Leukocyte Counts and T-Cell Frequencies Differ Between Novel Subgroups of Diabetes and Are Associated With Metabolic Parameters and Biomarkers of Inflammation

Jacqueline M. Ratter-Rieck,1,2 Haifa Maalmi,1,2 Sandra Trenkamp,1,2 Oana-Patricia Zaharia,1–3 Wolfgang Rathmann,2,4 Nanette C. Schloot,1 Klaus Straßburger,2,4 Julia Szendroedi,1–3 Christian Herder,1–3 and Michael Roden,1–3 for the German Diabetes Study (GDS) Group*

Diabetes 2021;70:2652–2662 | https://doi.org/10.2337/db21-0364

Frequencies of circulating immune cells are altered in those with type 1 and type 2 diabetes compared with healthy individuals and are associated with insulin sensitivity, glycemic control, and lipid levels. This study aimed to determine whether specific immune cell types are associated with novel diabetes subgroups. We analyzed automated white blood cell counts (n = 669) and flow cytometric data (n = 201) of participants in the German Diabetes Study with recent-onset (<1 year) diabetes, who were allocated to five subgroups based on data-driven analysis of clinical variables. Leukocyte numbers were highest in severe insulin-resistant diabetes (SIRD) and mild obesity-related diabetes (MOD) and lowest in severe autoimmune diabetes (SAID). CD4+ T-cell frequencies were higher in SIRD versus SAID, MOD, and mild age-related diabetes (MARD), and frequencies of CCR4+ regulatory T cells were higher in SIRD versus SAID and MOD and in MARD versus SAID. Pairwise differences between subgroups were partially explained by differences in clustering variables. Frequencies of CD4+ T cells were positively associated with age, BMI, HOMA2 estimate of β-cell function (HOMA2-B), and HOMA2 estimate of insulin resistance (HOMA2-IR), and frequencies of CCR4+ regulatory T cells with age, HOMA2-B, and HOMA2-IR. In conclusion, different leukocyte profiles exist between novel diabetes subgroups and suggest distinct inflammatory processes in these diabetes subgroups.

Immune cells play an important role in the pathogenesis of diabetes and diabetes-associated complications. In type 1 diabetes, T cell–mediated β-cell destruction drives hyperglycemia and disease onset (1). In addition to CD4+ and CD8+ T cells, which are autoreactive against islet antigens, regulatory T cells (Tregs) are also involved in the pathogenesis. Although frequencies of Tregs in peripheral blood do not seem to be altered in type 1 diabetes, their suppressive function is impaired (2), and restoring Treg function is one of the aims in the development of novel treatments for type 1 diabetes (3). There is further evidence for a role of B lymphocytes in the pathogenesis of type 1 diabetes, but depletion of B lymphocytes had only limited effects on the decline of β-cell function in type 1 diabetes (4).

In those with type 2 diabetes, subclinical inflammation is characterized by higher numbers of leukocytes in peripheral blood compared with individuals with type 1 diabetes and healthy controls (5). T cells isolated from peripheral blood (6) and adipose tissue (7) of individuals with type 2 diabetes are skewed toward a more proinflammatory phenotype and...
contribute to the development of insulin resistance and complications (8). Whether T cells also contribute to islet autoimmunity in type 2 diabetes remains controversial (9). Although the frequency of natural killer (NK) cells was reduced in peripheral blood of those with type 2 diabetes compared with healthy individuals (5), increased frequencies of circulating interleukin-6 receptor subunit α–positive (IL-6Rα⁺) NK cells are found in obese individuals and may contribute to insulin resistance (10). Whereas monocyte counts and the distribution of monocyte subpopulations in peripheral blood do not seem to be altered in those with diabetes compared with healthy controls (5, 11), monocyte function in patients with type 2 diabetes is characterized by increased adherence to endothelial cells and cytokine production, which promote the development of complications (12).

Altered frequencies of immune cells are associated not only with incident type 2 diabetes (13) but also with metabolic parameters, such as fasting glycemia, insulin resistance, and plasma lipid levels (5, 14), and with the risk of complications, such as cardiovascular disease (15) and peripheral neuropathy (16), in individuals with diabetes.

