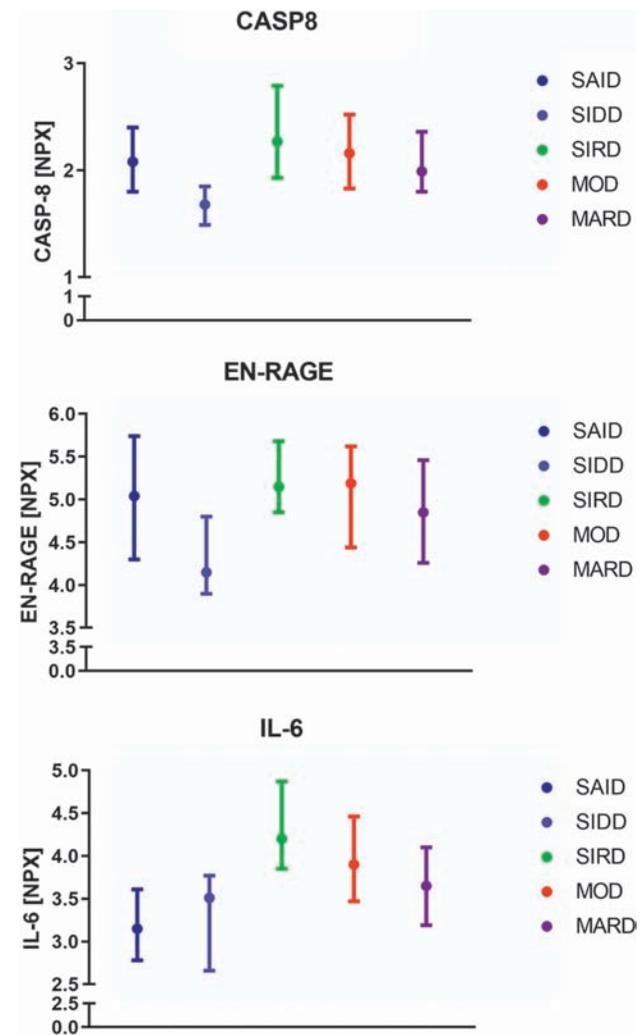

Figure 3—Serum concentrations (in NPX units) of the three biomarkers of inflammation which showed at least one pairwise difference between subgroups after adjustment for clustering variables. Data are shown as median ± 25th/75th percentiles. CASP8, caspase-8; EN-RAGE, S100 calcium-binding protein A12; IL-6, interleukin-6.

metabolism and type 2 diabetes (22). Of note, despite degree of obesity (based on BMI) being similar to that of MOD, SIRD may represent a state of dysfunctional adipose tissue, which is further supported by the higher prevalence of dyslipidemia. This along with hypertension would drive the onset of diabetes-related complications. After adjustment for clustering variables, SIRD had higher circulating levels of CASP-8 than SIDD. CASP-8 is a cytosolic cysteine protease that mediates programmed cell death. It is involved in β-cell apoptosis in diabetes (23) but also in the activation of T, B, and natural killer cells and macrophage differentiation. It is not well understood which processes lead to the release of this protein into the circulation, but it is noteworthy that higher circulating CASP-8 levels were associated with a higher risk of type 2 diabetes and coronary events (24,25).

SIDD represents the opposite extreme in comparison with SIRD with the most pronounced insulin deficiency and the lowest circulating levels of biomarkers of inflammation. Our exploratory PCA confirms the separation between SIRD and SIDD based on both the clustering variables and the biomarkers of inflammation. Three biomarkers were lower in SIDD than in all other subgroups after adjustment for clustering variables (CASP-8, IL-6, EN-RAGE). IL-6 is related to inflammation in adipose tissue. However, this cytokine has multiple sources and pleiotropic roles (26,27). IL-6 is released not only by immune cells and adipocytes but also by myocytes in response to exercise. In addition to proinflammatory properties, IL-6 mediates beneficial effects of exercise, stimulates insulin secretion through the release of glucagon-like peptide 1 (GLP-1), and may therefore represent an important cytokine that also counteracts metabolic stress and insulin resistance (28,29). EN-RAGE signals through the receptor for advanced glycation end products and the Toll-like receptor, thereby triggering cytokine production, chemotaxis, and increased oxidative stress (30). Higher circulating levels of EN-RAGE were associated with incident prediabetes and type 2 diabetes (31). Thus, it can be hypothesized that inflammation-related processes contribute to the development of diabetes more in the other subgroups than in SIDD, but this would require testing in a longitudinal study with blood samples also taken before the diagnosis of diabetes. It also appears that biomarkers of inflammation may not be relevant correlates of impaired β-cell function, although the role of inflammatory processes in β-cell demise in individuals with diabetes is well established (32). Given that advanced age and most lifestyle-related and environmental risk factors of type 2 diabetes are triggers of subclinical inflammation (9), it is tempting to speculate that the etiology of SIDD may have a stronger genetic component than that of SIRD, MARD, and MOD. So far, there is only evidence for differential associations between the gene variant rs7903146 in the *TCF7L2* gene and the type 2 diabetes–related subgroups with the nominally highest effect size for SIDD (2).

