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Cohort profile: the 'Biomarkers of heterogeneity in type 1 diabetes' study—a national prospective cohort study of clinical and metabolic phenotyping of individuals with long-standing type 1 diabetes in the Netherlands

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ABSTRACT

Purpose The 'Biomarkers of heterogeneity in type 1 diabetes' study cohort was set up to identify genetic, physiological and psychosocial factors explaining the observed heterogeneity in disease progression and the development of complications in people with long-standing type 1 diabetes (T1D).

Participants Data and samples were collected in two subsets. A prospective cohort of 611 participants aged ≥16 years with ≥5 years T1D duration from four Dutch Diabetes clinics between 2016 and 2021 (median age 32 years; median diabetes duration 12 years; 59% female; mean glycated haemoglobin (HbA1c) 61 mmol/mol (7.7%); 61% on insulin pump; 23% on continuous glucose monitoring (CGM)). Physical assessments were performed, blood and urine samples were collected, and participants completed questionnaires. A subgroup of participants underwent mixed-meal tolerance tests (MMTTs) at baseline (n=169) and at 1-year follow-up (n=104). Genetic data and linkage to medical and administrative records were also available. A second cross-sectional cohort included participants with ≥35 years of T1D duration (currently n=160; median age 64 years; median diabetes duration 45 years; 45% female; mean HbA1c 58 mmol/mol (7.4%); 51% on insulin pump; 83% on CGM), recruited from five centres and measurements, samples and 5-year retrospective data were collected.

Findings to date Stimulated residual C-peptide was detectable in an additional 10% of individuals compared with fasting residual C-peptide secretion. MMTT measurements at 90 min and 120 min showed good concordance with the MMTT total area under the curve. An overall decrease of C-peptide at 1-year follow-up was observed. Fasting residual C-peptide secretion is associated with a decreased risk of impaired awareness of hypoglycaemia.

Future plans Research groups are invited to consider the use of these data and the sample collection. Future work will include additional hormones, beta-cell-directed autoimmunity, specific immune markers, microRNAs,

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ The Biomarker cohort is a large longitudinal prospective cohort study with three time points, collecting biosamples and clinical data from participants with well-established and long-standing type 1 diabetes (≥5 years).
⇒ A subgroup with detailed clinical data underwent mixed meal tolerance tests at two time points, allowing further residual beta-cell marker studies.
⇒ The Biomarker and Long-term Type 1 Diabetes cohorts represent a 'real-world' population, also including participants from non-academic/specialised centres.
⇒ Despite the fact that data and biosamples were collected from more than 600 participants, this number may be too low for (sub)stratification of the data (eg, insulin delivery modality, different treating centres and therapies, etc).
⇒ In the prospective group, there was a relatively high dropout rate of 25% after 2 years, largely affected by the COVID-19 outbreak.

metabolomics and gene expression data, combined with glucometrics, anthropometric and clinical data, and additional markers of residual beta-cell function.

Trial registration number NCT04977635.

INTRODUCTION

Type 1 diabetes (T1D) is characterised by severe insulin deficiency caused by insulin-producing beta-cell dysfunction, followed by autoimmunity that damages beta cells .[1 2] This disease process frequently occurs in people with specific genetic backgrounds and is characterised by two presymptomatic phases (stages 1 and 2). Stage 3 is the phase of the appearance of clinical symptoms, the establishment of the clinical diagnosis and the initiation of insulin treatment.[3] Stage 4 is long-standing T1D.[4] The loss of beta cell function, as measured by C-peptide, starts in the presymptomatic phases and progresses through stages 3 and 4 T1D, resulting in the need for life-long insulin supplementation to survive. However, residual C-peptide production has been demonstrated in long-standing diabetes,[1 5 6] conferring clinical benefits in glycated haemoglobin (HbA1c) and long-term complications,[7 8] although the majority of persons with T1D (PWDs) with long-duration diabetes are microsecreters.[9 10] Despite the currently available treatments and promising developments in diabetes technology, reaching glycaemic targets is very difficult, if not impossible for many PWDs.[11] Consequently, exposure to suboptimal glucose levels causes microvascular and macrovascular, psychological and psychosocial complications in the long run in stage 4. This profoundly impacts life expectancy, leading to a loss of 10–18 life years, depending on the age of diagnosis.[12] Complications are strongly dependent on the PWD's historical glycaemic regulation, which in turn is determined by many factors and shows extensive heterogeneity between PWDs. Indeed, as illustrated by the Joslin Medalist cohort, there are PWDs with very long-duration T1D and very few complications, with one-third to one-half of them producing detectable C-peptide.[9 13] Cohorts of PWDs with long-standing T1D can thus contribute to elucidating the mechanisms and pathways involved in microvascular and macrovascular complications and comorbidities.[6 14 15] There are also indications for common protective factors between different complications.[16] Differences in quality of life between PWDs with and without complications have been reported to increase with diabetes duration.[17] In addition to biological factors (ie, genetic predisposition to insulin resistance and/or vascular damage and residual insulin production),[18] this heterogeneity also includes the availability and access to healthcare, medication and technology,[19] and psychosocial factors.[20] To prevent the development of T1D-related complications, international guidelines recommend HbA1c levels below 7% (53 mmol/mol)[21] or time in targeted blood glucose range (TIR) >70%.[22] However, only a minority of PWDs are currently achieving the recommended HbA1c levels.[11 23] This may partly be explained by the aforementioned extensive heterogeneity in pathology seen in all four stages of T1D. Clinical symptoms and severe metabolic disturbance (eg, diabetic ketoacidosis[24]) at onset, autoimmune markers before and after onset, initial glycaemic outcomes (HbA1c, acute glucose levels and TIR) and the efficacy of therapeutic interventions[18 25] all vary between PWDs. Furthermore, the T1D phenotype represents different distinct underlying functional or pathobiological mechanisms, also called endotypes.[26] Improving future outcomes will depend on the ability to further unravel this heterogeneity, dissect endotypes, develop individualised prediction, prevention and intervention strategies, and eventually even restore immunological tolerance and beta-cell mass.[27] Using combinations of (epi)genetic data (eg, genetic risk loci) and disease biomarkers (clinical data, metabolic markers and immunological markers) can provide a new integrative approach that will help to develop personalised T1D interventions.[2]