Recently, five novel diabetes subgroups were defined by Ahlqvist et al. (17) based on six variables: age at diagnosis, BMI, HbA₁₀, HOMA2 estimates of β-cell function (HOMA2-B) and insulin resistance (HOMA2-IR), and GAD antibodies (GADA). These subgroups differ not only in their metabolic phenotypes, such as whole-body and adipose tissue insulin resistance, but also in the prevalence and risk of diabetes complications (17–19). We recently showed that subgroups of individuals with recent-onset diabetes also differ in circulating levels of biomarkers of inflammation, with the highest levels of proinflammatory biomarkers in the severe insulin-resistant diabetes (SIRD) subgroup (20). These differences in biomarkers of inflammation may contribute to differences in the risk of complications between the subgroups. Whether diabetes subgroups are also characterized by different frequencies of immune cells is currently unknown but relevant to better understand the progression of diabetes.

We therefore aimed to comprehensively compare immune cell profiles among novel diabetes subgroups in individuals with recent-onset diabetes and test whether differences in immune cell profiles are partly explained by the between-cluster differences in anthropometric, clinical, and biochemical profiles. Furthermore, we aimed to analyze how specific immune cell types correlate with biomarkers of inflammation in the German Diabetes Study (GDS).

RESEARCH DESIGN AND METHODS
Study Participants
The analysis is based on data from individuals with recent adult-onset diabetes who participated in the GDS, an ongoing prospective observational cohort study (21), registered at ClinicalTrial.gov (NCT01055093). The study was approved by the ethics committee of Heinrich-Heine-University, Düsseldorf, Germany (reference 4508) and is performed in accordance with the Declaration of Helsinki. All participants provided written informed consent. Patients were allocated to predefined diabetes subgroups based on age, BMI, HbA₁₀, HOMA2-B, HOMA2-IR, and GADA using the nearest centroid approach with sex-specific classification rules published by Ahlqvist et al. (17). Participants positive for GADA were allocated to the severe autoimmune diabetes (SAID) subgroup. This analysis included 708 patients assigned to subgroups with available data from flow cytometric measurements (n = 201) and/or complete data from automated blood cell counts (n = 669) (Supplementary Fig. 1).

Measurement of Anthropometric and Clinical Variables
Measurements of anthropometric parameters and metabolic variables (including plasma glucose, cholesterol [total, high-density lipoprotein, low-density lipoprotein], serum triglycerides, HbA₁₀, and C-peptide) with standard laboratory procedures were described previously (21). HOMA2-B and HOMA2-IR were calculated using the HOMA2 calculator (University of Oxford) based on fasting C-peptide and glucose levels. Autoantibodies to full-length GAD were determined by a radioligand assay, and the estimated glomerular filtration rate was calculated based on serum creatinine and cystatin C levels as previously described (18). hs-CRP levels were determined with a Roche/Hitachi High-Technologies C311 analyzer. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or use of antihypertensive medication. Anti-inflammatory medication was paused for a minimum of 7 days prior to blood sampling.

Characterization of Leukocytes
Automated white blood cell counts were performed on the Sysmex KX21 or XP300 (Sysmex, Norderstedt, Germany). Flow cytometric analysis of leukocytes from fresh whole blood was performed as described (5). The following antibodies were used to identify the analyzed cell populations: CD3 (SK7), CD4 (SK3), CD8 (SK1), CD25 (2A3), CD194/CCR4 (1G1), CD56 (NCAM16.2), and CD19 (SJ25C1) from Becton Dickinson (Heidelberg, Germany) and CD127 (HCD127) and CD161 (HP-3G10) from BioLegend (San Diego, CA). Samples were measured on a dual laser FACSCalibur cytometer (Becton Dickinson). Acquisition of samples was performed using Cellquest software (Becton Dickinson). Samples were analyzed with FlowJo software (version 10). Gating strategies for the different cell populations are shown in Supplementary Fig. 2.

Proteomic Analysis
Protein biomarkers were measured in serum of fasting GDS participants using the Inflammation panel from Olink Proteomics (Uppsala, Sweden) (20, 22). This assay allows the relative quantification of 92 protein biomarkers by proximity extension assay technology. Protein concentrations
are given as normalized protein expression values on a log2 scale. Normalized protein expression values are calculated from cycle threshold values using normalization procedures to minimize intra- and interassay variations. One biomarker was excluded because of technical issues, and 17 biomarkers were excluded because they had >25% of values below the lower detection limit. Intra- and interassay coefficients of variation are described by Herder et al. (20).

