Towards Early Risk Stratification in Children and Adolescents with Type 1 Diabetes

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Towards Early Risk Stratification in Children and Adolescents with Type 1 Diabetes

Op weg naar een betere inschatting van het risico op het krijgen van complicaties bij kinderen en adolescenten met type 1 diabetes

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit te Rotterdam op gezag van de rector magnificus Prof.dr. R.C.M.E. Engels volgens besluit van het college voor promoties.

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Chapter 1

Introduction

Type 1 diabetes is a T-cell mediated auto-immune disease. Its pathogenesis has not yet completely been elucidated, but factors such as environmental triggers, genetic susceptibility, different age-dependent auto-immune responses, beta-cell subtypes more susceptible for inflammation, and certain viral infections are assumed to be involved in the process leading to diabetes (1). The incidence of type 1 diabetes is still increasing in childhood and adolescence (2-4), the largest increase being observed in the youngest age-group (age 0-4 years) (2, 3). From a lifetime perspective, diagnosis of type 1 diabetes at a younger age will result in longer disease duration, which has been convincingly demonstrated to be one of the major risk factors for the development of the micro- and macrovascular complications of diabetes: neuropathy, nephropathy, retinopathy and premature cardiovascular disease (5-9)(Table 1).

Table 1: Established risk factors for the development of micro- and macrovascular complications

Younger age at onset of diabetes*	(10, 11)
Puberty	(10, 12, 13)
Smoking	(5, 14)
Gender**	(7, 15-17)
Hypertension	(5, 14)
Dyslipidemia	(17, 18)
The presence of microvascular complications	(5, 7, 14, 16, 19)
Increased BMI	(14, 17, 20)

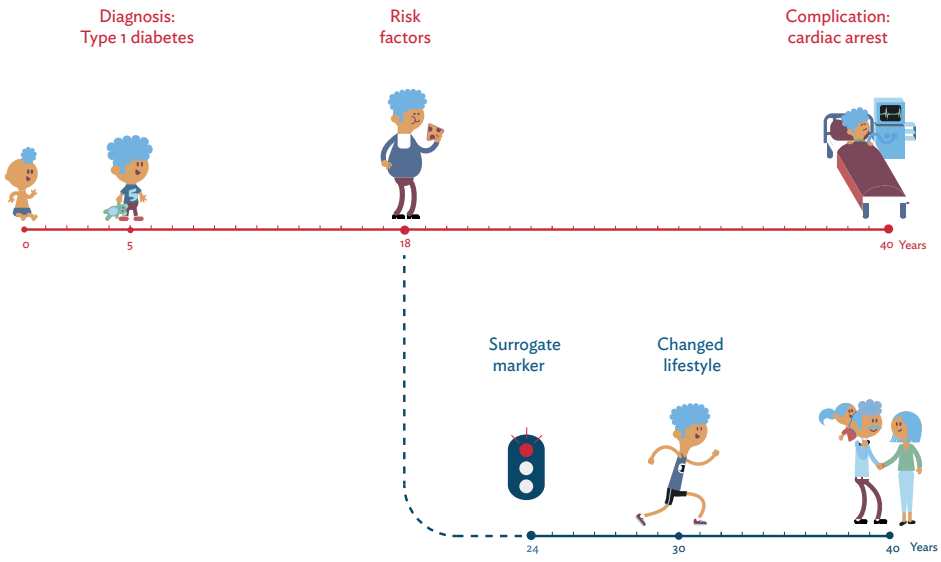
* The effect of young age at onset of diabetes has not consistently been described (10, 11)

** Gender may be of variable influence, depending on the complication studied (7, 15-17)

An increase in these complications is therefore expected to occur. However, whereas diabetes duration is a non-modifiable risk factor associated with complications, there are modifiable risk factors (16, 21-32), poor glycaemic control

(6-8, 16, 21, 22, 33-35) being the most important one. Improvement of glycaemic control has been shown to prevent or delay the development of these complications (7, 22, 35, 36). Controversially, despite improvements in glycaemic control over the last decades, the prevalence and incidence of micro- and macrovascular complications in patients with type 1 diabetes is still alarmingly high in both adults (6, 22, 33, 37-39) and adolescents (35, 40). As these complications are not only associated with decreased quality of life but also with reduced life expectancy up to 10 years (7, 15, 19), the challenge of present diabetes care is to improve this still unfavourable lifetime perspective in patients with type 1 diabetes. In our opinion, early and appropriate risk stratification is an important step towards this challenge. Appropriate risk stratification, i.e. identifying children and adolescents with type 1 diabetes that have one or more risk factor(s) and identifying those that already have early signs of micro- and/or macrovascular complications (Figure 1), may facilitate more individualized patient care. This will in turn allow both personalised screening protocols for the presence of other risk factors and complications and the initiation of intervention where possible.

Figure 1: Illustration of the current liftetime perspective of one patient in red. The expected change in lifetime perspective, based on an alarming result of a surrogate marker described in this thesis, is shown in blue.



Terminology:

Risk factor: each determinant in a determinant-endpoint relationship **Surrogate marker:** a substitute for a clinical endpoint which is associated with that endpoint or prognostic of that endpoint **Early sign of complication:** preclinical and/or clinical abnormality that may progress to a complication, i.e. endpoint **Complication:** clinical condition with medical /personal consequences and requiring treatment and/or medication and/or intensive follow-up

The general objective of this thesis is to improve this risk stratification in children and adolescents with type 1 diabetes, aiming to fill a scientific gap by studying some underexposed complication fields in pediatrics. In section 1.2 and Appendix 1 we review what was known in the literature in 2007 on tests in these fields that were not included in the current guidelines at the time (41). Literature from 2007 on other fields is outside the scope of this thesis but is discussed in Appendix 1. In section 1.3 we describe the research questions of the 'Early Detection of Diabetes Damage in Youth and Search for early prevention' (EDDDY-S) study, which was based on this review.

1.2 Risk factors and potential markers for micro- and macrovascular complications studied in the EDDDY-S study

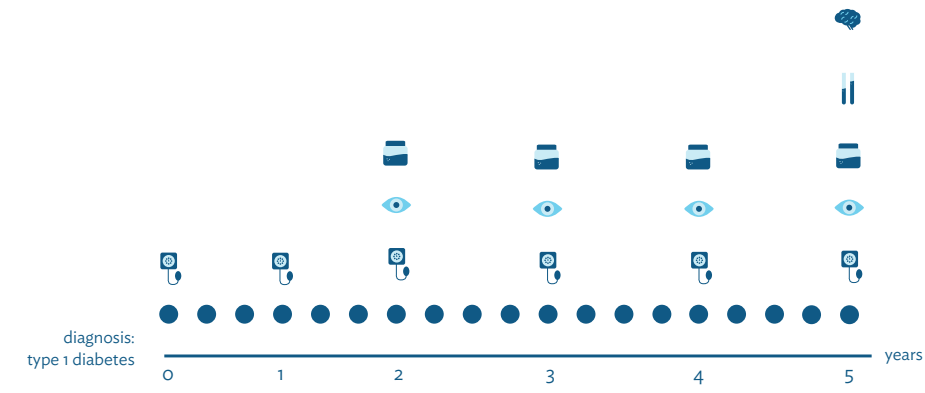
Two tests for diabetic peripheral neuropathy (DPN)

In 2007, the ISPAD guideline recommended the performance of history taking, physical examination and (optional) the measurement of the nerve conduction velocity by electrophysiologic study as screening tests for DPN (41). These tests were assumed to detect clinical DPN or, in case of abnormal nerve conduction velocity (NCV) only, to detect subclinical DPN. In daily practise, history taking and physical examination were performed.

Electrophysiologic studies, including NCV of sensory and motor nerves and sensory action potential (SNAP) amplitude, measure the function of large myelinated nerve fibers. In 2007, the diagnostic value of these electrophysiologic studies for the detection of subclinical DPN in children and adolescents with type 1 diabetes was debated (43). Besides, earlier detection of subclinical DPN by focus on

unmyelinated small fiber nerve dysfunction (assumed to precede dysfunction of the large myelinated nerve fibers) and more focus on axonal damage (assumed to precede myelin damage) was subject of debate as well (43-45). However, at that time, there was only a paucity of studies investigating damage of small unmyelinated nerve fibers in adults with type 1 diabetes and there were only few studies that focussed on other measures of dysfunction of the large myelinated nerve fibers than the conventional test of electrophysiologic study (13, 43, 45-50).

Figure 2: ISPAD Clinical Practice Consensus Guidelines 2006-2007. Microvascular and macrovascular complications. Pediatr Diabetes. 2007;8(3):163-70.



Neurological examination: history taking and physical examination.
When to commence screening is unclear.

Lipid profile: LDL-C, HDL-C, triglycerides after the age of 12 years.

AER/ACR or first morning albumin concentration from the age of 11 years with 2 years of diabetes duration and from the age of 9 years with 5 years of diabetes duration

Fundal photography or mydriatic ophthalmoscopy from the age of 11 years with 2 years of diabetes duration and from the age of 9 years with 5 years of diabetes duration

Blood pressure annually

Three monthly: HbA1c, BMI

Carotid intima media thickness (cIMT)

The 2007 ISPAD guideline recommended screening for the presence of risk factors for macrovascular complications (41). These recommendations lack assessment of any surrogate marker for macrovascular disease. By 2007, measurement of carotid intima media thickness (cIMT), endothelial dysfunction and arterial stiffness, were suggested to be surrogate markers for macrovascular disease in type 1 diabetes (51-53). Of these, most studies were on cIMT.

Atherosclerosis leads to premature cardiovascular disease (54) and was shown to develop, among others, in the carotid intima (55). Hyperglycaemia was found as one of the risk factors for atherosclerosis and increased cIMT (56-58). Indeed, increased cIMT had been found in adult patients with type 1 diabetes compared with healthy controls (52). In children and adolescents with type 1 diabetes, most, but not all, studies found an increased cIMT in the patients as compared with healthy controls (11, 59-62)

Summary of the pathophysiology of type 1 diabetes-related micro- and macrovascular complications

Over the last decades it has become apparent from several clinical studies (e.g DCCT and UKPDS) and preclinical observations that although diabetic complications in specific tissues may result from several pathological processes, hyperglycaemia is involved in most complications. Accompanied by glycaemic variability, hyperglycaemia is considered to be the central metabolic insult on tissues, resulting in activation of common pathways in vulnerable tissues. Aetiological co-factors including smoking, ageing, hyperinsulinaemia, dyslipidaemia and hypertension amplify this response. These common pathways include advanced glycation end products (AGEs) formation, reactive oxygen species overproduction, protein kinase C activation, mitochondrial dysfunction and activation of pro-inflammatory and pro-fibrotic signalling cascades and cytokines, causing the characteristic pathological and clinical abnormalities. Studies have shown that pathways resulting in diabetic complications are self-perpetuating once activated by the 'glycaemic insults'. Epigenetic changes and influences on microRNA expression patterns have been mentioned as potential mechanisms. Microvascular complications affect small vessels of the retina, kidney and nerves, with complications resulting from impaired autoregulation of blood flow, altered permeability, inflammation, extracellular matrix accumulation, hypoxia, cell loss, neovascularisation and fibrosis. Macrovascular complications result from arterial endothelial and smooth muscle inflammation and dysfunction leading to accelerated atherosclerosis, with resultant ischaemic heart disease, cerebrovascular disease and peripheral vascular disease (36, 39, 42).

Longitudinal lipid dynamics

The ISPAD guideline (41) advised annual screening for the risk factor hypertension and screening every 5 years for the risk factor dyslipidemia from the age of 12 years onwards. It also included target levels for body mass index (BMI), smoking behaviour and other known risk factors for macrovascular disease such as HbA1c. By 2007, two large longitudinal studies had determined the prevalence of dyslipidemia and lipid dynamics throughout childhood and adolescence. Prevalence was found to be high. Moreover, a considerable number of patients were found to change their lipid level already throughout childhood and adolescence from a low-risk lipid level to a less favourable one (63, 64).

Advanced glycation end products (AGEs) in skin

AGEs are products of reactive oxygen species and non-enzymatic reactions between sugars and amino groups of proteins ('Maillard reaction') (65, 66). Their production is enhanced in patients with higher glucose levels and other saccharide derivatives (67-69). Local accumulation of AGEs causes, among others, structural alteration of long-lived proteins such as collagen, fibrinogen and myelin through the formation of intermolecular and intramolecular cross-links (67-69). Associations between elevated AGEs and development of micro- and macrovascular complications in type 1 and type 2 diabetes were described in adults (68-71). Altogether, (elevated) AGEs may thus be a surrogate marker for the development of these micro- and macrovascular complications. Quantification of AGEs has been studied by measurement of serum levels of certain AGEs (72, 73), by measurement of collagen glycation by means of determining the plantar fascia thickness in children and adolescents (74) or by skin autofluorescence (SAF) in adults only (71, 75).

1.3 Research questions of the EDDDY-S study

In the design of the EDDDY-S study, we considered that any test to be studied in the pediatric age group should not only meet standard criteria to be suitable as (future) screening test (76), but should also be feasible for use in daily patient care, i.e. preferably being non-invasive, minimally time-consuming, and easy to perform. Furthermore, we aimed to fill a scientific gap by studying some under-exposed areas in complication-related research in pediatrics and in the ISPAD consensus guideline of 2007 (Figure 2). Consequently the EDDDY-S study did not cover tests in all fields of complications, but focused on three main areas (Figure 3).

Two tests for diabetic peripheral neuropathy (DPN)
Tests for the detection of subclinical DPN were found to be missing in the 2007 ISPAD guideline (41).

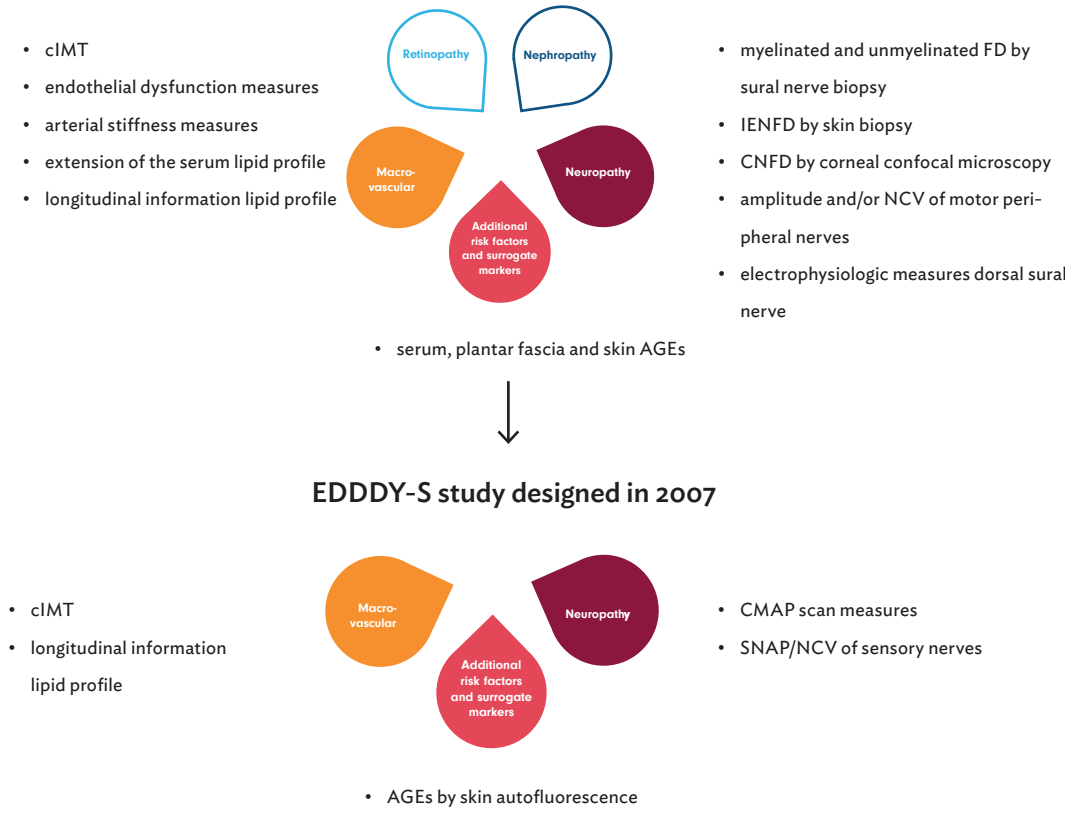
Hence we formulated research question 1:
what is the diagnostic value of measuring various compound muscle action potential (CMAP) scan variables, including measures of axonal excitability, axonal loss and reinnervation of the peroneal nerve by CMAP scan in children and adolescents with type 1 diabetes for the assessment of subclinical DPN? (Chapter 2)

Research question 2:
assesses the diagnostic value of measurement of the sensory nerve conduction velocity (NCV) and sensory nerve action potential (SNAP) amplitude of two distal sensory nerves in children and adolescents with type 1 diabetes for the assessment of subclinical DPN. (Chapter 3)

A response to the letter of Malik et al. was added, highlighting the importance of focussing on small nerve fiber function. (Chapter 4)

Carotid intima media thickness (cIMT)
The 2007 ISPAD guideline (41) did not include a surrogate marker(s) for macrovascular complications.