Concerning the MARD, MOD, and SAID subgroups, multiple differences in biomarkers of inflammation were seen in the unadjusted analysis, which, however, were abolished by adjustment for clustering variables. This observation suggests that these differences in inflammatory profiles are largely explained by age at diagnosis, anthropometry, and metabolic variables. Of note, MARD and MOD represent subgroups of type 2 diabetes, whereas SAID mainly reflects type 1 diabetes. Previous studies that compared circulating levels of biomarkers of inflammation found higher levels for some cytokines and soluble adhesion molecules in patients with type 2 diabetes than in those with autoimmune diabetes, but the distributions of biomarker concentrations overlapped widely (33,34). In our study, levels of FGF-21, IL-6, and CDCP1 (CUB

domain–containing protein 1) were lower in SAID compared with both MARD and MOD, but these differences were largely explained by adjustment for clustering variables. Therefore, biomarkers of inflammation do not appear to be better discriminators between type 1 diabetes and type 2 diabetes in the traditional classification system when age at diagnosis, anthropometry, metabolic variables, and autoantibodies are taken into account. However, it needs to be acknowledged that the biomarker panel used in this study did not contain potentially important cytokines such as IL-1β, whereas biomarkers such as FGF-21 are more closely related to glucose and lipid metabolism than to inflammatory processes (35).

Future studies need to investigate the extent to which differences in the profiles of inflammation-related biomarkers can explain the apparent differences between the diabetes subgroups regarding their risk of developing diabetes-related complications and comorbidities. Previous studies showed that SIRD was characterized by the highest risk of chronic kidney disease and different hepatic fat content and fibrosis (2,3). A recent multimarker study found that mainly TNF receptor superfamily members showed robust associations with incident end-stage renal disease in people with diabetes (7), which is reflected by our finding that SIRD had the highest levels of TNFRSF9. In a recent report, for which we used the same biomarker panel investigated in the current study, we showed multiple associations with estimated glomerular filtration rate (eGFR) in the GDS (14). Among the biomarkers associated with eGFR, 13 showed differences between diabetes subgroups in the unadjusted model of the current study (Fig. 1*A*) and 6 (CD5 [T-cell surface glycoprotein CD5], CSF-1, CST5 [cystatin D], IL-12B [IL-12 subunit β], TNFRSF9, and uPA [urokinase-type plasminogen activator]) showed at least one between-subgroup difference after the adjustment for clustering variables (Fig. 1*B*). The observations point toward a potential mediating role of these biomarkers in diabetes-related impairment of kidney function, which, however, would have to be investigated with use of a prospective design in a larger study sample. SIRD also showed the highest levels of biomarkers such as IL-6, IL-17C, CCL20 (C-C motif chemokine 20), CASP-8, and CD5 that have been implicated in different stages of nonalcoholic fatty liver disease (36–39) and merit further studies in this context.

In contrast, SIDD showed the strongest associations with retinopathy and diabetic sensorimotor polyneuropathy (DSPN) (2,3). Inflammatory mechanisms and multiple biomarkers of inflammation are implicated in the development of DSPN (40,41) so that an increased risk of DSPN in SIDD appears counterintuitive and needs to be replicated in additional cohorts before firm conclusions are possible. Finally, our data do not argue for biomarkers of inflammation as independent mediators of a differential risk of diabetes complications among MOD, MARD,

and SAID beyond the age at diagnosis and metabolic variables.