Together with the Juvenile Diabetes Research Foundation (JDRF) and the Dutch Diabetes Research Foundation ('Diabetes Fonds' (DF) in Dutch), we identified needs and research questions for a new project on evaluating existing and searching for new biomarkers, based on research gap analysis and existing knowledge of the field. Several studies on factors of heterogeneity and biomarkers in T1D around the onset of the disease (ie, stages 1–3) have been initiated.[25] However, unravelling the heterogeneity in long-standing T1D and identifying biomarkers to predict future complications require combining historical clinical data on PWDs with longitudinally collected biosamples. For instance, there are not many studies with longitudinal data on beta cell function in T1D of long duration.[9]

With funding from JDRF and DF, we developed the 'Biomarkers of heterogeneity in T1D' prospective cohort study. The main aim of this study was to prospectively collect data and biosamples from PWDs with long-standing T1D (≥5 years) in a real-world setting to detect changes in glycaemic markers, hormonal markers, immune/inflammatory markers and metabolic markers, including genetic, metabolomic and proteomic analyses. For example, certain medium-chain fatty acids and short-chain fatty acids have been suggested to be protective against albuminuria development in T1D.[28] Omics approaches, also applied in this Biomarker study, are very promising for biomarker development, but so far they have not been able to deliver validated biomarkers for clinical use, probably because of the fragmented nature of the information obtained through the single omics approach.[29 30] Novel computational approaches to data processing may help overcome challenges associated with the relatively small number of subjects in studies.[29] Additionally, psychosocial outcomes were measured, emphasising the impact of diabetes on psychosocial functioning and vice versa. All these data can be interlinked with anthropometric and clinical parameters. In this cohort profile paper, we provide a description of the study setup,

baseline characteristics of the participants, follow-up information, results of initial analyses and future aims. Data and samples are available for additional projects and collaborative research.

COHORT DESCRIPTION

The prospective 'Biomarkers of heterogeneity in T1D' cohort study (Clinicaltrials.gov/ NCT04977635; called 'Biomarker study' from hereon) was initiated by Diabeter Netherlands (Rotterdam, the Netherlands) and the University Medical Centre Groningen (UMCG; Groningen, the Netherlands). A total of around 600 participants was deemed sufficient to stratify for age and diabetes duration while ensuring practical and financial feasibility. Diabeter and UMCG provided the majority of patients (Diabeter, n=333; UMCG, n=185), limiting variation in treatment among participants. Additionally, in an attempt to reach planned inclusion numbers with enough participants from different age groups, PWDs from Haaglanden Medical Centre (The Hague, the Netherlands; n=78) and Ikazia Hospital (Rotterdam, the Netherlands; n=9) were recruited. Finally, six participants requested to be included on their own initiative, having heard of the study through social media channels. The study ran from 2016 to 2023. Between June 2016 and March 2021, a total of 611 PWDs aged ≥16 years and with a diabetes duration of ≥5 years (called the 'Biomarker complete study cohort' from hereon) were included (figure 1). The T1D diagnosis was determined by either the presence of diabetes autoantibodies, based on clinical and historical data, or both. Exclusion criteria were all types of diabetes that are not considered T1D according to American Diabetes Association criteria,[31] pregnancy (until 3 months after childbirth) and breastfeeding (until 3 months after breastfeeding), using experimental medication or participating in other studies with conflicting goals and schedules, decision against participation at the investigator's/physician's discretion and being unwilling to be informed on incidental findings. All PWDs who were included in the study were also invited to participate in additional mixed meal tolerance tests (MMTT). A group of around 150 participants (~50% with 5–15 years and 50% with >15 years of diabetes duration) was deemed sufficient for MMTT analyses to assess the reproducibility of C-peptide measurements over time and identify the additional value of an MMTT over a fasting C-peptide sample in our cohort. Finally, 169 participants (28%) positively responded (called the 'Biomarker MMTT-subcohort' from hereon). Participation in this substudy was voluntary, and no additional inclusion or exclusion criteria were applied; that is, this was not a selected group.

During this study, an opportunity arose to enrich the dataset with cross-sectional data and biosamples from PWDs with at least 35 years of diabetes duration: the 'Long-term T1D' cohort (NL62401.042.17; called the 'LTD cohort' from hereon). This cross-sectional cohort was also initiated by the UMCG and Diabeter Netherlands. This study included 160 PWDs with a diabetes duration of ≥35 years. T1D diagnosis and exclusion criteria are equivalent to the prospective Biomarker study. The cohort comprises participants from the Biomarker complete study cohort and PWDs recruited from the Martini Hospital (Groningen, the Netherlands), the Wilhelmina Hospital (Assen, the Netherlands) and the Treant Hospital Group (locations Emmen, Hoogeveen and Stadskanaal, the Netherlands). Recruitment started in 2019 and is expected to close by the end of 2023.