Figure 3: Promising tests studying some underexposed areas in complication-related research in pediatric diabetes and selected tests in these areas for the EDDDY-S study.



Abbrevations

FD: fiber density
IENFD: intraepidermal nerve fiber density
CNFD: corneal nerve fiber density
NCV: nerve conduction velocity
cIMT: carotid intima media thickness
AGEs: advanced glycation endproducts
EDDDY-S: Early Detection of Diabetes Damage in Youth and Search for early prevention
CMAP: compound muscle action potential
SNAP: sensory nerve action potential

Hence we formulated research question 3:
is the intima-media thickness of the carotid artery (cIMT) in children and adolescents with type 1 diabetes increased when compared with age- and gender-stratified healthy controls and what are the risk factors for increased cIMT in type 1 diabetes patients? (Chapter 5)

Advanced glycation end products (AGEs) in skin
We found that skin autofluorescence, a presumed surrogate marker for micro- and macrovascular complications, was not included in the 2007 ISPAD guideline (Figure 2).

This led to research question 5:
is skin autofluorescence (SAF) in children and adolescents with type 1 diabetes increased when compared with age and gender-stratified healthy controls and what are the risk factors for increased SAF in the type 1 diabetes patients? (Chapter 7)

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Chapter 2

Decreased excitability of the distal motor nerve of young patients with type 1 diabetes mellitus

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Abstract

Objective

The compound muscle action potential (CMAP) scan is a novel neurophysiological technique that appears more sensitive in detecting peripheral motor neuropathy than conventional methods. This study explores the value of the CMAP scan for the detection of subclinical diabetic peripheral motor neuropathy.

Methods

In this cross-sectional pilot study, CMAP scanning of the peroneal nerve was performed in a) 13 well-controlled patients (8-25 years old) with type 1 diabetes mellitus (T1DM) duration between 2.5 and 5 years, b) 17 patients (10-25 years old) with a duration of T1DM of at least 10 years, poorly controlled and/or with microvascular complications and c) 13 adults with T1DM and established clinical diabetic peripheral neuropathy. Various CMAP scan variables, including measures of axonal excitability and axonal loss and reinnervation, were compared between patients and healthy controls.

Results

Axonal excitability was significantly decreased in the young patient groups as compared with their controls. The CMAP scan measures of axonal loss and reinnervation differed only between patients with clinical diabetic peripheral neuropathy and their controls.

Conclusion

Motor nerve axonal excitability seems to be reduced early in T1DM, even in well-controlled young patients, and probably before (irreversible) axonal damage occurs. These changes can be measured by the CMAP scan, which makes this a promising tool for detecting nerve dysfunction in T1DM.

Introduction

Diabetic peripheral neuropathy (DPN) is a frequent complication in patients with type 1 diabetes mellitus (T1DM) (1, 2). Previous studies have shown that subclinical DPN already exists in children and teens with T1DM (3, 4), and that its incidence in this age group is increasing (5). An appropriate screening tool targeted at this age group is of paramount importance since glycemic control is a modifiable risk factor for DPN that may prevent ongoing nerve damage (1, 2, 6).

Presently, there is no established methodology to detect subclinical DPN in young patients (3, 4). The clinical benefit of the frequently applied nerve conduction studies of both motor and sensory nerves is an issue of ongoing debate (7, 8). Moreover, several papers suggest a different vulnerability and/or underlying pathophysiology for motor and sensory nerves (9-12). Nerve function should therefore be evaluated for motor and sensory nerves separately.

A frequently used measure in motor nerve conduction studies is the maximal compound muscle action potential (CMAP) amplitude of the extensor digitorum brevis (EDB) muscle (2, 13). A decreased CMAP amplitude is considered to indicate the loss of (functioning) peroneal nerve axons innervating the muscle fibers of the EDB. In the early stage of a neurogenic process, reinnervation of denervated muscle fibers by axonal sprouts of remaining intact axons results in normalization of the maximum CMAP amplitude (14). Therefore, patients with (an early stage of) subclinical DPN are unlikely to show an abnormal maximum CMAP amplitude, whereas more direct estimates of axonal loss and/or dysfunction may be valuable indicators of early nerve damage (14). This is supported by animal models of T1DM showing that an increased motor unit size and decreased motor unit number can be identified early in the disease course of DPN (11, 15). In these studies, the maximum CMAP amplitude remained unchanged (15) or changed later in the course of the disease (11).

The recently developed CMAP scan, basically a high-resolution stimulus-response curve, provides a visual and quantitative impression of the build-up of a muscle in terms of motor unit size and motor unit number (14, 16, 17). Furthermore, by evaluation of the stimulus intensities (SIs) applied, the CMAP scan can be used to assess the axonal excitability of the investigated motor nerve (16, 17).

Alterations in axonal excitability may precede axonal damage and loss, and may, therefore, be an even better marker of subclinical DPN than motor unit size and motor unit number (11, 18). The CMAP scan has a good reproducibility (19) and although it requires application of a large number of stimuli, the predictable, repetitive nature of the stimulus pattern and the mostly low intensities ensure that the technique is well-tolerated (20).

We hypothesized that the joint assessment of maximum CMAP amplitude, axonal excitability, motor unit size and motor unit loss by means of this CMAP scan can be used to identify pathological motor nerve changes typical for DPN in an early stage. In this pilot study, we performed CMAP scans in two groups of young patients with T1DM as well as in a group of adult patients with T1DM, with the three groups representing three degrees of DPN severity. In addition, CMAP scans were performed in healthy controls.

Patients and Methods

Patients and clinical measurements

For the purpose of the present study, we would preferably compare the results of the CMAP scan between T1DM patients with and without subclinical DPN and between these patients and patients with clinical DPN (as positive controls). Because the presence or absence of subclinical DPN cannot be established reliably by means of measurements other than biopsies (which we considered too invasive and hence unethical considering the pilot nature of the present study) (7, 8), we used sets of strict inclusion criteria related to established risk factors for DPN to define three groups of patients that were most likely to represent the three conditions (1, 6).

Included in the first patient group were patients, who were 8-25 years old, with a disease duration of 2.5-5 years and a haemoglobin A1c (HbA1c)(Vantage system, Siemens Medical Solutions Diagnostics, Tarrytown, NY) below 64 mmol/mol (8%) since the time of diagnosis (21). Eligible for the second group were patients, aged 10-25 years, who met the following criteria: I) disease duration of 10 years or more, II) evidence for poor glycemic control, e.g. at least three HbA1c levels above 69 mmol/mol (8.5%) between 2006 and 2009, and/or III) presence of

(early) signs of microvascular complications, e.g. retinopathy or microalbuminuria (22). All patients of the two forementioned groups were without clinical DPN, defined as: I) numbness of the feet and/or burning pain in the legs or feet, and II) reduced vibration sensation (determined with a 128-Hz tuning fork), and/or a decreased tactile perception threshold (defined as the inability to sense the 5.07 Semmes-Weinstein monofilament). Previous work suggests that the presence of subclinical DPN is unlikely in patients that meet the above criteria for inclusion in the first group (1, 6). Patients that were included in this group were called P1-patients. By contrast, subclinical DPN might be expected in at least some patients of the second group. These patients were called P2-patients. The third group included patients aged 20-70 years who were diagnosed with clinical symptoms of DPN according to the definition of DPN as described above (P3-patients).

The P1- and P2-patients were matched to age with a maximum age difference of 3.5 years with healthy controls (C1-controls and C2-controls). The controls of P3-patients were included from an existing cohort of healthy adult controls and were matched to age with a maximum difference of 3.17 years (C3-controls). Patients and controls had to be euthyroid and they had to have a negative medical history for renal failure and diseases known to cause peripheral neuropathy.

The P1- and P2-patients attended the outpatient clinic of Diabeter, a specialized pediatric and adolescent diabetes center in Rotterdam, the Netherlands. The (adult) P3-patients attended the Internal Medicine outpatient clinic of either the St. Franciscus Gasthuis or the Erasmus Medical Centre in Rotterdam, the Netherlands. The patients received medical care in agreement with the International Society for Pediatric and Adolescent Diabetes and American Diabetes Association guidelines (22, 23). Retrospective chart review was performed to obtain data on the exact disease duration and the most recently measured HbA1c.

The study was performed in agreement with the Declaration of Helsinki and approved by the Medical Ethical Board of the Erasmus MC Rotterdam. All subjects participated after signed informed consent.

Neurophysiological measurements

The CMAP scan is a non-invasive electrophysiological tool which records the electrical activity of a muscle in response to repetitive transcutaneous stimula-

tion of the motor nerve. Each axon (and related motor unit) has its own threshold for stimulation and will be activated when the stimulus intensity (SI) exceeds this threshold. When the SI is gradually increased from subthreshold to supramaximal values, all motor units in the muscle are successively activated.
CMAP scan recordings were performed from the EDB muscle using a Nicolet Viking Select EMG system (CareFusion, San Diego, CA) with the novel CMAP scan utility, as previously described (16, 20). The active recording electrode was placed over the belly of the EDB, at the point where the negative peak amplitude of the CMAP was maximal. The reference electrode was placed over the metatarsophalangeal joint of the fifth phalanx. Stimulation was applied approximately 8 cm proximal to the active recording electrode. After determination of the SI at which the lowest-threshold motor unit was activated (S0) and of the lowest SI that could elicit the maximum CMAP (S100), the CMAP scan was performed by decreasing the SI gradually from S100 to S0 of, in total, 500 consecutive stimuli (2 Hz, 0.1 ms pulse duration).

Plotting the recorded CMAP amplitude against the corresponding SI resulted in a stimulus-response curve, the so-called CMAP scan. In healthy subjects, the CMAP scan is usually smooth and sigmoid (Figure 1A and 1C). When large motor units are present, these tend to be visible as so-called “steps” (Figure 1B and 1D). These steps provide information on the extent of collateral reinnervation as well as an indication of motor unit loss (16, 17).

Figure 1: CMAP scan stimulus-response curve of a healthy subject and a patient

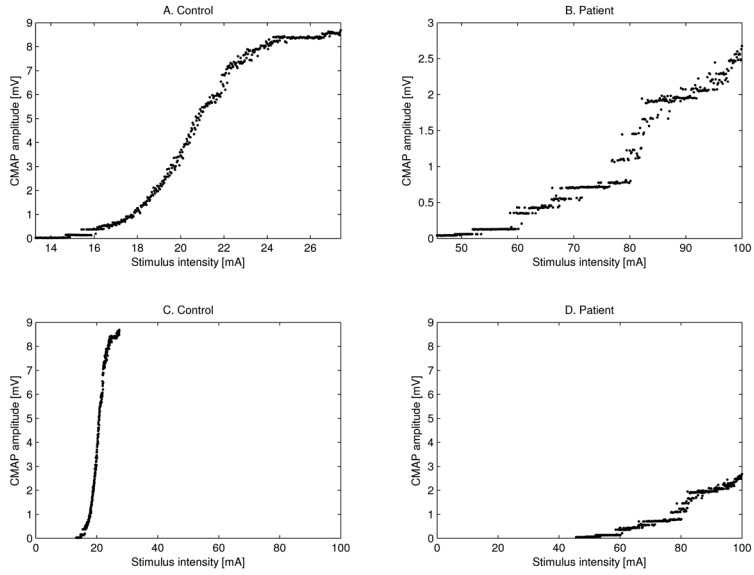


Figure 1A: CMAP scan obtained from a healthy subject: the stimulus-response curve is smooth and sigmoid. Figure 1B: CMAP scan obtained from a patient with clinical diabetic peripheral neuropathy: the stimulus-response curve is interrupted by large gaps and shifted toward abnormally high stimulus intensities, reflecting motor unit loss, the presence of enlarged motor unit potentials, and a reduced axonal excitability. Figure 1C and D: The same stimulus-response curves as in 1A and 1B, but now plotted on the same scale to allow direct visual comparison of the differences in maximum CMAP amplitude (y-axis) and stimulus intensities (x-axis) between control and patient.

CMAP scan data analysis

Data were imported in MATLAB (version R2008a; The MathWorks, Natick, MA). From each CMAP scan, we derived the SIs required to generate 5, 50, and 95 percent of the maximal CMAP amplitude (S5, S50, and S95, respectively). S5 and S95 provide an indication of the excitability of the most excitable and least excitable axons, respectively, whereas S50 provides an average value. As quantitative indicators of axonal loss and reinnervation, we used the maximum CMAP amplitude and the variable D50. D50 represents the number of size-ordered

gaps in the CMAP scan (starting with the largest one present) that need to be summed to exceed 50% of the maximal CMAP amplitude. For this purpose, a gap is defined as the difference between consecutive (sorted) CMAP sizes. That is, a CMAP scan resulting from 500 stimuli is built up from 499 gaps. In the hypothetical case that all gaps were equal, D50 would be 250 because 250 of these equal-sized gaps need to be summed to add up to (and just exceed) 50% of the maximum CMAP. In the presence of reinnervation, large motor units will contribute large motor unit potentials to the CMAP scan, resulting in large gaps. As a consequence, D50 will decline.

Statistical analyses

The patient and control group characteristics were described as proportions, medians, and interquartile ranges (IQR). The Mann-Whitney U test was used to test differences between two groups with skewed variables. In case of missing data, patients and their matched controls were excluded for that comparison. The significance level was set to $p=0.05$ (two-sided). All analyses were performed with SPSS version 17.0 for Windows (SPSS Inc, Chicago, Illinois).

Results

Our strict inclusion criteria for the P1- and P2-patient groups resulted in a limited number of patients that were eligible for participation in this study (30 out of 500 outpatients screened, n=13 in the group of P1-patients and n=17 in the group of P2-patients). Thirteen patients were eligible for the P3-group. Three young patients (two P2-patients and one P1-patient) and two P3-patients withdrew from the study after informed consent. One P2-patient and one P1-patient had to be excluded because of incomplete data. Table 1 shows the characteristics of the remaining patients and controls. P3-patients had a significantly longer disease duration than P2- and P1-patients (both $p<0.01$). The HbA1c of the P3-patients was similar to that of the P1-group.