**Statistical Analysis**

All statistical analyses were performed in RStudio (version 4.0.2). Data are presented as medians (25th percentile; 75th percentile) or percentages. Differences in the clinical characteristics of the study population across diabetes subgroups (Table 1) were analyzed with ANOVA or \( \chi^2 \) test. Pairwise differences between cell types across diabetes subgroups were estimated using linear models using the gls function from the nlme package without or with adjustment for clinical variables used to define the subgroups. The gls function fits a linear model using generalized least squares. Variances were allowed to be different between diabetes subgroups. All variables were log transformed (base e). \( P \) values of pairwise mean differences were adjusted for pairwise multiple comparisons using the Tukey-Kramer procedure (based on 10 different combinations of subgroups) of the emmeans package. \( P \) values <0.05 were considered to indicate statistically significant differences. Correlations between cell counts or frequencies and anthropometric or clinical variables were estimated for the whole study sample (i.e., all subgroups combined) using Spearman rank correlation coefficients and corresponding \( P \) values using the rcorr function from the Hmisc package. Some of these correlations have been previously analyzed in

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Data are presented as median (25th percentile, 75th percentile) or percentage (%). The frequency of missing data for all parameters was <3% per subgroup, except for estimated glomerular filtration rate (eGFR) (maximum 12.9% missing data).
an overlapping study sample stratified by type 1 and type 2 diabetes (14). For correlations with protein biomarkers within the diabetes subgroups, Bonferroni-corrected $P$ values $<0.0007$ (0.05/74) indicated significant differences (adjusted for multiple testing of different biomarkers). We did not adjust for testing of different cell types.

**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 M.R.).

**RESULTS**

**Study Population**
We analyzed automated blood cell counts in a subpopulation of 669 GDS participants with recent-onset diabetes who were assigned to diabetes subgroups (SAID $n = 232$; severe insulin-deficient diabetes [SIDD] $n = 17$; SIRD $n = 36$; mild obesity-related diabetes [MOD] $n = 190$; mild age-related diabetes [MARD] $n = 194$) based on age, BMI, HbA$_1c$, HOMA2-B, HOMA2-IR, and GADA (Supplementary Fig. 1 and Table 1, which lists participant characteristics). Participants of the different diabetes subgroups differed not only in the clustering variables but also in other clinical variables, such as plasma lipid levels. Additionally, we analyzed flow cytometric data, which were available for 201 participants (SAID $n = 59$; SIDD $n = 3$; SIRD $n = 13$; MOD $n = 68$; MARD $n = 58$) (Table 2 lists participant characteristics). Both study populations had similar proportions of participants assigned to specific subgroups (Supplementary Table 1). Proportions were also similar to the proportions found in all GDS participants assigned to subgroups in a previously published analysis (18).

**Leukocyte Counts and Neutrophil-to-Lymphocyte Ratios Are Different Between Diabetes Subgroups**
Unadjusted results of the automated blood cell counts are summarized in Supplementary Table 2. Blood leukocyte counts were highest in MOD and lowest in SAID (Fig. 1A, Fig. 2A, and Supplementary Table 3, which lists full results). Leukocyte counts were significantly higher in SIRD, MOD, and MARD versus SAID, SIRD versus MARD, and MOD versus MARD (Fig. 1A and Fig. 2A). The percentage of lymphocytes was lower in SIRD, MOD,

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Data are presented as median (25th percentile, 75th percentile) or percentage (%). The number of missing data for all parameters was no more than two missings per subgroup, except for estimated glomerular filtration rate (eGFR) (eight, zero, one, nine, and 15 missings for SAID, SIDD, SIRD, MOD, and MARD, respectively).
CD4⁺-to-CD8⁺ T-Cell Ratios and Frequencies of Treg Subpopulations Differ Between Diabetes Subgroups

We analyzed frequencies of multiple lymphocyte populations determined by flow cytometry in blood samples of 201 study participants with subgroup allocation (Supplementary Table 4). B-cell frequencies were higher in SIDD versus MARD (Fig. 3A, Fig. 4A, and Supplementary Table 5, which lists full results). No significant differences between diabetes subgroups were found for frequencies of NK cells, NK T cells, and CD4⁺CD8⁺ double-positive T cells (Fig. 4A). CD4⁺ T-cell frequencies were higher in SIRD versus MOD, MARD, and SAID, and CD8⁺ T-cell frequencies were lower in SIRD versus SAID and MOD, resulting in a higher CD4⁺-to-CD8⁺ T-cell ratio in SIRD versus SAID, MOD, and MARD (Fig. 3B–D and Fig. 4A). Furthermore, frequencies of CCR4⁺ Tregs were higher in MARD versus SAID and in SIRD versus MOD and SAID (Fig. 3E and Fig. 4A). No differences were observed for CD161⁺ Tregs (Fig. 3F and Fig. 4A).