The project and amendments for additional research and future research questions were approved by the Medical Ethics Review Board of the UMCG (Biomarker complete study cohort: METC 2015/493; LTD cohort: METC 2017/412).

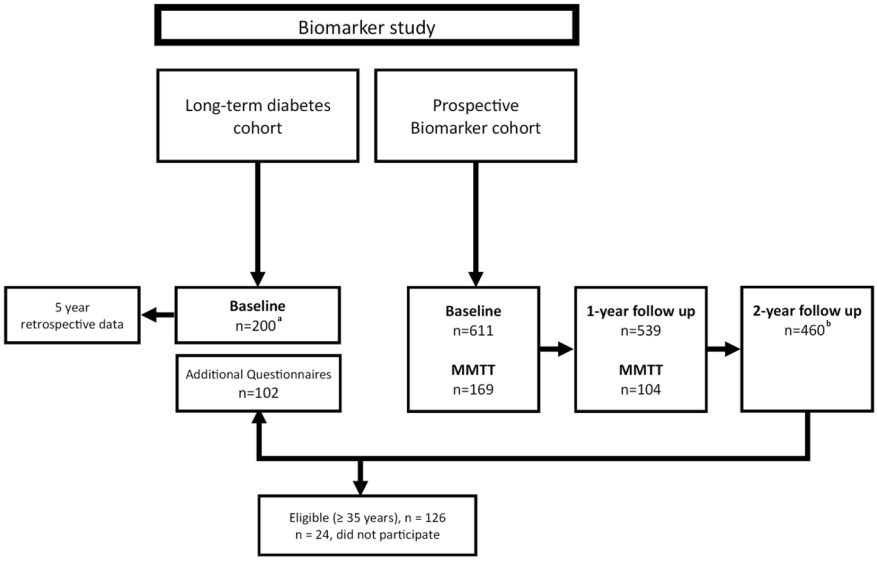

Figure 1 Flowchart of participant inclusion. The superscript letter 'a' refers to the aim to include 200 individuals. The superscript letter 'b' refers to the fact that n=23 of these n=460 skipped the 1-year follow-up.

Recruitment

Eligible PWDs received (electronic) flyers and notifications about the studies and were asked by their diabetes care providers about their interest in participating. Interested PWDs were contacted, provided with information and given the opportunity to ask questions to an independent physician. After written informed consent was provided, the participant was enrolled in the study and assigned a unique study number.

Data and sample collection

Online supplemental table 1 lists the parameters collected from the study participants.

Biomarker complete study cohort (prospective)

At the baseline visit, fasting blood and urine samples were collected. Part of the samples underwent immediate analysis of routine haematology and biochemistry, and the remainder of the samples were stored for future analysis. All participants completed six questionnaires: the World Health Organisation-Five Well-Being Index (WHO-5),[32] the Problem Areas In Diabetes Scale (PAID),[33 34] the WHO Quality of Life questionnaire (WHOQOL),[35] the Diabetic Neuropathy (DN4) questionnaire[36] and the Dutch version of the Clarke hypoglycaemic (impaired awareness of hypoglycaemia (IAH)) questionnaire,[37 38] either on paper or online. UMCG participants underwent anthropometric assessments and foot examinations, including arterial pulsation, tuning fork and monofilament evaluation,[39] by a trained physician assistant. For the participants attending the other participating clinics, anthropometric data were retrieved from their electronic health records (EHRs) by the study team after enrolment. All study procedures were repeated at the 1-year and 2-year follow-up visits, except for the questionnaires, which were repeated only at the 2-year follow-up.

Biomarker MMTT-subcohort (prospective)

Participants provided additional informed consent to participate in the MMTTs. They underwent an MMTT at baseline (n=169) and a 1-year follow-up (n=104). If possible, the MMTT was carried out during the same appointment as the fasting blood and urine sample collection. Alternatively, a separate appointment was made by the study team.

LTD cohort (cross-sectional)

At the study visit, fasting blood (when possible) and urine samples were collected. Part of the samples underwent immediate analysis of routine haematology and biochemistry and the remainder of the samples were stored for future analysis. Each participant completed paper-and-pencil questionnaires. Participants completed five questionnaires on quality of life (EuroQol-five dimension (EQ-5D)[40] and Patient Health Questionnaire-9 (PHQ-9)[41]), psychosocial burden and fears (Hypoglycaemia Fear Survey-II (HFS-II),[42] neuropathy (DN4),[36] IAH (Dutch version of the Clarke questionnaire[37 38]) and physical activity (International Physical Activity Questionnaire (IPAQ)[43]). Participants from all sites underwent anthropometric assessments and foot examinations, including arterial pulsation, tuning fork and monofilament evaluation,[39] by a trained physician. Retrospective data up to 5 years before inclusion were extracted from their EHRs by the study team after enrolment. Individuals included in the Biomarker complete study cohort with a diabetes duration of ≥35 years were contacted after the 2-year follow-up to fill out additional questionnaires (EQ-5D, PHQ-9, HFS-II and IPAQ) in order to harmonise the data between the LTD cohort participants derived from the Biomarker complete study cohort and those recruited additionally. Participants were only contacted if they had previously consented to be contacted for follow-up studies. When informed consent had been provided, the additional questionnaires were sent out. All study materials (eg, collected samples and paper questionnaires) and study data(sets), including medical record data and results from the questionnaires and sample analysis, were stored under the participant's unique study number.