To address the issue of causality in this context, we attempted a causal inference analysis using two-sample MR for CASP-8, EN-RAGE, and IL-6. We found suggestive evidence for a causal effect of EN-RAGE on renal complications in people with type 2 diabetes, but this finding was not significant after adjustment for multiple testing. Unfortunately, we were not able to check for horizontal pleiotropy, as we only had two IVs. Moreover, the causal effect was mainly driven by the SNP rs62143206, which is in trans with the protein-encoding gene. Overall, our analyses were limited by the availability of suitable IVs for estimating causal effects of these protein biomarkers on diabetes without and with complications (no SNP for CASP-8, only two SNPs each for EN-RAGE and IL-6). In the absence of larger GWAS for these protein biomarkers, our null findings need to be interpreted with caution. Additional IVs that could explain a larger proportion of variance in biomarker levels would increase the statistical power of such MR analyses and help to reveal potential causal effects.

It is important to note that our cross-sectional study focused on differences in biomarkers of inflammation and diabetes subgroups at only one time point during the 1st year after the diagnosis of diabetes. We have previously shown in the GDS that the cluster allocation can change during the first 5 years of follow-up and that these changes were mainly related to changes in glycemia and lipid levels (3). Studies comprising multiple measurements of clinical variables and biomarkers are needed to model trajectories of clustering variables and biomarkers of inflammation (see 42 and 43 for examples) ideally before and after the diagnosis of diabetes (44). These data would allow an analysis of the temporal relationships between changes in clustering variables and changes in biomarker levels, allowing further insights into aetiological pathways that might differ between the diabetes subgroups.

Our study has several strengths and limitations. A major strength is the unique cohort of people with recent-onset diabetes so that differences in biomarkers of inflammation between diabetes subgroups can be assessed before potential confounding by long-standing hyperglycemia and high prevalence of complications. Moreover, the use of a multimarker approach implicated comprehensive phenotyping of inflammation-related biomarkers, which is important because multiple parallel pathomechanisms can be expected to contribute to the pathophysiology of diabetes and its complications. Limitations of our study include the cross-sectional design and the low absolute number of individuals with SIDD and SIRD. Differences in the proportions of individuals in each subgroup compared with other European cohorts may partly be explained by the specific inclusion and exclusion criteria (e.g., limited age range, upper HbA_{1c} level, overrepresenta-

tion of individuals with type 1 diabetes) of the GDS. The exclusion of individuals with poor glycemic control might have led to the exclusion of the most extreme SIDD cases and thus to an underestimation of the differences in biomarkers between the SIDD and other subgroups. The oversampling of individuals with type 1 diabetes may also have resulted in a larger proportion of ICA-positive individuals among the GADA-negative subgroups than in other cohorts (45,46). The multimarker panel contained a range of biomarkers of inflammation, but interesting proteins such as further members of the IL-1 family were not included. The procedure to correct for multiple testing accounting for both the number of between-subgroup comparisons and the number of biomarkers may be too conservative given the correlation structure between the biomarkers. Therefore, the number of biomarkers that differ between diabetes subgroups may be underestimated. However, such a cautious approach appears preferable in the absence of external validation of our results. Not only should our results be replicated in external cohorts with participants with recent-onset diabetes but also future studies should assess the predictive value of biomarkers of inflammation for the risk of complications of diabetes in prospective analyses and test the extent to which these biomarkers may contribute to differences in diabetes-related complications among novel diabetes subgroups (e.g., using mediation analysis). Causal inference analyses such as MR studies can help with assessing the role of predictive biomarkers for disease etiology, but these analyses were limited by the scarcity of suitable IVs and thus low statistical power. Future studies might also revisit the definition of the SAID subgroup, which is currently based on GADA positivity only. The presence of ICA in some individuals from the other four subgroups suggests that the measurement of multiple autoantibodies may be better suited to assessing autoimmunity as a clustering criterion. Finally, our study sample consisted mainly of people of European descent and, consequently, results are not generalizable to other ethnicities.

In conclusion, our study identified multiple differences in biomarkers of inflammation between novel subgroups of diabetes. Circulating levels of biomarkers of inflammation were highest in SIRD and lowest in SIDD. Differences between subgroups remained significant for three biomarkers even after adjustment for clustering variables. Of note, there was no clear separation of SAID, reflecting type 1 diabetes, from MARD and MOD, currently classified as type 2 diabetes, with regard to biomarkers of inflammation. The link between high levels of inflammation-related biomarkers and pronounced insulin resistance points to a particular contribution of inflammatory processes to the SIRD subgroup. Future studies are warranted to investigate which of the biomarkers identified

in this study may explain differences in the risk of complications between the subgroups.