Frequencies of NK cells were positively correlated with age and negatively with HbA₁c. Frequencies of CD4⁺CD8⁺ double-positive T cells and CD4⁺ T cells and the CD4⁺-to-CD8⁺ T-cell ratio were positively correlated with age, BMI, HOMA2-B, and HOMA2-IR and negatively with GADA for CD4⁺ T cells and the CD4⁺-to-CD8⁺ T-cell ratio (Fig. 4B). CD8⁺ cells showed associations with the same clustering variables as CD4⁺ cells, but in the opposite direction (Fig. 4B). Frequencies of CCR4⁺ Tregs were positively correlated with age, HOMA2-B, and HOMA2-IR and negatively correlated with GADA. Frequencies of B cells, NK T cells, Treg, and CD161⁺ Tregs were not correlated with any of the clustering variables (Fig. 4B).

After adjustment for all variables defining the clusters, B-cell frequencies were lower in SAIĐ versus SIDD.
Leukocytes | Lymphocytes | Neutrophils | NLR
---|---|---|---
MOD | SIRD | MARD | SAID
NLR | 0.30 | 0.00 | -0.15 | -0.30

**DISCUSSION**

Our study demonstrates that leukocyte numbers and immune cell frequencies in peripheral blood differ among novel diabetes subgroups. Leukocyte counts and NLRs were highest in SIRD, MOD, and lowest in SAID. Furthermore, frequencies of CD4\(^+\) and CD8\(^+\) T cells and frequencies of CCR4\(^+\) T\(_{reg}\) differed between subgroups, suggesting that distinct lymphocyte populations govern the inflammatory processes in different diabetes subgroups. The attenuation of most pairwise differences in leukocyte numbers and immune cell frequencies between the subgroups after adjustment for the clustering variables indicates that these clustering variables explain a relevant proportion of these differences.

We found higher leukocyte counts in SIRD, MOD, and MARD compared with SAID, which is in line with previous studies reporting higher leukocyte counts in type 2 diabetes (5). Our study extends these findings by showing that leukocyte counts are also higher in SIRD and MOD compared with MARD and thus differ between different type 2 diabetes subgroups. Overall, there were fewer pairwise differences between any pair of type 2 diabetes subgroups than between SAID and the type 2 diabetes subgroups, highlighting the fact that immune cell profiles are more different between type 1 and type 2 diabetes than between different type 2 diabetes subgroups. Within the type 2 diabetes subgroups, SIDD, MOD, and MARD had rather similar immune cell profiles, whereas multiple differences between these subgroups and the SIRD subgroup were observed. Inflammation-related processes underlying complications may therefore be similar in SIDD, MOD, and MARD, but different in SAID and SIRD.

Our study shows that the SIRD subgroup had high leukocyte numbers and the highest NLR among the novel diabetes subgroups. These data extend previous analyses...
in the GDS showing that the SIRD subgroup was also characterized by the highest levels of hs-CRP (18) and other biomarkers of inflammation, such as IL-6 and S100 calcium-binding protein A12 (S100A12/EN-RAGE) (20). This highly proinflammatory profile in SIRD may play an important role in the development of complications of diabetes. The NLR, which was previously shown to predict cardiovascular events (23), was higher in SIRD, MOD, and MARD versus SAID. In accordance, SIRD had the highest cumulative evidence for coronary events in the ANDIS cohort (17). SIRD also has the highest prevalence of stage 2 and 3 nephropathies (18). Blood leukocyte counts were previously associated with inflammation, such as IL-6 and S100 calcium-binding protein A12 (S100A12/EN-RAGE) (20). Other biomarkers of inflammation, such as hs-CRP (18) and CD161+ T cells, CD4+-to-CD8+ T-cell ratio varies over a broad range in healthy individuals and is influenced by genetic factors, sex, and age (28). We observed a negative correlation of CD8+ T-cell frequencies with age. CD8+ T cells are reduced in number with age, but also show a higher degree of terminal differentiation (29). Although individuals with type 2 diabetes seem to have higher frequencies of terminally differentiated CD8+ T cells compared with healthy controls (30), it is currently unknown which metabolic parameters influence differentiation and senescence of T cells in diabetes. Furthermore, it would be interesting to determine the degree of T-cell differentiation in different diabetes subgroups and investigate whether the secretory phenotype of terminally differentiated T cells (31) contributes to diabetes complications.