Biochemical analyses and storage of samples and data

Biomarker complete study cohort

At each visit, blood samples were collected in coagulation (Becton-Dickinson, cat. no. 367953), lithium-heparin (Becton-Dickinson, cat. no. 367378), EDTA (Becton-Dickinson, cat. no. 367525), EDTA P800 (BD 366421) and PAXgene RNA (Qiagen, cat. no. 762165; at baseline only) blood collection tubes. PAXgene DNA tubes (Qiagen, cat. no. 761115) were collected for non-UMCG participants at one visit during the study. For UMCG participants, DNA was isolated from EDTA and EDTA-P800 pellets. The blood collected in coagulation tubes was allowed to coagulate for 30 min. Coagulation, lithium-heparin, EDTA, EDTA P800 and urine tubes were centrifuged at room temperature for 10 min at 1500 rpm. Fasting morning urine was collected in tubes without any additives (Becton-Dickinson, cat. no. 365000). DNA samples were genotyped using the Infinium Global Screening Array–24 v1 and v3 Illumina (San Diego, USA) as described earlier.[44]

Biomarker MMTT-subcohort

For the MMTT procedure, the target glucose level at the start of the test was between 3.3 and 12 mmol/L. If values were lower than 3.3 mmol/L, oral glucose was administered, and glucose levels were checked every 30 min until levels were in range again. If values were higher, a correction bolus was needed, and participants were retested after 30–45 min. If glucose values during the night prior to the test were continuously >12 mmol/mL, tests were rescheduled. An intravenous catheter was placed in a cubital vein or in the hand-wrist area, from which blood was sampled.

At time point 0, participants were given a dose of Resource Protein (Nestlé) mixed meal (@1.25 kcal/mL), comprising 6 mL/kg body weight to a maximum of 60 kg (=360 mL). The dose had to be consumed in no more than 5 min. Blood was collected at 0, 30, 60, 90 and 120 min in three types of tubes: coagulation tubes (Becton-Dickinson, cat. no. 367955), lithium-heparin tubes (Becton-Dickinson, cat. no. 367376) and EDTA P800 tubes (BD 366421). At each time point, glucose from the drawn blood was also measured using a point-of-care glucose meter.

LTD cohort

At the baseline visit, blood samples were collected in coagulation (Becton-Dickinson, cat. no. 367953), lithium-heparin (Becton-Dickinson, cat. no. 367378), EDTA (Becton-Dickinson, cat. no. 367525), trisodium citrate (Bectron-Dickinson, cat. No. 366575) and PAXgene RNA (Qiagen, cat. no. 762165) blood collection tubes. Fasting morning urine and 2-hour postprandial urine were collected in tubes without any additives (Becton-Dickinson, cat. no. 365000). The blood collected in coagulation tubes was allowed to coagulate for 30 min. Coagulation, lithium-heparin and EDTA tubes were centrifuged at room temperature for 10 min at 1300 rpm. DNA was isolated from EDTA pellets. Trisodium citrate tubes were centrifuged for 20 min at 1300 rpm. Urine sample tubes were centrifuged at 4° for 10 min at 2000 rpm.

The availability of biosamples is listed in table 1. All biosamples were aliquoted and stored at −80°C in dedicated freezers located in the clinical laboratory of the IJsselland Hospital (Capelle aan den IJssel, the Netherlands) and of the UMCG. Data from biochemical analyses and extracted from EHRs were collected in multiple databases stored on secure servers at the UMCG and at Diabeter.

Patient and public involvement

Participants, funders or the public were not involved in the design, conduct, reporting or dissemination plans of this study. Participants were updated via e-mail newsletters, the Diabeter Netherlands website (www.diabeter.nl) and on social media. We have presented initial results and will present future results of the studies at national and international conferences, in peer-reviewed research papers, various other channels, including local and social media, and via the research website: www.diabeterresearch.com.

Table 1 Biosample availability for baseline, 1-year and 2-year follow-ups

Biosamples	Biosamples baseline	First follow-up (T1)	Second follow-up (T2)
Biomarker complete study cohort			
Serum	✓	✓	✓
Lithium-heparin plasma	✓	✓	✓
EDTA (plasma)	✓	✓	✓
EDTA-P800 (plasma)	✓	✓	✓
RNA (whole blood)	✓		
DNA (from whole blood/buffy coat)	✓		
Urine (normal)	✓	✓	✓
Mixed meal tolerance tests cohort (additional samples to biosamples Biomarker complete study cohort)			
Serum (timepoints 0, 30, 60, 90 and 120 min)	✓	✓	
Lithium-heparin plasma (timepoints 0, 30, 60, 90 and 120 min)	✓	✓	
EDTA-P800 plasma (timepoints 0, 30, 60, 90 and 120 min)	✓	✓	
Long-term type 1 diabetes cohort			
Lithium-heparin	✓		
DNA (from whole blood/buffy coat)	✓		
Serum	✓		
Morning urine	✓		
Urine 2 hours postprandial	✓		
EDTA (plasma)	✓		
Citrate (plasma)	✓		
RNA (whole blood)	✓		