Table 1: Characteristics of patients and controls

	P1-patients	P2-patients	P3-patients	C1-controls	C2-controls	C3-controls
n=	11	14	11	11	14	11
Sex M/F	6/5	5/9	7/4	5/6	7/7	5/6
Age (yr)	13.92 (12.58-17.08)	18.75 (15.92-22.42)	47.50 (44.92-56.08)	14.00 (12.42-19.08)	19.13 (14.31-22.10)	51.00(47.83-55.25)
Disease duration (yr)	3.83 (3.50-4.50)	12.58 (11.40-16.46)	33.42 (24.58-41.42)	-	-	-
HbA1c (mmol/mol)	54 (52-60)	77 (68-90)	63 (53-70)	-	-	-
HbA1c (%)	7.1 (6.9-7.6)	9.2 (8.4-10.4)	7.9 (7.0-8.6)	-	-	-

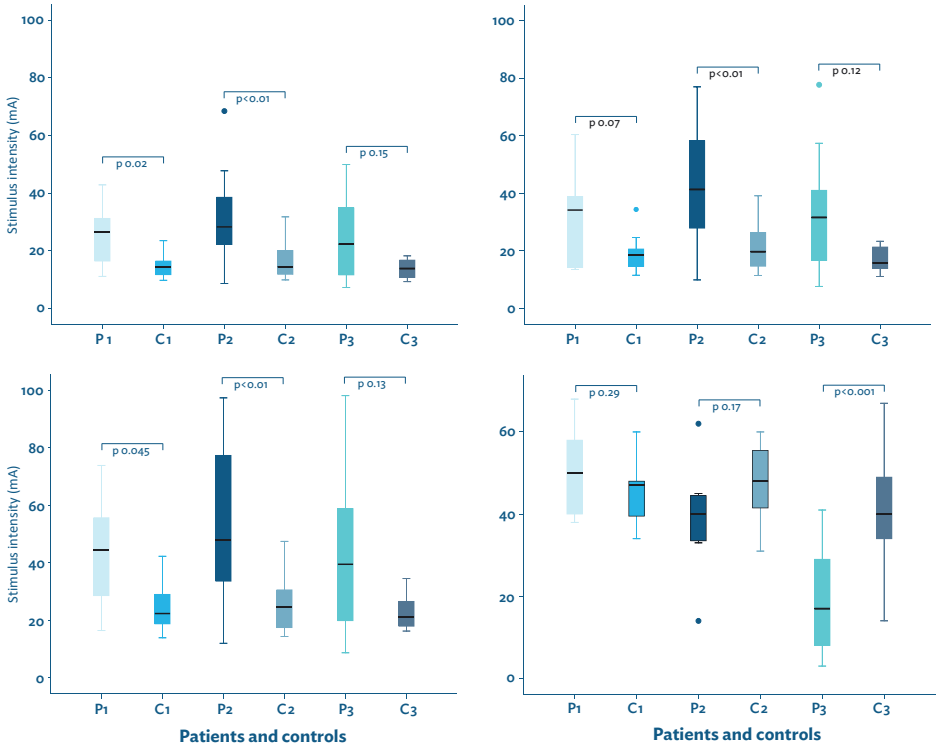
Data are expressed as median, interquartile range and proportion. HbA1c in mmol/mol = 10.93 x HbA1c (%)-23.5.

In the P3-patients, the recordings typically yielded a CMAP scan interrupted by large gaps shifted toward abnormally high SIs, reflecting motor unit loss, the presence of enlarged motor unit potentials, and a reduced axonal excitability.

The SI variables S5, S50, and S95 were significantly higher in P1- and P2-patients compared with their matched controls (all p values < 0.045) (Figure 2A-C), except the S50 for P1-patients vs. C1-controls (p=0.07). In the P3-patients, these SI variables were also increased compared with their controls, albeit not significantly (Figure 2A-C). The maximum CMAP amplitude was decreased in P2- and P3-patients compared with their controls (6.9 (5.9-8.4) mV vs 8.2 (6.8-10.0) mV (p 0.11), and 4.3 (1.5-8.2) mV vs 6.1 (5.4-7.5) mV (p 0.09), respectively). The CMAP amplitude of the P1-patients did not differ from the CMAP amplitude of the C1-controls (5.9 (3.6-8.5) mV vs 6.1 (4.2-8.7) mV (p 0.58)).

D50 was significantly decreased in P3-patients compared with their controls (p< 0.001)(Figure 2D).

Figure 2: Stimulus intensities



A: Stimulus intensities (y-axis) required to elicit responses of 5 percent of the maximum CMAP amplitude in patients and controls (x-axis). B: Stimulus intensities (y-axis) required to elicit responses of 50 percent of the maximum CMAP amplitude in patients and controls (x-axis). C: Stimulus intensities (y-axis) required to elicit responses of 95 percent of the maximum CMAP amplitude in patients and controls (x-axis).D: D50 (y-axis) in patients and controls (x-axis).

Discussion

Our study demonstrates increased stimulus intensities (SIs), reflecting reduced axonal excitability, in all three groups of patients with T1DM compared with their controls. Despite the small group sizes, these reductions in axonal excitability were found to be highly significant. This suggests that the CMAP scan is very sensitive to the axonal changes that occur with T1DM.

As a result of our inclusion strategy, we expected a trend toward higher SI values from the P1- via the P2- to the P3-group. Indeed, our results show higher SI values for the P2-patients than for the P1-patients. We did not expect to find aberrant values in the P1-group compared with their controls. The fact that we did indicates that reduced axonal excitability (whether stand-alone or as part of subclinical DPN) occurs in some individuals in a very early stage of T1DM, despite appropriate glycemic control and the absence of other microvascular complications. We may speculate that these patients are more prone to develop muscular atrophy, sensory neuropathy, and/or other microvascular complications. A larger, longitudinal study from the diagnosis of T1DM onwards is required to confirm that the early impairment of motor nerve excitability observed in this study is indeed a sign of subclinical DPN and/or identifies the high risk patients.

Another intriguing finding was that the SI values in the P3-group are similar to those in the P1-group. This may be an effect of a dramatically reduced number of remaining, functioning, axons in the P3-group with the most healthy ones surviving. The number of patients in the P3-group in particular is so small, however, that this may equally well be a chance finding that would not recur in a larger study.

Our findings of impaired axonal excitability are in agreement with a study in diabetic mice (11) and with a study in a patient group predominantly consisting of patients with type 2 diabetes (24). Axonal excitability is the result of the interplay between sodium and potassium currents in the axolemma, amongst many other factors (25). These currents are regulated by the Na+/K+- ATPase pump and free-standing sodium and potassium channels (26). Dysfunction of the Na+/K+ - ATPase pump is a well-known phenomenon in DPN (11, 18). In addition, several studies describe changes in free-standing sodium and potassium channels during the process of de- and remyelination (25, 27), a process that may occur both early

and late in the disease course of DPN (10, 28).

In addition to the excitability changes, our study has shown that the maximum CMAP amplitude was decreased in the patients with clinical DPN (P3-group) and to a lesser extent in the group likely to have subclinical DPN (P2-group). This supports the notion that the CMAP amplitude alters relatively late in the disease course of DPN (1, 12). As we anticipated this finding, we added D50 as CMAP scan measure of axonal loss and reinnervation. D50 was found to be decreased in the P3-group but not in the P2- and P1-group. Probably, the normal D50 in the P2-group results from the fact that D50 is sensitive only to changes in motor unit number in the range between 0 and 100 motor units. Near-normal numbers of motor units (200-300) due to mild axonal loss will not result in a decreased D50 (unpublished data, collected after the current pilot was performed). Hence, D50 is sensitive to detect the severe axonal damage in clinical DPN but cannot be used to assess subclinical DPN.

In designing our study, we used sets of strict inclusion criteria (related to established risk factors for DPN) to define different patient groups (1, 6). Hence, we hoped to establish that the P1-group would be unaffected and that at least some P2-patients would be affected by subclinical DPN. Use of biopsies, a reliable gold standard for establishing DPN (8, 29, 30), was not feasible for this pilot study format. Consequently, the extent to which conclusions can be drawn regarding the diagnostic possibilities of the CMAP scan variables is limited. However, as the CMAP scan provides a new type of information on the condition of the motor nerve in patients with T1DM, it opens avenues for further research. SI variables, reflecting axonal excitability, may be able to detect subclinical DPN in young patients, whereas a conventional measure such as the maximum CMAP amplitude is not.

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Compound muscle action potential (CMAP) scan variables

Chapter 3

Chapter 4

Comment on: Malik (2014) Which Test for Diagnosing Early Human Diabetic Neuropathy? Diabetes 63:2206–2208

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Recently, Professor Malik discussed the lack of an appropriate test for the early detection of diabetic neuropathy (DN) (1). He questioned the utility of conventional neurophysiological and symptom-based tests before outlining potential small-fiber-focused techniques as measures of subclinical DN (SDN), as illustrated by the elegant techniques of corneal confocal microscopy (1). Since improved glycemic control during the early stages of DN may prevent or delay nerve function deterioration, timely detection of SDN is important. The rapidly increasing prevalence of type 1 diabetes (T1D) in youth, resulting in longer disease duration and increased likelihood of developing SDN, underscores this unmet need for identifying early markers of SDN. We agree with Professor Malik that there are shortcomings in current markers. Indeed, the reliability of sensory nerve conduction velocity (NCV) as the 'gold standard' to detect SDN in children and adolescents with T1D has been questioned (2).

When we assessed sensory NCV, sensory nerve action potential (SNAP) amplitude of the superficial peroneal and sural nerves, and compound muscle action potential (CMAP) scans of the peroneal nerve in young patients with T1D and age-matched healthy controls, we found that motor neuron damage may coincide with or even precede sensory damage. While sensory NCV did not differ significantly between patients (range 12.5–19.9 years) and controls, nor between patients with well-controlled (duration < 5 years, HbA1c < 8.0%) and poorly controlled T1D (duration > 10 years, HbA1c > 8.5% and/or early signs of microvascular complications), SNAP amplitudes were lower in patients with poorly controlled T1D. Although diagnostic sensitivity was acceptable, accuracy and specificity were low (3). Compound muscle action potential (CMAP) scans revealed no difference in conventional motor nerve neurophysiological measures (axonal loss and re-innervation) between young T1D patients (range 8.08–23.58 years) and age-matched healthy controls (4). However, axonal excitability was significantly reduced in both well- and poorly controlled young patients and adults with T1D when compared with controls. This suggests that whereas early disturbances of motor neuronal function cannot be detected using conventional motor nerve neurophysiological measures, recently developed axonal excitability measures could prove useful in identifying the early signs of nerve function deterioration.

The abovementioned findings underscore the necessity for ongoing debate on appropriate surrogate measures. We caution against a singular focus on sensory

nerves as our results indicate that (early) motor nerve dysfunction may also potentially be a suitable marker of SDN.

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Chapter 5

Do traditional cardiovascular risk factors solely explain intima-media thickening in youth with type 1 diabetes?

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Abstract

Aims

To assess age-specific carotid intima-media thickness (cIMT) in children and adolescents with type 1 diabetes and to investigate associations between cIMT, age, classical cardiovascular disease (CVD) and other risk factors.

Methods

This study included a cross-sectional analysis of cIMT in 178 patients with type 1 diabetes and 208 healthy controls across age categories. In patients, the impact of gender, socio-economic status, ethnicity, current and historical , body mass index, blood pressure, haemoglobin A1c ,high-density lipoprotein and low-density lipoprotein cholesterol on cIMT was studied in a retrospective follow-up cohort study.

Results

Median cIMT was equally greater in patients versus controls across all age categories ($p \leq 0.03$). Regression models in patients confirmed a lack of association between cIMT and classical CVD risk factors.

Conclusions

Children and adolescents with type 1 diabetes showed greater cIMT than controls in all age categories. Increased cIMT did not seem to be consistently associated with classical adult CVD risk factors, adding to the current debate in pediatrics about the impact on classical CVD risk factors to the development of subclinical atherosclerosis in type 1 diabetes. Future studies are warranted to determine if cIMT could assist in predicting macrovascular complications of type 1 diabetes.

Introduction

Adult patients with type 1 diabetes have a considerably higher risk for cardiovascular disease (CVD) and cardiovascular mortality than healthy adults (1). As an increase in carotid intima-media thickness (cIMT) is associated with CVD, cIMT is widely used as a surrogate endpoint for subclinical atherosclerosis in adults (2-4). In addition to classical factors (e.g. hypertension, dyslipidaemia, smoking) (3), it is thought that glycaemic control, diabetes duration, and male gender (4, 5), as well as factors such as inflammation and genetic predisposition may contribute to cIMT increment and CVD risk in adults (6).

Although longitudinal data are lacking, cIMT is considered to also be a marker of subclinical atherosclerosis in children and adolescents with type 1 diabetes. However, in this group conflicting results on cIMT were observed when patients were compared with healthy controls (7-10). Little is known about age-specific differences in cIMT. In a study among children and adolescents with type 1 diabetes (aged 8–19 years), cIMT was increased during the first three age quartiles, but no further increase was observed in the fourth age quartile (9). Studies on the impact of covariables associated with increased cIMT in children and adolescents with type 1 diabetes are contradictory. One study found that cIMT was significantly associated with body mass index (BMI) and duration of diabetes (11), whereas another study found that age at diabetes onset, insulin dose, and total cholesterol level correlated with cIMT (7); systolic blood pressure (SBP) was the only factor found to be predictive in both studies (7, 11). In addition, Krantz et al. pointed to gender-specific differences in cIMT, with the high-density lipoprotein cholesterol (HDL-C)/low-density lipoprotein cholesterol (LDL-C) ratio being a significant factor in males, but not females (8) whereas Krebs et al. reported that diabetes duration and pulse pressure were significant factors in males; and LDL-C, glycated haemoglobin (HbA1c), and duration of diabetes being significant in females (12). These contradictory data of the impact of covariables on cIMT in children and adolescents with type 1 diabetes may point to a role other than the classical CVD risk factors, and also relevant heterogeneity between patients in the early stages of the pathophysiological process of subclinical atherosclerosis in youngsters.

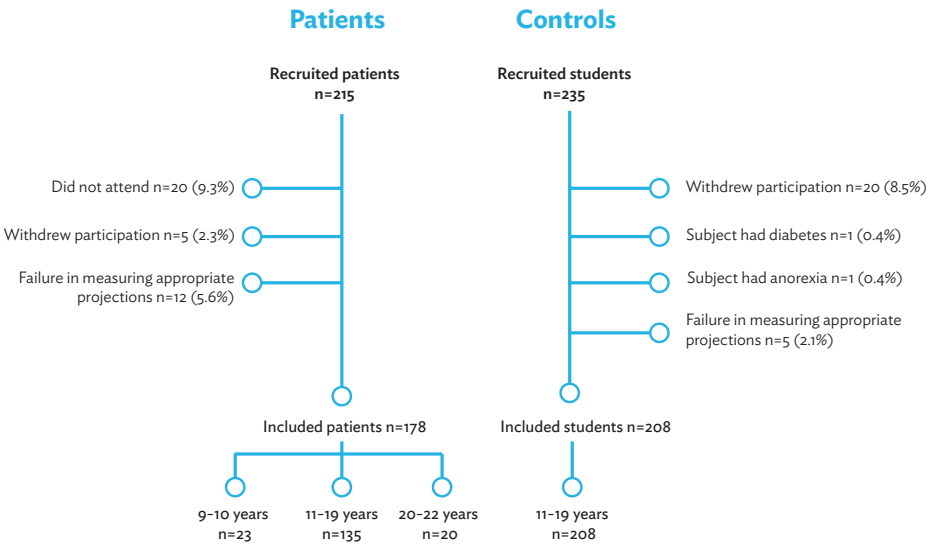
In this study, we aimed to investigate: (a) age-specific differences in cIMT between children and adolescents with type 1 diabetes versus healthy controls; and (b) associations between cIMT, disease duration, and current and historical (past) classical CVD risk factors in children and adolescents with type 1 diabetes. We hypothesize that cIMT increases with age and moreover that the difference between patients and controls in cIMT will be higher in the older age categories. Moreover, we postulate an association between cIMT and historical classical CVD risk factors as the development of subclinical atherosclerosis takes several years (5).