Higher frequencies of CD4+ T cells and lower frequencies of CD8+ T cells in SIRD versus SAID may also be

Figure 3—Distribution of lymphocyte frequencies among different diabetes subgroups. A–F: Percentage of B cells of total lymphocytes (A), percentage of CD4+ (B) and CD8+ (C) T cells of total CD3+ T cells, CD4+-to-CD8+ T-cell ratio (D), percentage of CCR4+ Tregs of total Tregs (E), and percentage of CD161+ Tregs of total Tregs (F) were assessed by flow cytometry in different diabetes subgroups. Upper and lower hinges of the boxplots correspond to the 75th and 25th percentiles, respectively. Whiskers extend to largest and smallest values (maximum 1.5 interquartile range) away from the hinge. P values were adjusted for pairwise multiple comparisons using the Tukey method.

Although SIRD and MOD both had similarly high leukocyte counts, the immune cell composition differed in these subgroups. Consequently, inflammation may be important in both subgroups, but different cell types may drive the underlying inflammatory processes. For future studies, it will be important to investigate whether immune cell distributions also change over time, because individuals may be allocated to different subgroups during the course of the disease (18).
driven by increased proliferation of CD4+ T cells. CD4+ T-cell proliferation is increased during infections but can also be modulated by metabolic factors. CD4+ T cells lacking insulin receptor expression showed decreased proliferation (32,33). Although it is unknown how insulin influences human T-cell proliferation, one could speculate that differences in insulin resistance and circulating insulin concentrations between subgroups may influence T-cell proliferation and frequencies.

CCR4+ Treg cells are antigen-primed Treg cells with immediate suppressive function (34), and their frequencies were increased in elderly (88–89 years) versus middle-aged (50–65 years) and young (18–25 years) individuals (35). How changes in Treg frequency and function may counteract systemic inflammation observed in aged individuals is mainly unexplored. It is possible that increasing CCR4+ Treg frequencies in old age (and in SIRD) reflect a counterregulation of altered immunosuppressive function of these cells. CCR4+ Treg cells can be recruited to various metabolic tissues, for instance to the gut (36), the adipose tissue (37), or the pancreas (38). Although CCR4+ Treg cells may play an important role in the protection of β-cells against autoreactive T cells (38), their role in the pathogenesis of type 2 diabetes is mainly unexplored.

We did not find consistent differences between subgroups for B cells and NK cells. These results are in line with the fact that data on the contribution of B cells to diabetes progression or development of complications are mostly lacking. Because IL-6Rα+ NK cells have been associated with insulin resistance and obesity-associated inflammation (10), a more detailed characterization of NK cells including various cell surface receptors may be necessary to discover differences in NK cell subpopulations between diabetes subgroups.

Our correlation analysis of leukocyte types with protein biomarkers revealed both associations that were similar in all groups (e.g., OSM correlated with leukocyte numbers in SAID, MOD, and MARD) and associations that were subgroup specific (e.g., IL-15Rα correlated with CCR4+ Treg only in MARD).

Leukocyte numbers were positively associated with OSM, HGF, and TGF-α, which can all be produced by granulocytes. These biomarkers were associated with leukocyte numbers in several diabetes subgroups and may therefore play a more general role in inflammation. OSM is produced by granulocytes, but also by the stromal vascular fraction in the adipose tissue (39), where it can act locally by inducing inflammation in adipocytes (40).