Table 2 Participants' characteristics at baseline (n=611), mean (SD) and range (unless stated otherwise)

	Biomarker complete study cohort		MMTT cohort		LTD cohort	
		n		n		n*
Age, median (IQR), range (years)	31.7 (23.2–52.4), 16.0–80.4	611	26.3 (21.5–47.9), 16.0–74.2	169	64 (56–70), 39–88	160
Age at diagnosis, median (IQR), range (years)	12.3 (7.9–20.6), 0.8–68.1	611	11.5 (7.9–17.0), 0.8–55.3	169	15 (8–23), 0–49	169
Diabetes duration, median (IQR), range (years)	18.5 (11.8–29.9), 3.8–72.6	611	17.2 (10.3–27.8), 5.0–64.8	169	45 (41–51), 35–72	160
Sex (% female)	59	611	59	169	45	175
Blood pressure (mm Hg)		588		163		155
Systolic	128 (13), 90–183		126 (12), 90–163		137 (18), 64–218	
Diastolic	72 (8), 45–102		71 (8), 52–101		70 (10), 41–99	
Height (cm)	175 (9), 145–200	610	174 (9), 156–193	169	175 (9), 146–203	160
Weight (kg)	79 (13), 52–123	592	78 (12), 54–113	166	81 (16), 38–134	161
Body mass index (kg/m^2)	26 (4), 18–40	592	26 (4), 19–37	166	27 (4), 15–40	159
Total daily insulin dose, median (IQR), range (U/day)	50 (40–64), 15–179	598	52 (42–64), 15–130	165	30 (18–45), 8–124	114
Glycated haemoglobin		609		169		112
mmol/mol	61 (13), 23–124		60 (12), 23–115		58 (9), 36–87	
%	7.7 (1.1), 4.3–13.5		7.7 (1.1), 4.3–12.7		7.4 (0.8), 5.4–9.1	
Glucose (mmol/L)	9.6 (3.9), 1.0–28.3	609			–	
C-peptide undetectable versus detectable (%)		609		169		
undetectable (<3.8 pmol/L)	74		75			
Detectable (≥3.8 pmol/L)	26		25			
C-peptide concentration in participants with detectable C-peptide, median (IQR), range (pmol/L)	30 (10–110), 3.9–1439	155	31 (10–129), 4–741	43	–	
Insulin administration (%)		610		169		150
multiple daily injections	39		33		49	
insulin pump	61		67		51	
Glucose monitoring method (%)		610		169		120
SMBG	77		82		17	
rt-CGM	19		14		47	
is-CGM	4		4		36	
Total cholesterol (mmol/L)	4.4 (0.8), 2.6–8.9	611	4.3 (0.8), 2.8–7.0	169	4.3 (0.9), 0.5–7.2	145
High-density lipoprotein-cholesterol (mmol/L)	1.7 (0.5), 0.6–3.6	611	1.7 (0.5), 0.9–3.6	169	1.7 (0.5), 0.3–3.1	146
Low-density lipoprotein-cholesterol, mmol/L	2.7 (0.7), 0.8–5.8)	611	2.6 (0.7), 1.3–5.2	169	2.4 (0.7), 0.9–5.4	147
Triglycerides, median (IQR), range (mmol/L)	0.9 (0.7–1.2), 0.2–5.4	606	0.8 (0.7–1.1), 0.3–4.7	164	1.0 (0.7), 0.4–6.4	147
Apolipoprotein B (mmol/L)	0.8 (0.2), 0.3–1.7	611	0.8 (0.2), 0.4–1.7	169	–	
Antihypertensive medication (%)	21	595	14	168	34	159
Lipid-lowering medication (%)	23	596	19	168	26	159

*Recruitment still ongoing.
is-CGM, intermittently scanned-continuous glucose monitoring; rt-CGM, real-time continuous glucose monitoring; SMBG, self-monitoring of blood glucose.

FINDINGS TO DATE

Baseline characteristics

The baseline characteristics of the study participants are shown in table 2. The median (IQR) age of the Biomarker complete study cohort participants at baseline was 31.7 (23.2–52.4) years, and 59% were women. The majority of participants were of Western European origin. The median age at diagnosis was 12.3 (7.9–20.6) years, and the median duration of diabetes was 18.5 (11.8–29.9) years. Participants used a median total daily dose of insulin of 50 (40–64) U/day and had a mean (SD) HbA1c of 61 (13) mmol/mol (7.7 (1.1)%). Fasting C-peptide levels were measured for the whole 'Biomarker complete study cohort'. The majority of participants (74%) did not produce any detectable fasting levels of C-peptide (<3.8 pmol/L). The 26% of participants who did showed a