Methods

Study population

The age-specific cIMT values of 178 children and adolescents with type 1 diabetes were compared with those of 208 healthy controls (hereafter referred to as 'controls') in a cross-sectional study (Figure 1). All eligible patients with type 1 diabetes aged 9–22 years (inclusive) were recruited between August 2008 and October 2010 at the outpatient clinic of Diabeter, a large, certified diabetes centre in Rotterdam, the Netherlands. Diabeter provides comprehensive and advanced management of children and adolescents with type 1 diabetes. Patients with inadequately controlled or treated coeliac disease and hypothyroidism, and patients treated for growth hormone deficiency or on lipid-lowering medication were excluded.

Figure 1: Study profile.



The age- and gender-stratified control group included healthy children aged 11–19 years, and was recruited from a secondary school in Rotterdam in October and November 2011. Information on gender, birth date, ethnic background, and concomitant diseases was obtained. Controls with concomitant diseases other than attention deficit/hyperactivity disorder were excluded. Study participants and parents of minors provided signed informed consent. The study was performed in agreement with the Declaration of Helsinki and approved by the Medical Ethical Board of the Erasmus Medical Center Rotterdam in Rotterdam, the Netherlands.

Age-specific cIMT data were determined by stratifying the patient and control groups into several age categories 11–12, 13–14, 15–16, and 17–19 years for patients and controls; and age categories 9–10 and 20–22 years for patients only. The impact of anthropometric, HbA1c, and lipid data on cIMT values in the children and adolescents with type 1 diabetes was studied using retrospective data from this cohort. Longitudinal anthropometric, HbA1c, and lipid data were retrieved from electronic patient charts and grouped according to time of measurement. Data obtained at a date closest to the date at which cIMT was measured were

included in the time frame 'current'. The time frame during which inclusion of 'current' data was assumed to be reliable was 6 months for anthropometrics, 3 months for HbA1c, and 12 months for lipids. Past data, obtained between the first clinic visit at Diabeter until 12 months before cIMT measurement, were included in the time frame 'historical'. Data on HbA1c and lipids were included if they were obtained ≥3 months after diagnosis of type 1 diabetes. For anthropometrics, data obtained at a date closest to the first clinic visit (but ≥1 month after diagnosis) were included. Diabetes duration at time of cIMT measurement, age at onset of type 1 diabetes, gender, and a family history for premature CVD were considered independent of the time of measurement. Family history of premature CVD (heart attack, coronary bypass surgery, and/or stroke before age 55 years) in first- and second-degree relatives was updated at the end of the study period.

Carotid ultrasonography

Common cIMT was measured at the outpatient clinic of the Sint Franciscus Gasthuis, Rotterdam, with cIMT in the control group of students was measured at their school. All carotid ultrasound scans were performed with an ART-LAB 411240 arterial analyser (Esaote; Genova, Italy) and carried out by one experienced sonographer (Noelle van der Meulen; author). Measurements were performed according to previously described guidelines (13). Briefly, participants lay in a supine position, the head resting comfortably and the neck slightly hyperextended and rotated in the opposite direction of the probe. Ultrasound images were obtained of the distal 1 cm of the far wall of each common carotid artery (CCA) using B-mode ultrasound, resulting in two echogenic lines. These lines represent the combined thickness of the intima and media layers of the arterial wall. Until September 2, 2009, each CCA was imaged in two different projections: CCA right side 120–180° and CCA left side 180–240°. Mean cIMT was calculated from these four projections. From September 23, 2009, onwards, each CCA was imaged in three different projections (CCA right side 90–120–180°, and CCA left side 180–240–270°) and, as a consequence of this protocol change, mean cIMT was calculated as the mean of six projections. If information on one projection or one projection of each CCA was missing, mean cIMT was calculated as the mean of the four or five remaining projections.

Anthropometric data

BMI data were converted to standard deviation scores (SDS) and SBP to percentiles on the basis of commonly applied reference values (14-17). A high BMI was defined as a BMI ≥ +2 SDS. A normal BMI was defined as a BMI < +2 SDS.

Laboratory data

HbA1c was measured at every clinic visit by an immunochemical assay (Vantage System; Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) with intra- and inter-assay coefficient of variation (CV) values of < 3.7% and < 4.3%, respectively.

LDL-C, HDL-C, and total cholesterol (TC) were assessed in non-fasting state at a frequency recommended by the International Society for Pediatric and Adolescent Diabetes guidelines (18). LDL-C, HDL-C, and TC were measured by enzymatic colorimetric assay on a Hitachi Cobas C501 analyser (Roche Diagnostics; Mannheim, Germany) with an intra- and inter-assay CV deemed acceptable according to the External Quality Assurance Services in the Netherlands: intra-assay CV: LDL-C 0.90%, HDL-C 0.70%, and TC 1.10%; and inter-assay CV: LDL-C 1.90%, HDL-C 0.90%, and TC 1.60%. Until 2006 LDL-C was calculated using the Friedewald formula; from 2006 onwards, a direct measurement for LDL-C was used. For the historical data, intra-individual median LDL-C, HDL-C, TC, and HbA1c were used, and the CV of HbA1c (ratio of intrapersonal HbA1c standard deviation [SD]/mean HbA1c) was utilized as a measure of historical HbA1c variability (19).

Statistical analysis

Categorical variables were expressed as proportions and percentages. Continuous variables were expressed as mean ± SD for normally distributed variables, or median with interquartile range (IQR) for skewed data. Differences were tested by $-^2$ test or Fisher's Exact test in the case of categorical variables, and independent Student t-test or Mann-Whitney U test in the case of continuous variables.

Carotid intima media thickness (cIMT)

Table 1: Demographic, anthropometric, and laboratory data of the patients, stratified by age

	Patients aged < 15.3 years (n = 89)	Patients aged ≥ 15.3 years (n = 89)
Age, median (IQR), years	12.5 (10.9–14.2)	18.6 (17.3–20.0)
Diabetes duration, median (IQR), years	5.6 (3.6–8.3)	10.0 (6.3–14.0)
Female, n (%)	37 (42)	44 (49)
Family history premature CVD, n (%)		
Positive	23 (26)	17 (19)
Negative	60 (67)	63 (71)
Unknown	6 (7)	9 (10)
BMI-current, n (%)		
BMI ≤ −2 SDS	1 (1)	–
BMI > −2 SDS and < 0 SDS	11 (12)	13 (15)
BMI ≥ 0 SDS and BMI < +2 SDS	67 (75)	65 (73)
BMI ≥ +2 SDS	10 (11)	9 (10)
BMI-historical, n (%)		
BMI ≤ −2 SDS	-	-
BMI > −2 SDS and < 0 SDS	20 (23)	15 (17)
BMI ≥ 0 SDS and BMI < +2 SDS	59 (66)	67 (75)
BMI ≥ +2 SDS	7 (8)	3 (3)
SBP-current percentile, median (IQR)	79.0 (56.5–91.0)	88.0 (68.0–98.0)
SBP-historical percentile, median (IQR)	73.0 (56.5–92.3)	87.0 (68.0–94.0)
HbA1c-current		
Median (IQR), mmol/mol	64 (57–72)	68 (58–77)
Median (IQR), %	8.0 (7.4–8.7)	8.4 (7.5–9.2)
HbA1c-historical,		
Median (IQR), mmol/mol	61 (57–65)	66 (58–73)
Median (IQR), %	7.7 (7.4–8.1)	8.2 (7.5–8.8)
HbA1c CV-historical, median (IQR)	0.070 (0.051–0.088)	0.073 (0.058–0.095)
LDL-C-current, median (IQR), mmol/l	2.3 (2.1–2.7)	2.6 (2.1–3.0)
LDL-C-historical, median (IQR), mmol/l	2.2 (1.8–2.5)	2.5 (2.0–2.8)
HDL-C-current, median (IQR), mmol/l	1.6 (1.4–1.8)	1.4 (1.2–1.7)
HDL-C-historical, median (IQR), mmol/l	1.5 (1.2–1.7)	1.4 (1.1–1.6)
cIMT, median (IQR), mm	0.423 (0.390–0.446)	0.413 (0.386–0.449)

Missing values (patients aged < 15.3 years/ patients aged ≥ 15.3 years): BMI-current (0/2); BMI-historical (3/4); SBP-current (0/1); SBP-historical (3/4); HbA1c-current (0/2); HbA1c-historical (5/3); HbA1c CV-historical (7/6); LDL-C-current (13/13); LDL-C-historical (27/16); HDL-C-current (15/10); HDL-C-historical (25/18). BMI, body mass index; cIMT, carotid intima-media thickness; CV, coefficient of variation; CVD, cardiovascular disease; HbA1c, glycated haemoglobin; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SDS, standard deviation score.

We found that diabetes duration and age at onset were confounding factors (multicollinearity). Diabetes duration was included as an independent variable, using age as a stratifying variable, and excluded age at onset for further statistical analysis. Weight for height SDS, diastolic blood pressure, and TC were not analysed further because these variables were highly correlated with BMI, SBP, and LDL-C, respectively.

Median cIMT values and differences in cIMT between patients with type 1 diabetes and controls were analysed across several age categories (11–12, 13–14, 15–16, and 17–19 years). The age categories 9–10 years and 20–22 years were included for patients only, as there were no controls available in these age ranges.

For the patient group, two multiple-regression analyses were performed to identify any associations between cIMT and duration of type 1 diabetes, socio-economic status (SES), gender, ethnicity (Western/non-Western) and various classical CVD risk factors. Model 1 included an 'enter' analysis (in which we specified the set of variables that make up the model) whereas model 2 included a backward stepwise regression analysis. To adjust for confounding, we stratified patients by median age: patients aged < 15.3 years and patients aged ≥ 15.3 years. Median age was chosen to ensure equal group sizes.

The significance level was set at $p < 0.05$ (two-sided). All analyses were performed with IBM SPSS Statistics 20.0 for Windows (SPSS Inc.; Chicago, IL, USA).

Results

Characteristics of patients with type 1 diabetes and controls

Patients with type 1 diabetes (n = 135 patients age-matched to n = 208 controls for age categories available for both patients and controls: 11–12, 13–14, 15–16, and 17–19 years) were older than controls (patients: median age 15.3 [IQR 13.4–18.2] years; controls: median age 14.6 [IQR 13.4–15.7] years; p = 0.001). The proportion of females was lower in the patient group compared with the control group (45% vs. 63%; p = 0.001). The ethnic distribution was different for patients when compared with controls: 73% of patients originated from Western Europe, 16% were of Mediterranean origin, and 11% had another non-Western European background. For the control group these proportions were 79%, 4%, and 17%, respectively (Fisher's Exact test; p = 0.001). Table 1 presents the demographic, anthropometric, and laboratory data of patients with type 1 diabetes after stratification by age < 15.3 or ≥ 15.3 years. The supplemental table presents the same data for the stratification in the various age categories.

Age-specific cIMT in patients with type 1 diabetes and controls

The overall cIMT was significantly greater in patients with type 1 diabetes when compared with controls (mean ± SD: 0.420 ± 0.047 mm vs. 0.390 ± 0.033 mm; P < 0.001). The same applied to the separate age categories (Figure 2).There were no gender differences in cIMT within age categories (see Table 2B).

Association of cIMT with classical CVD risk factors in patients with type 1 diabetes

Maximum follow-up time was 15.4 years for the laboratory data, with a median follow-up of 3.1 years. No associations were found between cIMT and HbA1c and LDL-C (Figure 3). Results of the regression analyses are presented in Tables 2A (enter analysis) and 2B (backward analysis). While some variables in these analyses were significant, beta values were generally low and the predictive power of these variables was low. These variables did not contribute substantially to cIMT.

Table 2A: Regression analyses: Model 1 (enter analysis).

	Patients < 15.3 years old ('Younger' group) Adjusted R2: 0.130 (P = 0.19)			Patients ≥ 15.3 years old ('Older' group) Adjusted R2: 0.105 (P = 0.18)		
	B	95%CI	P value	B	95% CI	P value
Duration of type 1 diabetes (years)	0.003	-0.003 / 0.009	0.27	0.006	0.002 / 0.010	0.01
Gender (female vs male)	-0.003	-0.033 / 0.027	0.85	0.005	-0.028 /0.029	0.74
Ethnicity (Non-Western vs Western)	-0.003	-0.035 / 0.030	.86	-0.005	-0.046 /0.036	0.82
Socio-economic status	-0.005	-0.018 / 0.009	0.48	0.007	-0.007 / 0.021	0.33
Family history unknown vs negative/positive	-0.004	-0.056 / 0.048	0.88	-0.043	-0.107 / 0.022	0.19
BMI high vs normal-current	0.032	-0.010 / 0.075	0.13	0.008	-0.05 / 0.067	0.77
BMI high vs normal-historical	-0.009	-0.064 / 0.046	0.74	-0.038	-0.148 / 0.072	0.49
SBP-current	0.00	-0.001 / 0.001	0.96	0.00	0.00 / 0.001	0.24
SBP-historical	0.00	0.00 / 0.001	0.12	0.00	-0.001 / 0.001	0.78
LDL-C-current	0.010	-0.026 / 0.046	0.58	-0.010	-0.038 / 0.018	0.49
LDL-C-historical	0.016	-0.028 / 0.060	0.47	0.024	-0.007 /0.054	0.13
HDL-C current	-0.019	-0.080 / 0.041	0.52	-0.002	-0.068 /0.063	0.94
HDL-C historical	0.005	-0,052 / 0.062	0.86	0.002	-0.055 /0.059	0.94
HbA1c-current	-0.005	-0.018 / 0.008	0.44	0.002	-0.011 / 0.016	0.76
HbA1c-historical	0.008	-0.013 /0.028	0.46	-0.022	-0.044 / 0.00	0.052
HbA1c CV-historical	-0.167	-0.493 / 0.160	0.31	0.347	-0.095 / 0.788	0.12

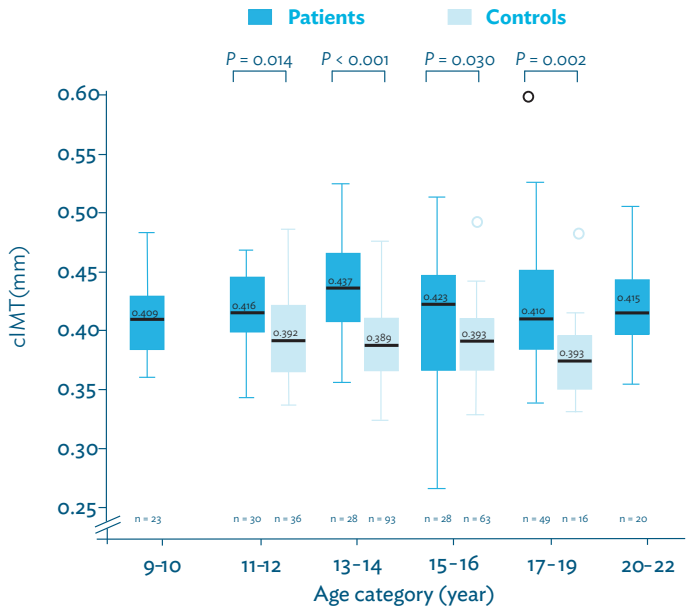
Carotid intima media thickness (cIMT)

Table 2B: Regression analyses: Model 2 (backward stepwise analysis).