Whether OSM is a cause or consequence of inflammation in diabetes and how it contributes to complications in different diabetes subgroups remain elusive. HGF, in contrast, was already associated with incident distal sensorimotor polyneuropathy in a population-based cohort (41), whereas TGF-α was inversely associated with estimated glomerular filtration rate in patients with diabetes (42).
TWEAK and DNER were negatively correlated with NLR only in the MARD subgroup. Circulating concentrations of TWEAK were negatively correlated with fasting glucose and are decreased in those with type 2 diabetes and in individuals with end-stage renal disease (43). TWEAK concentrations were also negatively correlated with subclinical atherosclerosis (44), and therefore, participants in the MARD subgroup with high NLRs and low serum levels of TWEAK may be at higher risk for cardiovascular complications. Higher levels of TWEAK and DNER were also associated with better nerve conduction in patients with type 2 diabetes (45).

We found a strong correlation between frequencies of CCR4+ Tregs and IL-15Rα in MARD, but not in the other subgroups. In human blood, IL-15Rα forms a complex
with IL-15, thereby stabilizing the cytokine, which has been shown to influence the expansion of CD8+ T cells (46) and FoxP3+ Treggs (47). Circulating levels of IL-15 were associated with adipose tissue distribution (48) and were higher in patients with coronary artery disease (48). It is currently unknown whether differences in CCR4+ Treggs in subgroups are associated with differences in adipose tissue physiology.

A key strength of our study is the recent onset of diabetes (<1 year since diagnosis of diabetes) in our study population. This limits confounding effects of long-term hyperglycemia or presence of long-term diabetes-associated complications on immune cell populations and biomarkers of inflammation. In addition, the extent of the phenotyping allows a substantially more detailed immunologic characterization of the novel diabetes subgroups. One limitation of our study is the small number of individuals in the SIDD and SIRD subgroups, especially for the flow cytometric data. Furthermore, we performed an exploratory study and therefore did not apply Bonferroni correction for multiple testing of different cell types. Although we had flow cytometric data available for several lymphocyte populations, we did not have information available on the myeloid cell compartment (classic vs. alternative monocytes, dendritic cells) and therefore cannot estimate the contribution of these cell types to complications in the different subgroups. Our study also lacks functional characterization of immune cell types (e.g., by analysis of cytokine production), which would be desirable in future studies to clarify the functional role of immune cells in the development of diabetes and its complications. Therefore, it is not known yet whether the detailed characterization of immune cell types will identify targets that could affect the specific clinical treatment of individuals in the novel diabetes subgroups.

In conclusion, our study highlights that immune cell frequencies in the circulation differ between subgroups of diabetes. Different T-cell frequencies in different subgroups of type 2 diabetes suggest distinct inflammatory processes related to adaptive immunity in these diabetes subgroups. Several of the pairwise differences between the diabetes subgroups are in part explained by the phenotypic variables underlying the clustering algorithm to define the subgroups. Future studies investigating functional properties of circulating immune cells and immune cell composition in tissues may help to clarify how different immune cell types contribute to inflammation in different subgroups of diabetes.

**Acknowledgments.** The authors appreciate the contribution of all study participants. The authors thank the staff of the Clinical Research Center at the Institute for Clinical Diabetology, German Diabetes Center, for their excellent work.

**Funding.** The GDS was initiated and financed by the German Diabetes Center, which is funded by the German Federal Ministry of Health (Berlin, Germany), the Ministry of Culture and Science of the state of North Rhine-Westphalia (Düsseldorf, Germany), and grants from the German Federal Ministry of Education and Research (Berlin, Germany) to the German Center for Diabetes Research e.V. (DZD).

The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

**Duality of Interest.** N.C.S. is currently employed by Lilly Germany GmbH, Bad Homburg, Germany. No other potential conflicts of interest relevant to this article were reported.

**Author Contributions.** J.M.R.-R. performed the statistical analysis and wrote the manuscript. J.M.R.-R. and C.H. designed the study, drafted the analysis plan, and interpreted data. H.M., W.R., K.S., and C.H. contributed to the statistical analysis. S.T., O.-P.Z., N.C.S., J.S., C.H., and M.R. contributed data. C.H. and M.R. contributed to the draft of the manuscript. M.R. contributed to data interpretation. All authors reviewed and edited the manuscript and approved its submission. J.M.R.-R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Prior presentation.** Data from this study were presented at the virtual Diabetes Congress of the German Diabetes Association (DDG), 12–15 May 2021, and the virtual Annual Meeting of the European Association for the Study of Diabetes (EASD), 28 September–1 October 2021.

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