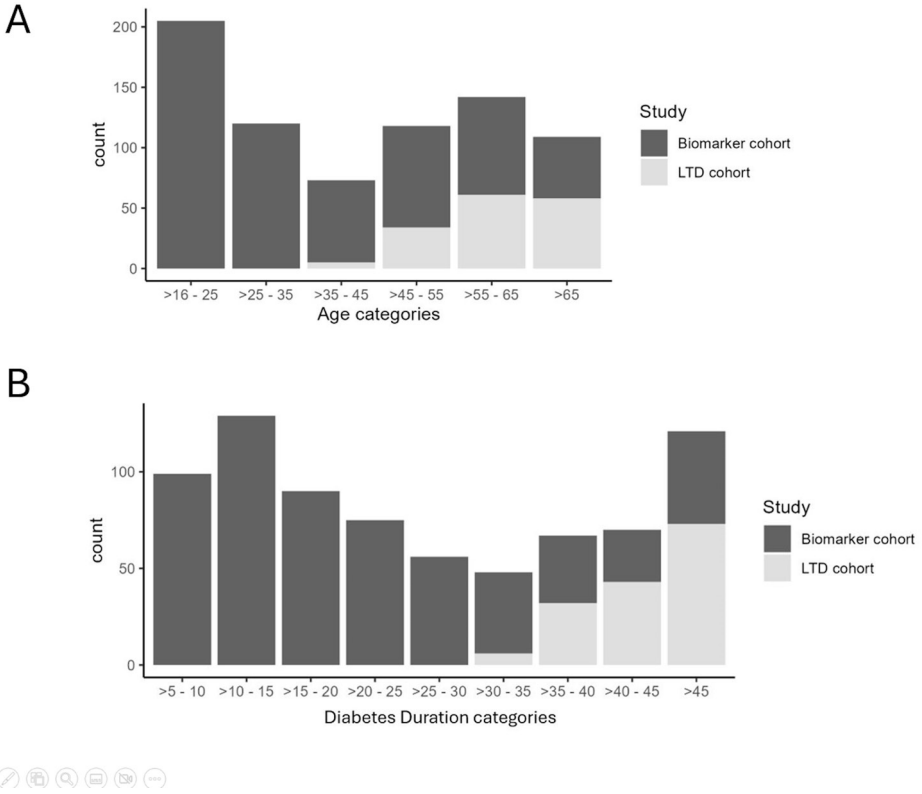

Figure 2 Distributions of (A) age and (B) diabetes duration.

median fasting C-peptide level of 30 (10–110) pmol/L. The individuals in the LTD cohort had a median age and diabetes duration of 63 and 45 years, respectively (see also figure 2). Almost half the participants were women, with a mean body mass index (BMI) of 27 kg/m². The mean HbA1c was 58 mmol/mol (7.4%), with a median total daily dose of 30 U/day. Around half the participants used an insulin pump, with 47% using real-time-CGM (rt-CGM) and 36% using intermittently scanned-CGM. This is higher when compared with the Biomarker complete study cohort, likely because rt-CGM was reimbursed at the time this study started. Antihypertensive and lipid-lowering drugs were used by 21% and 16% of the study participants, respectively.

Follow-up information

Biomarker complete study cohort

After baseline recruitment of 611 participants (T0), 539 (88%) returned for a 1-year follow-up visit between May 2017 and March 2020 (T1), after a median (IQR) follow-up time of 12.6 (11.9–13.5) months after T0 (range: 8.9–20.3). Two-year follow-up visits were completed by 460 participants (75%) between April 2018 and March 2021 (T2), after a median of 13.0 (11.7–13.6) months after T1 (range: 4.8–35.4). Reasons for loss to follow-up included, but were not restricted to, COVID-19 (40%), own request/no time (23%), diabetes treatment at another hospital (17%), pregnancy/breastfeeding (7%), illness/medication use/accident (5%), death (2%) and other reasons (6%).

MMTT cohort

169 participants (28%) underwent an MMTT between August 2016 and February 2019 and 2020, of whom 104 underwent a second MMTT (62% of the 169 participants who underwent an MMTT at baseline) between August 2017 and March 2019, after a median follow-up time of 12.4 (11.9–13.4) months (range: 8.6–25.3).

Publications to date

Many different commercially available C-peptide assays are used for research and routine analysis, but not all assays are suitable for measurements in the lower picomolar range and those which are not equally sensitive.[45] Samples from the cohort (only from one centre) were first used to compare and verify the ultrasensitive Mercodia enzyme-linked immunosorbent C-peptide assay (ELISA) with the Beckman immunoradiometric assay (IRMA) for C-peptide. Reproducibility (coefficient of variation), limit of blank, limit of detection and limit of quantitation were compared.[46] Because only the IRMA met the specifications claimed by the manufacturer, providing the lowest threshold for quantification of serum C-peptide, we chose this assay for our C-peptide analyses.[46]

Second, in an analysis of the longitudinal cohort, we assessed the association between fasting serum C-peptide levels and the presence of IAH in PWDs.[47] Residual C-peptide secretion was associated with a lower risk of IAH and a higher BMI, the presence of microvascular complications and a higher age at diabetes onset were independent risk factors for IAH in PWDs. Specific signalling

and metabolic pathways involved in the counterregulatory response to hypoglycaemia are especially affected in PWDs with IAH and are suggested to result in adaptive changes in the brain. In the most recent publication resulting from this study,[48] metabolomics and genome-wide association methodologies were combined to look for metabolites that are expressed differentially between PWDs with IAH and PWDs without IAH. Compared with controls, PWDs with IAH were significantly older, had longer diabetes duration, a lower daily insulin dose and used more antihypertensive and lipid-lowering medications. Twelve metabolites were identified that showed higher expression in PWDs with IAH. These were sphingomyelins and glycerophospholipids, suggesting differences in nerve functioning.