	Patients < 15.3 years old ('Younger' group) Adjusted R2: 0.246, (P = 0.002)			Patients ≥ 15.3 years old ('Older' group) Adjusted R2: 0.202, (P = 0.003)		
	B	95%CI	P value	B	95% CI	P value
Duration of type 1 diabetes (y)	0.005	0.001 / 0.009	0.02	0.006	0.002 / 0.009	0.001
BMI high vs normal-current	0.037	0.008 / 0.066	0.014	na	na	na
LDL-C-historical	0.024	0.002 / 0.046	0.031	na	na	na
SBP	na	na	na	0.000	0.00 / 0.001	0.063
HbA1c - historical	na	na	na	-0.021	-0.037 / -0.05	0.010
HbA1c CV - historical	na	na	na	0.373	0.031 / 0.715	0.033

'NA': was not included in the backward stepwise analysis CVD, cardiovascular disease; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; SBP, systolic blood pressure; HbA1c CV, HbA1c coefficient of variation
BMI-high: BMI ≥ + 2 SDS; BMI-normal: BMI < +2 SDS.

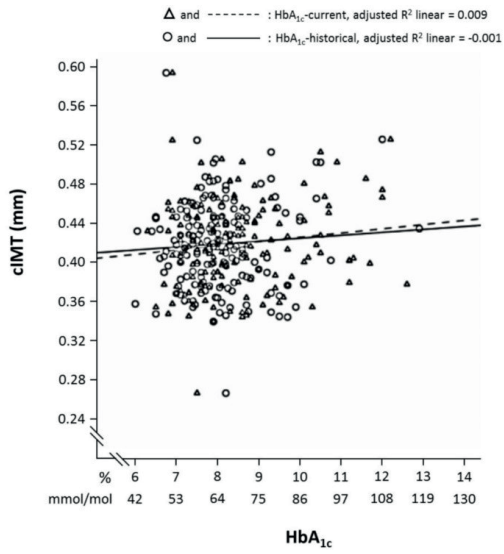
Figure 2: Age-specific median cIMT of children and adolescents with type 1 diabetes and controls.



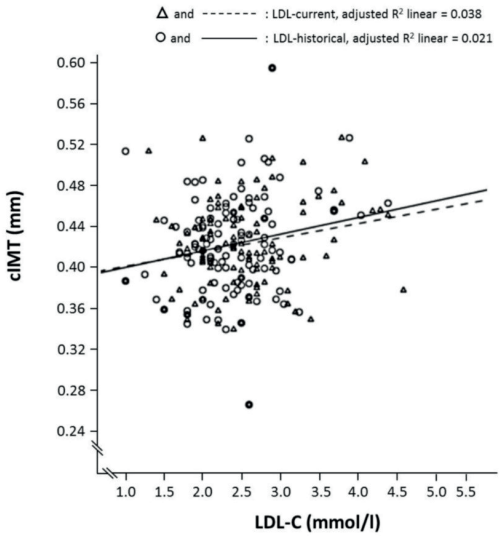
The mean cIMT for each patient was calculated from 4 projections in 103 patients and 1 control, from 5 projections in 7 patients and 13 controls, and from 6 projections in 68 patients and 194 controls. Open circles are outliers. cIMT, carotid intima-media thickness.

Figure 3: Association of cIMT with HbA1c (A) and LDL-C (B).

A



B



cIMT, carotid intima-media thickness; HbA1c, glycated haemoglobin; LDL-C, low-density lipoprotein cholesterol.

Discussion

Mean cIMT of the entire patient group was greater than in the control group. These results are consistent with some previous studies (7, 8, 10), but not with other studies (9, 20). In our age-specific analysis of cIMT, we found that median cIMT was significantly and equally greater in children and adolescents with type 1 diabetes when compared with controls across all age categories. Our analysis on factors contributing to increased cIMT in patients showed only limited contribution of classical adult CVD risk factors, glycaemic variability, and a positive family history for premature CVD; despite some significant results, effect sizes were small and predictive power was low.

The lack of a clear contribution of classical CVD risk factors to cIMT in our study and in previously published pediatric studies (7, 8, 11, 12) may be explained by differences in age, diabetes duration, length of follow-up, and CVD risk categorisation (current vs. historical data). Alternatively, the interplay between adult classical CVD risk factors may change over time and/or may contribute in a more complex way to the vascular changes and cIMT increase seen in type 1 diabetes. This may be the case in children and adolescents in particular, but also in adults with type 1 diabetes as outlined in the recently published American Heart Association and American Diabetes Association scientific statement (4), in which doubts are raised on the relative contributions of classical CVD risk factors to CVD in adults with type 1 diabetes (6). We postulate that multiple factors such as: immune and inflammatory status (21, 22); varying glycation due to, as yet unravelled, genetic factors (23); epigenetic factors (24); increased glucose variability, specifically in children and adolescents (25, 26); glycaemic variability (19); and possible age-related factors, such as different timing of pathophysiological events — several vascular changes only occur during puberty (27) — are involved in increases of cIMT in type 1 diabetes. Prospective longitudinal studies including (at least) more sequential measurements of cIMT, Tanner stages, (early signs of) microvascular complications, classical CVD risk factors, glycaemic variability, and markers of immune status and inflammation are needed to better assess patient heterogeneity and identify factors contributing to vascular changes.

An important strength of this study is that we compared the median cIMT of patients and controls in different age categories, showing that the median cIMT of

patients is consistently higher than in controls for all age categories. In addition, we showed that cIMT does not appear to increase with age. However, it should be noted that the relationship with age was studied cross-sectionally instead of longitudinally: previous studies, that were cross-sectional as well, were also not consistent with respect to the effect of age on cIMT. Dalla Pozza et al (7) found an association between age and cIMT in young patients with type 1 diabetes, whereas Margeirsdottir et al only found this for the first three age quartiles in young patients with type 1 diabetes (9). Jourdan et al (28) found only a slight increase of cIMT with age in controls. Our data indicated that significant heterogeneity exists between patients and that a subset of the children and young adolescents with type 1 diabetes may be prone to developing CVD at a very young age. These findings are worrisome, and underscore the need for further research into (modifiable) factors that affect cIMT. Another strength of this study is that both current and historical data of CVD risk factors were available for regression analyses, as were SES, ethnicity and an updated family history for premature CVD. The latter did not affect cIMT, which is consistent with results from previous studies in patients with type 1 diabetes (8, 9). However, this is in contrast with studies in healthy volunteers in which 'positive family history for premature CVD' affects cIMT (29, 30). This may be explained by the inclusion of older adolescents in one of these studies (30), correlating with older parents and subsequently a higher likelihood of CVD events, and also with a positive family history in the other study (29).

A limitation of the study could be that, despite a maximum follow-up window of 15.4 years for the laboratory data, the median follow-up of only 3.1 years may be too short to detect the detrimental effects of CVD risk factors on cIMT. Also we did not include patient smoking status in the analysis due to low prevalence, possibly as a result of underreporting and life style education. Furthermore, we did not investigate if CVD risk factors, SES, smoking, and other lifestyle factors were different in the control group, complicating the interpretation of the age-specific comparison of cIMT between patients and controls. Indeed, several assumed CVD risk factors may be more unfavourable in children and adolescents with type 1 diabetes than in controls (7, 9, 10). However, reported differences in these risk factors between patients and controls are small (9, 10). In addition, as already mentioned, the contribution of CVD risk factors to cIMT is controversial (6, 7, 9, 10, 12). Therefore, we feel that the lack of data on CVD risk factors of controls has

not affected our conclusion that the age-specific cIMT is greater in patients with type 1 diabetes when compared with controls. Another point to note is that prospective repeated measurements of cIMT in the same patient group would have strengthened the results. Although the switch in measurement protocol (number of projections) did not significantly influence the mean cIMT measurements (median difference 0.003 mm), future studies should have consistent methodology throughout the study. Finally, data on intra-rater cIMT measurement error was unavailable. In our study, the mean difference in cIMT between the study and control groups was mean 0.030 mm (varying between 0.023–0.048 mm for the separate age groups), which is comparable to the results found by others: approximately 0.010–0.04 mm (7-10). We assume that the published coefficients of variation (7, 8, 10) also apply to our study, this would imply that differences in mean cIMT of approximately 0.022 mm or smaller are differences potentially attributable to intra-rater measurement error.

Conclusions

In summary, this study shows that cIMT, a surrogate marker for subclinical atherosclerosis, is greater in children and adolescents with type 1 diabetes than in controls across all age categories. Increases in cIMT seem independent of both current and historical classical CVD risk factors and, therefore, the increases in cIMT seem to be driven, at least in part, by a different pathophysiological process in children and adolescents with type 1 diabetes when compared with adult patients. To help identify those patients who are at-risk of macrovascular complications, larger studies with repeated measurements of cIMT in children and adolescents with type 1 diabetes, including classical adult CVD risk factors and other potentially contributing factors such as immune status and genetic predisposition, are warranted.

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Supplemental table: Demographic, anthropometric, and laboratory data in the patients, stratified for age

Age category (years)	9–10 (n = 23)	11–12 (n = 30)	13–14 (n = 28)	15–16 (n = 28)	17–19 (n = 49)	20–22 (n = 20)
Diabetes duration in years, median (IQR)	5.5 (3.5–6.8)	6.4 (4.6–8.6)	6.1 (3.5–9.3)	6.2 (3.2–11.4)	9.9 (6.4–14.1)	12.4 (9.1–14.5)
Gender: Female, n (%)	9 (39)	13 (43)	12 (43)	10 (36)	26 (53)	11 (55)
Family history premature CVD, n (%) Positive Negative Unknown	 6 16 3	 9 20 1	 6 20 2	 7 17 4	 11 34 4	 1 16 3
BMI current: BMI ≤ -2 SDS BMI > -2 SDs and < 0 SDS BMI ≥0 SDs and BMI < +2 SDS BMI ≥ +2 SDs	 - 3 18 2	 - 7 21 2	 - - 23 5	 1 4 20 3	 - 6 36 6 n = 1 missing	 - 4 14 1 n = 1 missing
BMI historical: BMI ≤ -2 SDS BMI > -2 SDS and < 0 SDS BMI ≥ 0 SDS and BMI < +2 SDS BMI ≥ +2 SDS	 - 5 13 4 n = 1 missing	 - 9 20 - n = 1 missing	 - 4 21 2 n = 1 missing	 - 6 17 2 n = 1 missing	 - 8 38 2 n = 1 missing	 - 3 17 -
SBP-current percentile, median (IQR)	64 (36–93)	69 (57–84)	84 (66–92)	89 (70–99)	88 (62–96) n = 1 missing	88 (72–99)
SBP-historical percentile, median (IQR)	78 (61–91) n = 1 missing	73 (45–91) n = 1 missing	73 (57–95) n = 1 missing	84 (56–94) n = 1 missing	88 (68–95) n = 1 missing	84 (69–88)
DBP current	50 (32–63)	30 (16–46)	33 (20–66)	53 (22–84)	47 (30–72) n = 1 missing	59 (44–82)
DBP historical	65 (30–81) n = 1 missing	36 (20–59) n = 1 missing	64 (42–82) n = 1 missing	51 (26–74) n = 3 missing	61 (47–80) n = 1 missing	62 (46–75)
HbA1c-current, Median, mmol/mol (IQR) Median, % (IQR)	61 (57–66) 7.7 (7.4–8.2)	64 (55–70) 8.0 (7.2–8.6)	65 (61–74) 8.1 (7.7–8.9)	67 (58–86) 8.3 (7.5–10)	70 (61–84) 8.6 (7.7–9.8) n = 2 missing	64 (56–73) 8.0 (7.3–8.8)
HbA1c-historical, Median, mmol/mol (IQR) Median, % (IQR)	60 (55–63) 7.6 (7.2–7.9) n = 1 missing	62 (55–66) 7.8 (7.2–8.2) n = 2 missing	62 (57–66) 7.8 (7.4–8.2) n = 2 missing	65 (57–77) 8.1 (7.4–9.2) n = 2 missing	67 (60–73) 8.3 (7.6–8.8) n = 1 missing	66 (63–74) 8.2 (7.9–8.9)
LDL-C-current, median mmol/l (IQR)	2.4 (2.1–2.7) n = 4 missing	2.3 (2.1–2.6) n = 4 missing	2.4 (2–2.7) n = 3 missing	2.5 (2.1–2.9) n = 7 missing	2.7 (2.1–3.1) n = 5 missing	2.6 (2.0–2.9) n = 3 missing
LDL-C-historical, median mmol/l (IQR)	2.2 (1.9–2.5) n = 6 missing	2.1 (1.8–2.5) n = 6 missing	2.5 (1.8–2.8) n = 10 missing	2.4 (1.9–2.6) n = 11 missing	2.5 (2.2–2.9) n = 9 missing	2.3 (2.0–2.9) n = 1 missing
HDL-C-current, median mmol/l (IQR)	1.7 (1.3–1.8) n = 5 missing	1.7 (1.5–2) n = 5 missing	1.5 (1.4–1.7) n = 3 missing	1.5 (1.3–1.6) n = 5 missing	1.3 (1.2–1.6) n = 5 missing	1.6 (1.3–1.8) n = 2 missing
HDL-C-historical, median mmol/l (IQR)	1.4 (1.2–1.6) n = 5 missing	1.5 (1.4–1.9) n = 6 missing	1.5 (1.2–1.8) n = 10 missing	1.4 (1.2–1.7) n = 10 missing	1.4 (1.1–1.6) n = 11 missing	1.3 (1.1–1.8) n = 1 missing
HbA1c CV-historical, median (IQR)	0.070 (0.06–0.08) n = 1 missing	0.07 (0.05–0.09) n = 2 missing	0.08 (0.05–0.10) n = 3 missing	0.06 (0.05–0.09) n = 4 missing	0.08 (0.06–0.10) n = 3 missing	0.06 (0.05–0.08)
cIMT, median mm (IQR)	0.409 (0.385–0.430)	0.416 (0.398–0.446)	0.437 (0.404–0.466)	0.423 (0.366–0.447)	0.410 (0.384–0.452)	0.415 (0.395–0.445)

Carotid intima media thickness (cIMT)

Chapter 6

Chapter 7

Increased skin autofluorescence of children and adolescents with type 1 diabetes despite a well-controlled HbA1c: results from a cohort study

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Abstract

Background

Early identification of children and adolescents with type 1 diabetes at high risk for development of complications is important, as early intervention may prevent further deterioration. Here we investigate the applicability of assessing skin advanced glycation end products (sAGEs) by skin autofluorescence (SAF) as a potential surrogate risk marker.

Methods

This study included a cross-sectional analysis of SAF in 77 patients with type 1 diabetes mellitus and 118 healthy controls across age categories (11–12, 13–14, 15–16, and 17–19 years old). In patients, the impact of current and historical glycated hemoglobin (HbA1c) values, age, and duration of diabetes on SAF was studied in a retrospective cohort study and analyzed with multivariable analyses.

Results

SAF was significantly and similarly higher in patients when compared with controls across all age categories ($P \leq 0.009$). For patients, age, duration of diabetes, and current and historical HbA1c were associated with SAF in univariate analysis. Multivariate analysis showed no association between HbA1c and SAF. A subgroup of patients with a HbA1c-within-target (≤ 7.5%/59 mmol/mol) were observed to have high SAF.