Appendix

GDS Group. The GDS Group consists of H. Al-Hasani, V. Burkart, A.E. Buyken, G. Geerling, C. Herder, A. Icks, K. Jandeleit-Dahm, J. Kotzka, O. Kuss, E. Lammert, W. Rathmann, V. Schrauwen-Hinderling, J. Szendroedi, S. Trenkamp, D. Ziegler, and M. Roden (speaker).

References

1. Pearson ER. Type 2 diabetes: a multifaceted disease. Diabetologia 2019; 62:1107–1112

2. Ahlqvist E, Storm P, Käräjämäki A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. Lancet Diabetes Endocrinol 2018;6:361–369

3. Zaharia OP, Strassburger K, Strom A, et al.; German Diabetes Study Group. Risk of diabetes-associated diseases in subgroups of patients with recent-onset diabetes: a 5-year follow-up study. Lancet Diabetes Endocrinol 2019;7:684–694

4. Zou X, Zhou X, Zhu Z, Ji L. Novel subgroups of patients with adult-onset diabetes in Chinese and US populations. Lancet Diabetes Endocrinol 2019;7: 9–11

5. Dennis JM, Shields BM, Henley WE, Jones AG, Hattersley AT. Disease progression and treatment response in data-driven subgroups of type 2 diabetes compared with models based on simple clinical features: an analysis using clinical trial data. Lancet Diabetes Endocrinol 2019;7:442–451

6. Herder C, Kannenberg JM, Carstensen-Kirberg M, et al. A systemic inflammatory signature reflecting cross talk between innate and adaptive immunity is associated with incident polyneuropathy: KORA F4/FF4 study. Diabetes 2018;67:2434–2442

7. Niewczas MA, Pavkov ME, Skupien J, et al. A signature of circulating inflammatory proteins and development of end-stage renal disease in diabetes. Nat Med 2019;25:805–813

8. Ridker PM. Anticytokine agents: targeting interleukin signaling pathways for the treatment of atherothrombosis. Circ Res 2019;124:437–450

9. Furman D, Campisi J, Verdin E, et al. Chronic inflammation in the etiology of disease across the life span. Nat Med 2019;25:1822–1832

10. Mesquida M, Drawnel F, Fauser S. The role of inflammation in diabetic eye disease. Semin Immunopathol 2019;41:427–445

11. Szendroedi J, Saxena A, Weber KS, et al.; GDS Group. Cohort profile: the German Diabetes Study (GDS). Cardiovasc Diabetol 2016;15:59

12. American Diabetes Association. 2. Classification and diagnosis of diabetes: *Standards of Medical Care in Diabetes—2018*. Diabetes Care 2018; 41(Suppl. 1):S13–S27

13. HOMA2 Calculator. Accessed 1 December 2020. Available from https://www.dtu.ox.ac.uk/homacalculator/download.php

14. Maalmi H, Herder C, Strassburger K, et al. Biomarkers of inflammation and glomerular filtration rate in individuals with recent-onset type 1 and type 2 diabetes. J Clin Endocrinol Metab 2020;105:dgaa622

15. Schamarek I, Herder C, Nowotny B, et al.; German Diabetes Study Group. Adiponectin, markers of subclinical inflammation and nerve conduction in individuals with recently diagnosed type 1 and type 2 diabetes. Eur J Endocrinol 2016;174:433–443

16. Herder C, Schamarek I, Nowotny B, et al.; German Diabetes Study Group. Inflammatory markers are associated with cardiac autonomic dysfunction in recent-onset type 2 diabetes. Heart 2017;103:63–70

17. Hemani G, Zheng J, Elsworth B, et al. The MR-Base platform supports systematic causal inference across the human phenome. eLife 2018;7:e34408

18. Sun BB, Maranville JC, Peters JE, et al. Genomic atlas of the human plasma proteome. Nature 2018;558:73–79

19. Elsworth B, Lyon M, Alexander T, et al. The MRC IEU OpenGWAS data infrastructure. 10 August 2020 [preprint]. bioRxiv:2020.08.10.244293

20. Ahlqvist E, Prasad RB, Groop L. Subtypes of type 2 diabetes determined from clinical parameters. Diabetes 2020;69:2086–2093

21. Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders. Nature 2017;542:177–185

22. Roden M, Shulman GI. The integrative biology of type 2 diabetes. Nature 2019;576:51–60

23. Liadis N, Salmena L, Kwan E, et al. Distinct in vivo roles of caspase-8 in β-cells in physiological and diabetes models. Diabetes 2007;56:2302–2311