Third, the stability of residual C-peptide production over time was assessed.[49] About 25% of PWDs still showed some residual C-peptide production (table 2). About 10% of participants who did not show fasting residual C-peptide production did still show meal-stimulated residual C-peptide production. Because the MMTT procedure constitutes a burden to PWDs, an easier way to reliably assess residual C-peptide secretion is required, for example, a simplified MMTT that tests only one time point. To assess if fasting residual C-peptide production or residual C-peptide production at 90 min or 120 min after an MMTT may be sufficient to identify residual C-peptide production, we compared these variables with the C-peptide area under the curve (AUC) of the complete MMTTs. The 90-min and 120-min MMTT time points showed good concordance with the MMTT total AUC. Overall, there was a decrease in C-peptide at 1-year follow-up.[49]

Next steps

Diabetes research on the intersection of immunology and metabolism is a field that is developing at a fast pace.[50] While research is increasingly focusing on the role of the innate immune system in the earliest stages of the disease and its sequelae,[51 52] our main goal was to focus on heterogeneity in hormonal, immune, inflammatory, and metabolic markers and insulin resistance in PWDs with long-standing T1D. With regard to hormones, the samples have already been analysed for fasting and stimulated C-peptide. In addition to being a proxy for residual insulin production, there is evidence that C-peptide is a biologically active peptide,[13] which is an interesting avenue for additional research. Next, we plan to measure additional relevant hormones (eg, glucagon, glucagon-like peptide (GLP)-1, GLP-2, leptin, growth hormone and proinsulin), innate immune markers (eg, C reactive protein, fibrinogen and complement component C3) and specific immune markers like cytokines (eg, interleukin (IL)-6, IL-10, tumour necrosis factor (TNF)-alpha) and adipokines (eg, chemerin, CCL2 and adiponectin). Immune markers have been described to be differentially expressed in PWDs with different durations of T1D.[53]

The innate immune system is able to sense metabolic stress induced by factors such as nutritional components and changes in the intestinal microbiota, refocusing the problem of diabetes on other organs, such as the liver and the gut.[54] We aim to explore some of the involved pathways in an integrated systems biology approach to assess clinical heterogeneity and improve clinical phenotyping in T1D. While metabolic biomarkers associated with the early pathogenesis of T1D are established,[55] there is a need for similar markers that exist beyond the clinical onset period. Currently, samples from the Biomarker cohorts are undergoing genetic, metabolomic and proteomic analyses. These data can then be linked to the hormonal data. We also plan to estimate additional features of insulin resistance based on blood pressure, insulin dose per kg of lean body mass, markers of lipid metabolism (fasting free fatty acids, triglycerides and high-density lipoprotein cholesterol), liver enzymes and adipokines (IL6, TNF-alpha and adiponectin). In addition, new markers such as microRNA (miRNAs) and exosomes (miRNA containing vesicles) have been identified as promising markers of disease and of complications in T1D.[56–60] miRNA markers related to autoimmunity in T1D have been shown to be maintained or even increased in long-duration T1D.[61] All these data can be interlinked with data like residual beta-cell function (as measured by C-peptide levels), anthropometric and clinical parameters like, glycaemic outcomes, and beta-cell-directed autoimmunity.

Strengths and limitations

With regard to the prospective part of the study, the main strengths of this cohort are the prospective nature of the measurements and the collection of biosamples from participants with established T1D, whereas most longitudinal studies collect biosamples in the context of the early phases of T1D. To test the stability of C-peptide assessments, a subgroup of participants underwent an MMTT at two time points. Clinical and biochemical data can be linked to the results of ancillary genetic studies on DNA and mRNA and a large pool of data, and samples are available for collaborative projects. For both cohorts, detailed clinical data are available for all participants and for a large subgroup of PWDs historical glucose and HbA1c data are also available. Furthermore, PWDs were recruited from multiple clinics, including non-academic/specialised centres, representing a 'real-world' population.

This study also has some limitations. First, while data and biosamples were collected for more than 600 participants, this number may be too low for detailed analyses in specific PWD subgroups (eg, insulin delivery modality, different treating centres and therapies). However, although biomarker research indeed often uses large retrospective EHR data sets, there are a number of caveats to the use of large data sets.[62] It is difficult to collect good quality data in real-world healthcare practice settings, with the quality of data in EHRs depending on which information is collected and how. Social and behavioural factors, also collected in our Biomarker study, are rarely recorded in EHRs while they drive more than half of the variance

in health outcomes, with medical care explaining only about 10%. It is often assumed that large sample sizes will mitigate systematic biases (eg, in information collection, missing data in EHRs, measurement error and unreliable measures) and other issues in samples of people from EHR systems, but ignoring these biases may compromise the applicability of research findings as results will largely be based on random variation. The effect sizes found in small trials are much more meaningful and relevant for individual patients. Indeed, pilot studies with smaller sample sizes, like our Biomarker study, are often meant to assess if it is worthwhile to initiate larger studies and also to get a feel for the required sample size.[63] A second limitation is that participants were followed for only 2 years, which is relatively short, especially in long-standing T1D. Third, this cohort may not be representative of the general Dutch population of PWDs, considering the high percentage of mainly younger participants using technology. It is likely that these participants feel more engaged in looking for solutions for T1D (management), potentially introducing participation bias. This is likely due to Diabeter's history, starting out as a paediatric centre for T1D care and research. Over the years, many adult PWDs were transferred to the Diabeter at their own initiative. Also, clustering of care for specific diseases is currently a trend in the Netherlands, and Diabeter has taken over the care of adult PWDs from a number of hospitals. Fourth, our study included relatively few people with adult-onset T1D, which relates to the points just discussed. PWDs with adult-onset T1D can develop certain comorbidities more frequently than PWDs with childhood-onset T1D, for example, coeliac disease.[15] Fifth, the inclusion age of ≥16 years prevents extrapolation of the data to younger cohorts, because the pathophysiological processes in younger patients are different and more rapid at onset, resulting in less C-peptide reserve, which may relate to different disease processes later in the course of the disease. Sixth, in this study, cellular immunity was not assessed so it is not possible to investigate possible associations between beta-cell function and beta-cell autoimmunity. For instance, it is known that the function and phenotype of innate-like T lymphocytes are different in PWDs with long-term versus recent-onset T1D.[64] Finally, there was a relatively high dropout rate of 25% after 2 years, largely influenced by the COVID-19 outbreak.