Conclusion

Children and adolescents with type 1 diabetes show higher SAF than controls. The presumed correlation of high HbA1c with high SAF does not exist in all patients. Thus, use of this non-invasive measure may provide a surrogate marker for diabetic complications, additional to HbA1c.

Introduction

Reactive oxygen species and non-enzymatic reactions between sugars and amino groups of proteins ('Maillard reaction') are involved in the formation of advanced glycation end products (AGEs) (1). AGEs cause oxidative stress-related tissue damage (1), which plays an important role in microvascular and macrovascular complications in diabetes (2).

As skin collagen has a half-life of 10–15 years (3), skin AGEs (sAGEs) represent long-term glycemia. Accumulation of sAGEs can be assessed easily and non-invasively by measuring skin autofluorescence (SAF) (4). sAGEs have been proposed as a surrogate measure for risk assessment additional to HbA1c in patients with diabetes (5). In adults, increased levels of sAGEs were found in patients who developed complications (5–7). As early intervention may prevent damage later in the disease course of diabetes (8), identification of young patients at high risk for micro- and macrovascular complications is of paramount importance (9). SAF may be an effective measurement to identify this disadvantaged group. Previous studies assessed SAF in a rather heterogeneous group of children and adolescents with type 1 diabetes (10, 11), but some lacked a proper control group (12, 13). Felipe et al. (14) found SAF to be weakly associated with mean HbA1c of the preceding period and with diabetes duration.

Here we investigate if SAF reflects glycemic control expressed by HbA1c in a homogeneous study population. SAF in Dutch Caucasian children and adolescents with type 1 diabetes was compared with SAF in healthy Caucasian controls. In patients, associations of SAF with age, diabetes duration, gender, and current and historical (past) HbA1c as a reflection of long-term glycemic control were determined. We hypothesize that SAF will be higher in patients compared with controls and that it will associate with the variables age, historical HbA1c, and diabetes duration.

Methods

Study design and population

The study design was a retrospective cohort study of patients with type 1 diabetes and healthy controls. Patients aged 11–19 years with type 1 diabetes ≥ 3 months were recruited between April 2010 and January 2013 while visiting the outpatient clinic of Diabeter, a certified center of reference for diabetes care in Rotterdam, Netherlands. Diabeter provides comprehensive and advanced management for children and adolescents with type 1 diabetes. Patients were only included if they were Caucasian and if measurements of SAF and HbA1c were performed on the same day. Patients with inadequately controlled celiac disease, hypothyroidism, and those using lipid-lowering therapy were excluded.

The healthy control group was recruited from a secondary school in Rotterdam in October and November 2011. Controls with missing data, non-Caucasian ethnicity, and concomitant diseases other than attention deficit/hyperactivity disorder were excluded. Study participants and parents of minors provided signed informed consent. The study was approved by the Medical Ethical Board of the Erasmus Medical Center, Rotterdam, Netherlands, and performed in accordance with the Declaration of Helsinki.

Anthropometric and laboratory data

In controls, information on gender, ethnic background, and concomitant diseases was collected. Anthropometric and laboratory data were not obtained for this group. In patients, information on duration of diabetes, gender, body mass index (BMI), blood pressure, current HbA1c, and HbA1c values in the past (called 'historical HbA1c' from here on) was retrieved from electronic patient charts. Information on BMI and blood pressure was included if measurements were performed within a time interval of 1 month around the SAF measurement. BMI was converted to standard deviation scores (SDS): a high BMI was defined as a BMI ≥ +2 SDS. A normal BMI was defined as a BMI < +2 SDS (15–17). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were converted to percentiles (18). HbA1c was measured at every clinic visit by immunochemical assay (Vantage System, Siemens Medical Solutions Diagnostics, Tarrytown, NY) with intra- and inter-assay coefficients of variation of < 3.7% and < 4.3%, respectively. Current HbA1c was defined as the HbA1c measured on the same day as

SAF. Historical HbA1c was defined as the median intrapersonal HbA1c value of multiple HbA1c data included from the first clinic visit onwards. Historical HbA1c was only determined if the first HbA1c measurement was done ≥ 3 months after the diagnosis of type 1 diabetes and if ≥ 3 HbA1c measurements were done in the period from the first measurement to the current HbA1c measurement (current HbA1c measurement not included). A current or historical HbA1c > 7.5%/59 mmol/mol was defined as 'HbA1c-above-target' HbA1c whereas an HbA1c of ≤ 7.5%/59 mmol/mol was defined as (relatively) 'Hba1c-within-target' HbA1c (19).

SAF measurements

Patient SAF measurements were performed at Diabeter. Measurements of controls were performed at their school. The volar side of the forearm was measured with the AGE Reader CU autofluorescence reader (Diagnoptics BV, Groningen, Netherlands), making sure that the site of measurement was clean. The autofluorescence reader illuminates a skin surface of approximately 1 cm^2 with an excitation light source between 300 and 420 nm (peak excitation ~350 nm) (20). Three independent measurements were performed in approximately 30 seconds: the arm was repositioned between measurements. The mean of the three measurements was displayed by the SAF reader in arbitrary units. Previously, Sugisawa et al. (21) reported a coefficient of diurnal variation of 3.7% and a coefficient of daily variance of 4.6%.

Statistical methods

Due to the lack of prior data on SAF measurements in patients and controls as well as absence of data on the relationship between HbA1c and SAF in DM patients, formal sample size could not be calculated at study onset. Instead, our aim was to include at least 100 students and at least 70 diabetics during the study period.

Normal distributions were expressed as mean with SD. Continuous variables with skewed distributions were expressed as median with interquartile range (IQR). Categorical variables were expressed as proportions and percentages. Differences in continuous variables between groups were tested with the Mann-Whitney U test. Differences in categorical variables between groups were tested by the chi-squared test or Fisher's exact test. Correlations were tested with Pearson's rho in case of normal distribution and Spearman's rho in case of skewed distribution.

Patients with type 1 diabetes and controls were stratified into multiple age categories (11–12, 13–14, 15–16, and 17–19 years). For each age category, patients were stratified according to HbA1c-above-target and HbA1c-within-target. For both patients with HbA1c-above-target and HbA1c-within-target, SAF was descriptively compared with SAF of controls.

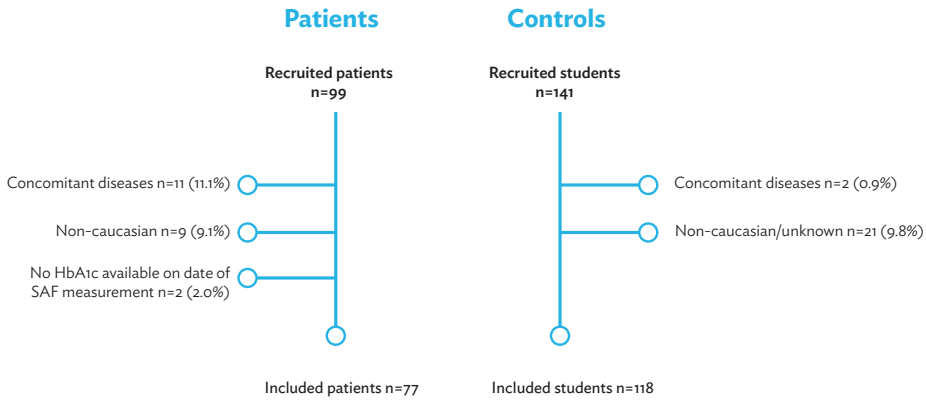
Univariate and multiple linear regression analyses were performed. Univariate linear regression analyses were used to assess the impact of the following covariables on SAF: age; diabetes duration (≤ 4 years, 4 to <10 years, and ≥ 10 years); gender; current HbA1c (current value, and HbA1c-above-target/HbA1c-within-target); and historical HbA1c (historical value, and HbA1c-above-target/HbA1c-within-target). BMI was not included due to low variability in BMI values. SBP and DBP were not included because no associations with SAF were found. Multiple linear regression analyses were performed using two models. Model 1 assessed the impact of age and diabetes duration on SAF. In model 2, current and historical HbA1c were added to the model. A *P* value < 0.05 was considered statistically significant.

Results

Study population

A total of 99 patients with type 1 diabetes and 141 controls were recruited, of whom 77 patients and 118 healthy controls were included (Figure 1). Baseline characteristics of patients and controls are presented in table 1. The median age of patients with type 1 diabetes was higher than controls (P = 0.004). Females were significantly overrepresented in the control group (P = 0.042). Historical HbA1c was determined in 73 patients (median 26 HbA1c measurements; IQR 17–37; range 3–71) in a period of 4.12 years (IQR 2.43–6.07 years; range 0.75–13.88 years).

Figure 1: Study profile.



SAF, skin autofluorescence.

SAF measurements in controls and patients

Figure 2 shows SAF measurements per age category for the controls, and for the patients with historical HbA1c-above-target and HbA1c-within-target. Both the overall SAF (patients: median SAF 1.40 [1.23-1.54]; controls: mean SAF 1.14 [0.14], $P < 0.001$) and the median SAF in the different age categories were significantly higher in patients when compared with controls (Figure 2). In the control group, median SAF increased from 1.10 [IQR 1.00–1.20] in the age category 11–12 years to 1.40 [IQR 1.10-1.40] in the age category 17–19 years. In the patient group, the same pattern was seen: in the age category 11–12 years, median SAF was 1.24 [IQR 1.19-1.40], and in the age category 17–19 years, median SAF was 1.53 [IQR 1.48-1.66] (Figure 2).

Table 1: Baseline characteristics of patients with type 1 diabetes and healthy controls

	Patients: n=77	Controls: n=118
Age, years	15.3 (13.6–17.0)	14.4 (13.1–15.4)
Gender: female, n (%)	39 (50.6%)	77 (65.3)
Diabetes duration, years (IQR)	6.6 (3.5-9.5)	NA
Age at diabetes onset, years (IQR)	8.9 (5.7–11.4)	NA
SBP (percentile)	78 (49–91)[a]	NA
DBP (percentile	37 (21–66)[a]	
BMI, n (%) ≤ -2 SDS > -2 SDS and < 0 SDS ≥ 0 SDS and < +2 SDS ≥ +2 SDS	- 16 (21.6)[b] 55 (74.3)[b] 3 (4.1)[b]	NA
HbA1c Current	8.46 (1.35)	NA
Median Intrapersonal Historical HbA1c, % (IQR)	8.0 (7.48–8.60)[c]	NA

[a] n = 69, [b] n = 74, [c] n = 73; DBP, diastolic blood pressure; IQR, interquartile range; SBP, systolic blood pressure; SDS, standard deviations score.

Impact of HbA1c on SAF in patients

Within the patient group, median SAF of patients with a current HbA1c-within-target (n=13) was 1.32 [IQR 1.19-1.52] and of patients with a historical HbA1c-above-target (n=64) 1.40 [IQR 1.22-1.54](*P* = 0.654). In the same group, median SAF of patients with a historical HbA1c-within-target (n=19) was 1.36 [IQR 1.23-1.50] and of patients with a historical HbA1c-above-target (n=54) 1.41 [1.23-1.54] (*P* = 0.580). There were also no statistically significant differences in SAF between the HbA1c-above-target and HbA1c-within-target patients for the various age categories (Figure 2).

Figure 2: Age-specific median skin autofluorescence (SAF) in patients with a HbA1c-above-target (> 7.5%/59 mmol/mol) and a HbA1c-within-target (≤ 7.5%/59 mmol/mol), compared with controls. P-values: comparison of patients vs controls per age category by Mann-Whitney U test. Error bars: interquartile range.

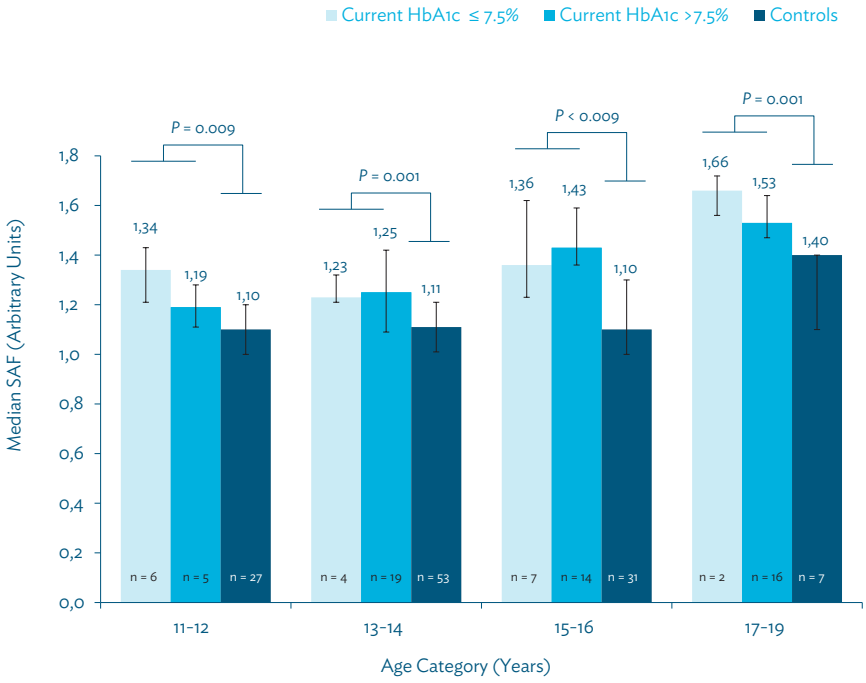
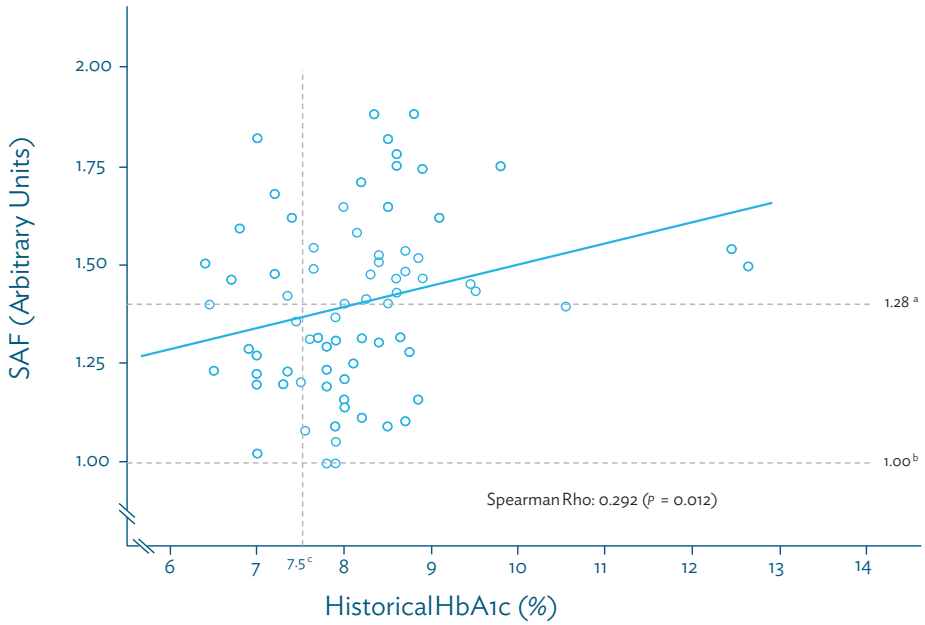


Figure 3 shows the association between historical HbA1c and SAF. The Spearman correlation coefficient between historical HbA1c and SAF was 0.292 (P = 0.012). Figure 3 also shows that 11 patients with a historical HbA1c-within-target had an elevated SAF > 1.28 (mean SAF of controls + 1 SD), whereas 1 patient with a historical HbA1c-above-target had a decreased SAF < 1.00 (mean SAF of controls -1 SD).