24. Svensson T, Svensson AK, Kitlinski M, et al. Plasma concentration of caspase-8 is associated with short sleep duration and the risk of incident diabetes mellitus. J Clin Endocrinol Metab 2018;103:1592–1600

25. Xue L, Borné Y, Mattisson IY, et al. FADD, caspase-3, and caspase-8 and incidence of coronary events. Arterioscler Thromb Vasc Biol 2017;37: 983–989

26. Donath MY, Böni-Schnetzler M, Ellingsgaard H, Halban PA, Ehses JA. Cytokine production by islets in health and diabetes: cellular origin, regulation and function. Trends Endocrinol Metab 2010;21:261–267

27. Cron L, Allen T, Febbraio MA. The role of gp130 receptor cytokines in the regulation of metabolic homeostasis. J Exp Biol 2016;219:259–265

28. Ellingsgaard H, Hauselmann I, Schuler B, et al. Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. Nat Med 2011;17:1481–1489

29. Pedersen BK. Physical activity and muscle-brain crosstalk. Nat Rev Endocrinol 2019;15:383–392

30. Xiao X, Yang C, Qu SL, et al. S100 proteins in atherosclerosis. Clin Chim Acta 2020;502:293–304

31. Brahimaj A, Ligthart S, Ghanbari M, et al. Novel inflammatory markers for incident pre-diabetes and type 2 diabetes: the Rotterdam Study. Eur J Epidemiol 2017;32:217–226

32. Donath MY, Dalmas É, Sauter NS, Böni-Schnetzler M. Inflammation in obesity and diabetes: islet dysfunction and therapeutic opportunity. Cell Metab 2013;17:860–872

33. Pham MN, Hawa MI, Pfleger C, et al.; Action LADA Study Group. Pro- and anti-inflammatory cytokines in latent autoimmune diabetes in adults, type 1 and type 2 diabetes patients: Action LADA 4 [published correction appears in Diabetologia 2012;55:534]. Diabetologia 2011;54:1630–1638

34. Pham MN, Hawa MI, Roden M, et al.; Action LADA Study Group. Increased serum concentrations of adhesion molecules but not of chemokines in patients with type 2 diabetes compared with patients with type 1 diabetes and latent autoimmune diabetes in adult age: action LADA 5. Diabet Med 2012;29:470–478

35. Staiger H, Keuper M, Berti L, Hrabe de Angelis M, Häring HU. Fibroblast growth factor 21-metabolic role in mice and men. Endocr Rev 2017;38:468–488

36. Koliaki C, Szendroedi J, Kaul K, et al. Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. Cell Metab 2015;21:739–746

37. Paquissi FC. Immune imbalances in non-alcoholic fatty liver disease: from general biomarkers and neutrophils to interleukin-17 axis activation and new therapeutic targets. Front Immunol 2016;7:490

38. Hatting M, Zhao G, Schumacher F, et al. Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents. Hepatology 2013;57:2189–2201

39. Chu X, Jin Q, Chen H, et al. CCL20 is up-regulated in non-alcoholic fatty liver disease fibrosis and is produced by hepatic stellate cells in response to fatty acid loading. J Transl Med 2018;16:108

40. Bönhof GJ, Herder C, Strom A, Papanas N, Roden M, Ziegler D. Emerging biomarkers, tools, and treatments for diabetic polyneuropathy. Endocr Rev 2019;40:153–192

41. Herder C, Roden M, Ziegler D. Novel insights into sensorimotor and cardiovascular autonomic neuropathy from recent-onset diabetes and population-based cohorts. Trends Endocrinol Metab 2019;30:286–298

42. Tabák AG, Jokela M, Akbaraly TN, Brunner EJ, Kivimäki M, Witte DR. Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study. Lancet 2009;373:2215–2221

43. Carstensen M, Herder C, Kivimäki M, et al. Accelerated increase in serum interleukin-1 receptor antagonist starts 6 years before diagnosis of type 2 diabetes: Whitehall II prospective cohort study. Diabetes 2010;59:1222–1227

44. Herder C, Kowall B, Tabak AG, Rathmann W. The potential of novel biomarkers to improve risk prediction of type 2 diabetes. Diabetologia 2014;57:16–29

45. Turner R, Stratton I, Horton V, et al.; UK Prospective Diabetes Study Group. UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. Lancet 1997;350:1288–1293

46. Tuomi T, Carlsson A, Li H, et al. Clinical and genetic characteristics of type 2 diabetes with and without GAD antibodies. Diabetes 1999;48:150–157