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Competing interests Diabeter Netherlands is an independent clinic, which was acquired by Medtronic. The research presented here was independently performed and there is no conflict of interest.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

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Ethics approval This study involves human participants and was approved by the project and amendments for additional research and future research questions were approved by the Medical Ethics Review Board of the University Medical Center Groningen (Biomarker complete study cohort: METC 2015/493; LTD cohort: METC 2017/412). Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available upon reasonable request. We welcome collaboration with other research groups interested in the data/samples of this cohort. Researchers can visit the Diabeter Research website (https://www.diabeterresearch.com/biomarker-study/) for additional information and can contact us at research@diabeter.nl.

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Supplemental table 1: Summary of parameters collected from the cohort.

	Biomarker complete study cohort			**Biomarker MMTT cohort[a]**		**LTD cohort**
	Baseline	**Year 1**	**Year 2**	**Baseline**	**Year 1**	**Baseline**
Sex	✓					✓
Ethnicity	✓					✓
Age at diagnosis	✓					✓
Diabetes duration	✓	✓	✓			✓
Age	✓	✓	✓			✓
Systolic and diastolic blood pressure	✓	✓	✓			✓
Pulse	✓	✓	✓			✓
Height	✓	✓	✓			✓
Weight	✓	✓	✓			✓
BMI	✓	✓	✓			✓
Waist circumference						✓
Hip circumference						✓
Total daily insulin dose	✓	✓	✓			✓
Mode of insulin administration (MDI or insulin pump)	✓	✓	✓			✓
Glucose monitoring method (SMBG, rt-CGM or is-CGM)	✓	✓	✓			✓
Medication use	✓	✓	✓			✓
Co-morbidities/complications (kidney, eye and macrovascular)[b]	✓	✓[c]	✓[c]			✓
HbA1c	✓	✓	✓			✓
Fasting glucose	✓	✓	✓			
Fasting C-peptide	✓	✓	✓			
Glucose during MMTT				✓[d]	✓[e]	
MMTT stimulated C-peptide				✓[d]	✓[e]	

Routine laboratory parameters measured from samples[f]	✓	✓	✓			✓
Routine laboratory parameters extracted from EHRs[f]	✓	✓	✓			✓
Genotyping data from global screening array	✓					
Quality of life (WHO-5)	✓		✓			
Problem Areas in Diabetes (PAID) scale	✓		✓			
World Health Organization Quality of Life (WHOQOL) questionnaire	✓		✓			
Diabetic Neuropathy (DN) questionnaire	✓		✓			✓
Clarke hypoglycaemic questionnaire	✓		✓			✓
EuroQol (EQ-5D)			✓[h]			✓
Patient Health Questionnaire (PHQ-9)			✓[h]			✓
Hypoglycaemia Fear Survey			✓[h]			✓
International Physical Activity Questionnaire			✓[h]			✓

[a] Same as for 'Biomarker complete study cohort', except for MMTT samples

[b] Addison's disease, Angina pectoris, Autonomic Neuropathy, Cerebrovascular Accident (stroke), Chronic Kidney Disease and Dialysis, Coeliac disease, Erectile Dysfunction, Hypothyroidism, Hyperthyroidism, Hypertension, Hypercholesterolemia, Laser treatment, Lower Limb Amputation, Lower limb ulcer, Myocardial Infarction, Peripheral artery disease, Peripheral Neuropathy, Pre-proliferative Retinopathy, Proliferative Retinopathy.

[c] Only at baseline for UMCG participants

[d] n=169

[e] n-104, only Diabeter Netherlands participants

[f] Alanine aminotransferase, Aspartate Aminotransferase, C-reactive protein, Gamma-glutamyltransferase, Total cholesterol, HDL-cholesterol, LDL-Cholesterol, Triglycerides, Apolipoprotein B, Vit D3. In UMCG for all freshly measured on same day as blood draw.

[g] TSH, Free T4, Thyroid peroxidase antibodies, Total cholesterol/HDL-cholesterol ratio, Non-HDL cholesterol, Urine creatinine, Urine albumin, Albumin/creatinine ration, Serum creatinine, Tissue transglutaminase IgA antibodies. In UMCG for all freshly measured on same day as blood draw.

[h] In individuals with ≥35 years T1D in the 'Biomarker complete study cohort' who also agreed to complete the questionnaires of the LTD study.

BMI, body-mass index; EHR, electronic health record; HbA1c, glycated haemoglobin; is-CGM, intermittent scanning continuous glucose monitoring; MDI, multiple daily injection; MMTT, mixed-meal tolerance test; rt-CGM, real-time continuous glucose monitoring; SMBG, self-monitoring of blood glucose; WHO-5, World Health Organisation- Five Well-Being Index.