Univariate linear regression analyses (Table 2) showed a significant impact of age, diabetes duration, current HbA1c, historical HbA1c, current HbA1c (HbA1c-above-target/HbA1c-within-target), and historical HbA1c (HbA1c-above-target/

HbA1c-within-target) on SAF. We found no significant impact of gender on SAF. Adjusted R^2 did not exceed 0.207.

Figure 3: Correlation between historical HbA1c and skin autofluorescence (SAF) in children and adolescents with type 1 diabetes.



[a]mean SAF of controls + 1 SD, [b]mean SAF of controls -1SD cHbA1c-within-target (≤ 7.5%/59 mmol/mol) vs HbA1c-above-target (> 7.5%/59 mmol/mol)

For the multiple linear regression analyses (Table 2), Model 1 showed a significant effect of 'diabetes duration ≥ 10 years' and age on SAF. In Model 2 the addition of the current HbA1c (HbA1c-above-target/HbA1c-within-target) and historical HbA1c (HbA1c-above-target/HbA1c-within-target) showed a significant impact of age and diabetes duration but not of current or historical HbA1c on SAF. The adjusted R^2 increased to 0.235.

Table 2: Univariate and multiple linear regression analyses (n = 77) to assess the impact of the following covariables on SAF: age; diabetes duration; gender; current HbA1c (current value, and HbA1c-above-target/ HbA1c-within-target); and historical HbA1c (historical value, and HbA1c-above-target/ HbA1c-within-target).

	Beta coefficient	95% CI	P-value	Adjusted R2
Univariate analysis				
Age (years)	0.047	0.026, 0.067	< 0.001	0.207
Duration of diabetes (years)	0.025	0.013, 0.038	< 0.001	0.172
Sex (male/female)	0.047	−0.056, 0.150	0.366	−0.002
Current HbA1c (%)	0.056	0.019, 0.092	0.003	0.097
Historical HbA1c (%) (n = 73)	0.057	0.003, 0.111	0.040	0.045
Current HbA1c: HbA1c-above-target vs. Hba1c-within-target	0.034	-0.104, 0.172	0.628	-0.010
Historical HbA1c: HbA1c-above-target vs. Hba1c-within-target (n = 73)	0.028	-0.092, 0.147	0.645	-0.011
Multiple linear regression analysis				
Model 1 (n=77)				0.265
Age (years)	0.040	0.019, 0.061	< 0.001	
Diabetes duration 4 to < 10 years vs. ≤ 4 years	0.065	−0.044, 0.172	0.239	
Diabetes duration ≥ 10 years vs. ≤ 4 years	0.187	0.053, 0.320	0.007	
Model 2 (n=73)				0.235
Age (years)	0.043	0.022, 0.065	< 0.001	
Diabetes duration 4 to < 10 years vs. ≤ 4 years	0.056	−0.064, 0.175	0.356	
Diabetes duration ≥ 10 years vs. ≤ 4 years	0.179	0.030, 0.328	0.019	
Current HbA1c: HbA1c-above-target vs. Hba1c-within-target	0.012	-0.138, 0.162	0.872	
Historical HbA1c: HbA1c-above-target vs. Hba1c-within-target	-0.048	−0.172, 0.077	0.446	

HbA1c-above-target: > 7.5%/59 mmol/mol; HbA1c-within-target HbA1c: ≤ 7.5%/59 mmol/mol.

Discussion

Consistent with previous studies (12–14), patients with type 1 diabetes showed significantly higher SAF than controls, both for the group as a whole and across all age categories. It is important to note that this was already apparent for the lowest age category (age 11–12 years). SAF appeared to increase faster in the elder adolescents for both patients and controls and with diabetes duration in patients. Differences in SAF between current HbA1c-within-target and HbA1c-above-target patients and between historical HbA1c-within-target and HbA1c-above-target patients were small. In addition, SAF was only weakly associated with diabetes duration and HbA1c (both current and historical) in our homogeneous group of Dutch Caucasians, consistent with findings from Felipe et al. (14). This association disappeared when adjusting for diabetes duration and age. Interestingly, a subgroup of patients with a HbA1c-within-target had an elevated SAF.

Previous studies showed conflicting results on the association between historical HbA1c and SAF (5, 21). A strong correlation between historical HbA1c and SAF would be expected, as SAF is believed to be at least partly caused by hyperglycemia-induced superoxide and carbonyl damage, resulting in permanent damage to long-lived proteins such as collagen (3, 22). However, in this study, a strong association between historical HbA1c and SAF could not be demonstrated. An explanation may be that the period during which historical HbA1c was determined was too short or that median intra-individual HbA1c is an inadequate parameter to express historical HbA1c. Alternatively, as sAGEs are considered to be formed by various pathways (5, 22, 23), the influence of hyperglycemia on SAF may also be overestimated. It is intriguing to see that some patients show an elevated SAF despite having a HbA1c-within-target. This may be explained by genetic factors influencing either the level of glycation of HbA1c or by factors that influence AGE formation such as polymorphisms of the AGE-receptor (RAGE) gene (24) or the NAT2 acetylator (25). Also, oxidative/carbonyl stress may play a role (23, 26), which may be hypoglycemia-related (27). An outstanding question is if this heterogeneity in patients reflects differences in risk for complications. If so, then SAF measurement in this subgroup may provide information on risk for complications independent of HbA1c.

A strength of this study was that adjustment for skin color was not necessary, as measured patients and controls were from the same ethnic background (Caucasian). Homogeneity of the patient population supports internal validity. However, the results cannot be applied to non-Caucasians and therefore generalizability is lower. Also, we studied the age range 11–19 years in more detail when compared with previous studies (12, 14, 28), showing clearly that children and adolescents with type 1 diabetes in the age category 11–12 years already have elevated SAF. We took into account the use of skin care products, as these can affect SAF readings (29). One limitation of our study as well as previous studies (13,14) is that we were unable to quantify measurement errors in terms of coefficient of variation. To reduce measurement error as much as possible, only one type of AGE-reader was used and SAF measurements were performed in triplicate. However, when measuring SAF is to be of use in routine clinical practice, precision CV of these measurements will have to be assessed to be able to distinguish measurement error from clinically meaningful SAF measurements. SAF readings may be confounded by a number of behavioral factors such as dietary factors and fasting state (30). Additional factors such as smoking and exercise are implicated in the accumulation of SAF (20, 28). We could not adjust for these factors. BMI may influence SAF, in particular in individuals with central obesity (31). We did not extend the BMI analyses as only 4 patients had a BMI > +2 SDS.

In summary, children and adolescents with type 1 diabetes show higher SAF than controls. Age and duration of diabetes are weakly associated with SAF. In e majority of patients, SAF does not seem to provide information additional to HbA1c. However, in a subgroup of patients with HbA1c-within-target an elevated SAF was observed . For this subgroup, measuring SAF may have added value in identifying patients that are at high risk for complications. Further longitudinal, prospective studies should provide insight into whether SAF measurement in youngsters has predictive value for the development of complications during the disease course of type 1 diabetes and how this is related to glycemic control.

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Chapter 8

General Discussion

8.1 Introduction

The incidence of type 1 diabetes is still increasing (1-3). Despite advances in care and increasing knowledge about modifiable risk factors and potential interventions (4-7), the incidence and prevalence of the microvascular (nephropathy, neuropathy and retinopathy) and macrovascular complications of diabetes are still substantially increased compared with the general population (8, 9). Taking into account that there are more determinants than those involved in the classical hyperglycaemia paradigm, two factors are thought to contribute to this worrisome perspective for patients with type 1 diabetes: 1) a lack of awareness of the impact of (certain) risk factors and 2) the suboptimal early detection of the high-risk patient, e.g. the patient with risk factors and/or early signs of complications (10-12). We reasoned that improving the identification of children and adolescents with type 1 diabetes with (one or more) risk factors and/or early signs of complications, by improving early risk stratification, will allow adaptation of screening procedures and/or implementation of earlier interventions. This, in turn, would hopefully result in an improved lifetime perspective for the patients with type 1 diabetes.

The EDDDY-S study was conducted between 2007 and 2018 with the goal to improve this early risk stratification in children and adolescents with type 1 diabetes. This study was designed to focus on areas in complication-related research underexposed in 2007 for this age-group and therefore does not cover tests in all complication fields.
The Early Detection of Diabetes Damage in Youth and Search for early prevention (EDDDY-S) study assessed:

- the diagnostic value of two parameters for the detection of subclinical peripheral diabetic neuropathy (DPN): compound muscle action potential (CMAP) scan by CMAP scan of the peroneal motor nerve; and measurement of nerve conduction velocity (NCV) and sensory nerve action potential (SNAP) of two sensory nerves by electrophysiologic tests (Chapters 2-4).
- a surrogate marker for macrovascular complications: carotid intima media thickness (cIMT) by means of carotid ultrasound (Chapter 5).
- longitudinal lipid dynamics of patients with type 1 diabetes: predominantly the change to an unfavourable level ('losing track of lipids')

through childhood and adolescence was studied because dyslipidemia is a risk factor for macrovascular complications (Chapter 6).
- a presumed surrogate marker for both micro- and macrovascular complications: assessment of skin autofluorescence (SAF) by measuring advanced glycation end products (AGEs) in the skin using the AGE-reader (Chapter 7).

The outline of this general discussion, in which we will further elaborate on early risk stratification in children and adolescents with type 1 diabetes, is as follows: in section 8.2 for each field of complication studied in the EDDDY-S study, the results will be summarized followed by a short review of the literature (Figure 1). After putting the results into context of the literature, feasibility for future use in a proposed screening protocol will be discussed. As this screening protocol would also include tests which are currently still in the research setting, we allocated the tests in the proposed screening protocol to one of the following 3 parameter categories:

A. tests included in the recommendations according to international guidelines (13, 14) and as such in general assumed to be established diagnostic tests
B. diagnostic tests other than the ones recommended in the current guidelines
C. tests in research context

After some methodological considerations in section 8.3, we conclude the general discussion with integrating our proposals for screening into a suggested screening protocol in section 8.4.

8.2 Summary and implications of results from the EDDDY-S study

Two tests for diabetic peripheral neuropathy (DPN)

EDDDY-S

Large nerve fiber dysfunction is a presumed late(r) sign of DPN (15, 16). Because screening recommended in the guideline of 2007 (17) predominantly tests for large nerve fiber dysfunction, two pilot-studies of the EDDDY-S study assessed the detection of subclinical DPN. One pilot study measured the nerve conduction velocity (NCV) and sensory nerve action potential (SNAP) amplitude of two sensory nerves, investigating if the frequently-used reference test for DPN (electrophysiologic study of the NCV) indeed fails to detect subclinical DPN. In the other pilot study, several variables of the novel compound muscle action potential (CMAP) scan were measured in the peroneal motor nerve. The first pilot study showed that neither the NCV nor the SNAP amplitude of the two sensory nerves did accurately detect subclinical DPN in the children and adolescents with type 1 diabetes. The second pilot study showed the CMAP-variable 'stimulus intensity' (reflecting axonal excitability) to be abnormal in both young patients with well-controlled type 1 diabetes and a relatively short disease duration, and in patients with poorly controlled type 1 diabetes and quite long disease duration, when compared with healthy controls. We therefore conclude that the CMAP scan is a promising test for the detection of early DPN.

2007-2018

Small nerve fiber dysfunction as measure of subclinical DPN is typically performed by measuring corneal nerve fiber density (CNFD) and corneal nerve fiber branch density (CNFBD) by corneal confocal microscopy (CCM). In adult patients with type 1 diabetes, CNFD and CNFBD were shown to correlate with intra-epidermal nerve fiber (branch) density in skin biopsies (reference test) (18). Associations between several corneal nerve measurements and severity of clinical DPN (18-20), other small nerve fiber function tests (18, 21) and the development of future DPN (22) were found. In children and adolescents with type 1 diabetes, only one study was performed. In this pilot study, no difference was found in corneal confocal microscopy (CCM) measures between adolescents

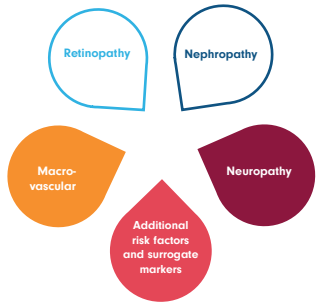
with type 1 diabetes (n=6) and healthy age-matched controls (23), perhaps due to small sample size.

Screening tests for the detection of large nerve fiber dysfunction were evaluated as well (24). Determination of the vibration perception threshold (VPT), by the Biothesiometer or Vibratory Sensory Analyser, appeared to have a better diagnostic utility in detecting subclinical DPN in children and adolescents with type 1 diabetes than the tuning fork or Vibratron II (24-27). Evaluation of the diagnostic value of testing the tactile function with smaller monofilaments (1 mN) showed controversial results (16, 24, 28). The debate on the diagnostic value of electrophysiologic studies in detecting subclinical DPN continued. A small percentage of patients with clinical DPN at young adult age had an impaired peroneal and median motor nerve conduction velocity and/or sural sensory nerve action potential at baseline (mean age at baseline 15.5 years)(29). Hyperglycaemie was suggested to affect nerve conduction velocity but not sensory nerve action potential when present during electrophysiologic testing (30) in contrary to a previous study on this subject (31).

Finally, several studies evaluated the diagnostic value in detecting subclinical DPN of CCM and/or other small nerve fiber function tests such as quantitative sensory testing together with or compared with large fiber function tests (22, 32). In adults with type 1 diabetes, Breiner and Pritchard showed one or more small nerve fiber function tests to be abnormal in case of (future development of) abnormal large nerve fiber function (22, 33). However, these studies, did not show that small nerve fiber dysfunction per se precedes large nerve fiber dysfunction. In children, adolescents and young adults with type 1 diabetes, only cross-sectional studies were performed. VPTs (large nerve fiber) by means of Biothesiometer/ Vibratory Sensory Analyser and/or thermal thresholds (small nerve fibers) by means of Thermal Threshold Tester/ Neurosensory TSA-II were shown to be abnormal, and thus suggested to detect subclinical DPN, in 14-28% of the children and adolescents with type 1 diabetes (25, 26). It was not specified which of the two performed tests were abnormal in their patient group. Weintrob and Blankenburg showed that more young adults and adolescents with type 1 diabetes had small nerve fiber dysfunction then large nerve fiber dysfunction (32, 34). However, both studies do not show if the patients with large nerve fiber dysfunction had small nerve fiber as well, which would have strengthened the hypothesis

Figure 1: Promising tests between 2007-2018 studying some underexposed areas in complication-related research in pediatric diabetes and tests in these areas that were included in the EDDDY-S study (with in bold most promising tests).

- cIMT
- endothelial dysfunction measures
- arterial stiffness measures
- extension of the serum lipid profile
- longitudinal information lipid profile



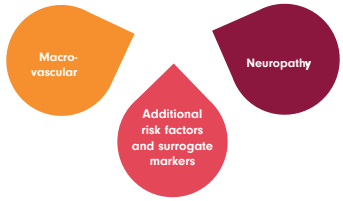
- CNFD, CNFBD by corneal confocal microscopy
- VPT by Biothesiometer or Vibratory Sensory Analyser
- tactile function testing with smaller monofilaments
- electrophysiologic measures of motor and sensory nerves

- AGEs by skin autofluorescence

EDDDY-S study 2007 - 2018

Macrovascular

- cIMT
- longitudinal information lipid profile



Neuropathy

- CMAP scan measures
- SNAP/NCV of sensory nerves

- AGEs by skin autofluorescence

Abbrevations

CNFBD: corneal nerve fiber branch density
cIMT: carotid intima media thickness
CNFD: corneal nerve fiber density
VPT vibration perception threshold
AGEs: advanced glycation endproducts
NCV: nerve conduction velocity
EDDDY-S: Early Detection of Diabetes Damage in Youth and Search for early prevention
CMAP: compound muscle action potential
SNAP: sensory nerve action potential

of small nerve fiber dysfunction preceding dysfunction of the large nerve fibers. Additionally, longitudinal data are lacking.

Thus, at present it remains to be elucidated if the preferred approach in detecting subclinical DPN appropriately in children and adolescents with type 1 diabetes should include only (one of the) small nerve fiber function tests, or a combination of large and small nerve fiber function tests.

Implications

Our research showed a low diagnostic value of measurement of the sensory NCV and SNAP amplitude for the detection of subclinical DPN in children and adolescents with type 1 diabetes. Despite the small study population, we predicted that a larger sample is unlikely to alter this main conclusion. Whereas these study results strengthen the idea and that of others that more focus on other nerve fibers than the large nerve fibers (only) is indicated in the development of screening tests for subclinical DPN, recent studies suggested that measurement of the VPT by Biothesiometer/ Vibratory Sensory Analyser detects subclinical DPN and thus inclusion of this test should be considered as subject of the screening protocol. In addition, the CMAP scan study results showed that another approach to large nerve fiber damage, e.g. focus on axonal damage, may also be helpful in the detection of subclinical DPN. New scientific insights from 2007 to 2018 show that more focus on small fiber nerve function should be considered as part of the screening protocol for subclinical DPN as well. CCM appears most promising.

Our ultimate goal is that the screening protocol for children and adolescents with type 1 diabetes includes one screening test for subclinical DPN. However, with the current paucity of studies that assess promising screening tests based on determination of the VPT by Biothesiometer/ Vibratory Sensory Analyser, the CMAP scan and CCM, we propose to study these three tests in a combined diagnostic and research screening protocol. We allocate the VPT by Biothesiometer/ Vibratory Sensory Analyser to category B because abnormal VPT has been found in type 1 diabetes in several pediatric studies (24-26). We allocate measurement of stimulus intensities of the peroneal nerve by the CMAP scan and CNFD and CNFBD by CCM to parameter category C because there is not much experience yet with both tests in pediatrics.

Carotid intima media thickness (cIMT)

EDDDY-S

cIMT is considered to be a surrogate marker of subclinical atherosclerosis and associated with macrovascular complications (35-37). It gained our attention because a surrogate marker and/or early sign for macrovascular complications was lacking in the childhood and adolescent age-group with type 1 diabetes. In 2007, the existing studies on this subject showed controversial results on cIMT increase in children and adolescents with type 1 diabetes compared with healthy controls and on the impact of contributing factors to cIMT increase (38-40). Additionally, information about age-specific differences between children and adolescents with type 1 diabetes and their peers was lacking (38-40). Across all age categories we found cIMT to be equally increased in patients with type 1 diabetes compared with healthy controls, i.e. to already be abnormal in the youngest age category (9-10 years old). No significant contribution of poor glycaemic control and other classical risk factors was found.

2007-2018

Several studies showed cIMT to be increased in children and adolescents with type 1 diabetes as compared with healthy peers (38, 41, 42). cIMT progression was detected in high-risk patients within only a short follow up period (41, 43). Controversially, a review of observational studies showed that cIMT is not always increased in pediatric patients with type 1 diabetes when compared with healthy controls (44). Small study populations, young age, a short duration of diabetes (and thus short follow-up) were suggested as explanations, in addition to more general comments such as wide reference ranges for cIMT in children and adolescents and the heterogeneity in cIMT study protocols (44). Several studies suggested aortic IMT (aIMT) to be a more sensitive surrogate marker for subclinical atherosclerosis (35, 45-48). However, replacing cIMT by aIMT measurement is questionable because measuring aIMT is suggested to be more difficult in case of increased tissue penetrance and takes more time (46-48).

Endothelial function was measured by flow-mediated dilation (FMD) of the brachial artery as a result of reactive hyperaemia after shear stress using different techniques. In children and adolescents with type 1 diabetes, FMD was shown to be abnormal when compared with healthy controls (48-52). Associations

with HbA1c and microalbuminuria were found (49, 51, 52). Despite FMD being a non-invasive objective measure of endothelial function, the required conditions of measurement in a regulated room temperature (48-50), the possible influence of acute hyperglycaemia on FMD (50, 53) and the absence of pediatric reference ranges, makes its implementation in routine outpatient care challenging.

Several measures for arterial stiffness, such as small artery elasticity (SAE), pulse pressure (PP), pulse wave velocity (PWV), augmentation index (A175) and brachial distensibility (BrachD) were studied between 2007-2018. An earlier SAE peak in life, a diminished SAE and an increased PP beyond the age of 30-40 years old was found in patients with type 1 diabetes and complications as compared with the other patients without complications and healthy controls (54). Another study found an increased PP already in childhood in patients with type 1 diabetes (55). PWV was shown to increase throughout adolescence in patients with type 1 diabetes (56), whereas two studies comparing PWV to reference ranges found contrary results (57, 58). One of these studies evaluated A175 and BrachD as well and found both to be abnormal in the patients with type 1 diabetes when compared with healthy controls (58, 59). Reference values for European children and adolescents have been determined for PWV (60), PP (61) and SAE (62). To our knowledge, reference values of augmentation index (A175) and a diminished brachial distensibility have not been established yet. Less accurate measurement of PP and PWC have been suggested in case of a very young age and an increased waist circumference respectively (56, 57).

Implications

cIMT has been clearly shown to be abnormal in children and adolescents with type 1 diabetes (38, 40-43, 63). Appropriate reference ranges are available (63). Also taking into account the shown associations of increased cIMT and macrovascular complications (36, 37), we think cIMT should be included in the future screening protocol (parameter category B). Due to a lack of appropriate reference ranges and the incapability of measuring aIMT in case of obesity, aIMT will not be added although it might show abnormalities earlier than cIMT. Abnormal PP and PWV, reflecting increased arterial stiffness, are both promising surrogate markers for macrovascular complications. We hypothesize these markers to replace the cIMT measurement because both tests are less observer-dependent and easily applicable in the outpatient clinic patient care. Reference ranges are

available (60, 61). Because there is not enough experience with these tests in the pediatric age group, we allocate PP and PWV to parameter category C. We propose to include both cIMT and PP and PWV in a proposed screening protocol. Future evaluation of the three presumed surrogate markers in this screening protocol should address if PP and/or PWV can replace cIMT measurement.

Longitudinal lipid dynamics

EDDDY-S

The values of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and triglycerides (TG) of healthy children and adolescents are shown to be quite stable from childhood through young adulthood (64). A longitudinal study by Maahs evaluated the dynamics of these lipids and of non-HDL cholesterol (non-HDL-C) in children and adolescents with type 1 diabetes (65). This study showed that a considerable proportion of patients changed from a low-risk lipid level into a borderline-high risk or high-risk level or changed from a borderline-high risk level into a high-risk level of lipids ('lose track of lipids'). As dyslipidemia is a known risk factor for macrovascular complications, detection of patients that lose track of lipids is important. Our retrospective longitudinal study, including 651 children and adolescents with type 1 diabetes, studied the dynamics of LDL-C, HDL-C, non-HDL-C, TC and TG and showed that a considerable number of the patients switched to borderline-high risk or high-risk levels of >1 of the studied lipids within a median follow up duration of 3.24 years. Of the patients with a LDL-C or HDL-C low-risk level at baseline, 5-10% lost track of these lipids within a screening interval of two years. With regard to the other lipid parameters, 25-30% lost track of lipids. The use of a prognostic index, including age, gender, BMI and HbA1c, diabetes duration and ethnicity, was only moderately able to predict this change on the short term.

2007-2018

In addition to the early studies of Maahs (65) and Edge (66), more recent studies strengthen the finding that children and adolescents with type 1 diabetes 'lose track of lipids' (67-69) and show predominantly the determinants HbA1c and BMI to influence lipid dynamics (65, 67, 68). Additionally, several cross-sectional studies in this age-group with type 1 diabetes showed added value in risk stratification of extending the lipid profile (LDL-C, HDL-C, non-HDL-C, TC and TG)

with lipids such as apolipoprotein B and LDL-C particle size that are supposed to inform better about the atherogenicity (70-72).

Implications

Our longitudinal study in lipid dynamics together with study of the literature 2007-2018 showed that inclusion of apoB, among others, can be of additional value in interpreting the lipid profile. Therefore, we suggest to add apoB to the lipid screening protocol (parameter category B). It also revealed that 5% of the patients lost track of non-HDL-C levels within 16.5 months; TC levels within 13.5 months; and TG levels within 13 months. This was in contrast with 22 months for LDL-C and 20 months for HDL-C. With conventional treatment of dyslipidemia focussing on LDL-C in this age group (10, 14), we suggest to continue the 2 year lipid screening protocol (parameter category B). Future study should address if this interval should be adapted to the losing track of one of the other fore mentioned lipids, but only if relevant treatment options exist. Whereas our tracking study only showed moderate power of a combination of several determinants in predicting losing track of lipids on the short term, one or more of those determinants were shown to predict losing track of lipids if used separately in other studies (65, 67, 68). We propose to study the impact of the determinants age, gender, HbA1c, BMI, ethnicity and diabetes duration, separately and in combinations, on the likelihood of losing track of lipids. Additionally we suggest to study the impact of other determinants such as smoking, puberty, non-alcoholic fatty liver disease and positive family history of premature cardiovascular disease on the likelihood of losing track of lipids.

Advanced glycation end products (AGEs) in skin

EDDDY-S

Advanced glycation end products (AGEs) in the skin, measured by skin autofluorescence (SAF), were shown to associate with both micro- and macrovascular complications in adults with type 1 diabetes and therefore appeared a promising surrogate marker for these complications (73, 74). In 2007, measurement of SAF had not yet been performed in children and adolescents with type 1 diabetes. In the EDDDY-S study, SAF was shown to be elevated similarly across all age categories in the patients with type 1 diabetes, i.e. also in the youngest age group already (11-12 years). Glycaemic control was not shown to associate with SAF ele-

vation. Interestingly, a subgroup of patients showed increased SAF despite having good glycaemic control.

2007-2018

AGE levels were determined by skin autofluorescence (SAF) or skin intrinsic fluorescence (SIF) in several studies in the period 2007-2018 (75-78). In adults with type 1 diabetes, previously found associations between increased SAF/SIF and the prevalence of micro- and macrovascular complications were confirmed (75-77, 79, 80). In children and adolescents with type 1 diabetes, SAF/SIF were shown to be increased in this age-group when compared with healthy controls (45, 81-83). Associations of SAF/SIF with early signs of micro- and macrovascular complications in this age-group were shown with regard to retinopathy, a measure of heart rate variability and aIMT, but not with peripheral nerve abnormalities and albumin excretion rate or glomerular filtration rate (26, 45). Studies that examined correlations between SAF/SIF and (long-term) HbA1c showed conflicting results (26, 75, 77, 78, 80, 84, 85). Moreover, increased SAF/SIF was already shown in children that were just recently (< 1 week) diagnosed with type 1 diabetes (82) and was shown to be present in a subgroup of patients with normal HbA1c levels (26, 78).

Implications

Measuring SAF is a presumed surrogate marker for micro- and macrovascular complications in patients with type 1 diabetes (73-77, 79, 80, 86, 87). With controversial results on long-term HbA1c and SAF and a subgroup of patients showing elevated SAF despite appropriate (long-term) glycaemic control, it remains to be elucidated if SAF is of additional value to HbA1c in predicting micro-and macrovascular complications. Future studies should show if HbA1c and SAF can be interchangeable surrogate markers for the average patient, but also if SAF is a better surrogate marker in a subgroup of patients. Longitudinal prospective studies, starting SAF measurement at diagnosis of type 1 diabetes, followed by 3-monthly measurements (parameter category C) combined with simultaneous assessment of (early) signs of complications at regular intervals, should help answering the following outstanding issues:

- The intrapersonal variability of SAF over a relatively short period of time, possibly explained by confounding factors such as dietary factors, fasting and

smoking the day before measurement (88-91), or an association with short-term glycaemic control or other glucose related measures (such as glucose variability).
- What should become the future SAF measurement scheme, e.g. which SAF measurement(s) in the scheme associate best with (early) signs of long-term complications?
- Does SAF indeed identify a subgroup of patients that develop (early) signs of complications despite appropriate glycaemic control? Moreover, are regular SAF instead of HbA1c measurements warranted for these patients?

The EDDDY-S study reveals two intriguing findings that need further study:

Firstly, in at least a subgroup of children and adolescents with type 1 diabetes, glycaemic control was not a contributor to the development of complications; no significant contribution of HbA1c to the increase in cIMT was found; a subgroup of patients had an elevated SAF despite an HbA1c < 7.5% (58 mmol/mol); decreased axonal excitability in a patient-group with well-controlled diabetes was shown; and HbA1c did not contribute to 'losing track of lipids' for LDL-C, HDL-C, non-HDL-C, TC and TG, possibly due to heterogeneity in the patient group.

There is overwhelming evidence to suggest that poor glycaemic control (i.e. high HbA1c) is a dominant risk factor for the development of complications in the entire cohort of patient with type 1 diabetes (4-6, 8, 92-98). However, this may not be the case for the individual patient. In the EDDDY-S study we showed that poor glycaemic control did not contribute significantly to decreased axonal excitability, cIMT increase, elevation of SAF, and only moderately to losing track of lipids (Chapters 2,5,6 and 7). The EDDDY-S study is not the only study that takes a more individualized approach. Cho showed a higher prevalence of sub-clinical retinopathy in adolescents with type 1 diabetes with well-controlled diabetes but increased SAF in the highest tertile (26), whereas Lilje did not find an association between elevated skin intrinsic fluorescence (SIF), poor glycaemic control and increased aortic intima-media thickness (45). Appropriate glycaemic control may have mitigated the (relative) contribution to study results in the EDDDY-S study. The findings in our study and the Cho and Lilje studies may be explained by heterogeneity among patients with type 1 diabetes for at least a proportion of the patients. There might be a subgroup of patients that will never develop

complications and a subgroup of patients that will develop complications despite good glycaemic control. We do indeed think such subgroups exist. Alternatively, other established risk factors not included in these studies (5, 8, 9, 40, 93-95, 99-107), or yet unidentified risk factors may play a role and their effects may (partly) overrule the effect of glycaemic control on complications.

In the last few years, the relevance of heterogeneity among patients with diabetes has gained more attention. Heterogeneity in the disease diabetes is thought to be caused by, among others, genetic and environmental factors, which not only affect the course of the disease and its subtypes(108-110), but will also pose different risks for the development and/or progression of complications (11, 111). Presumable risk factors that may diminish the (relative) contribution of HbA1c to the development of complications are listed in Table 1.

Table 1: Risk factors described to increase complication risk in type 1 diabetes

The presence of certain inflammation markers	(112, 113)
Less pre-diabetes beta cell mass (originated in utero, environmentally or genetically)	(114)
Increased beta cell vulnerability to the auto-immune attack	(114)
More biological variation in HbA1c, measured by haemoglobin glycation index	(115)
Genotypes related to presence or development of risk factors	(116-120)
Unfavourable microRNA expression	(121)
Increased dysregulation of epigenetic mechanisms	(122)
Influence of 'metabolic memory' related to the timing of hyperglycaemia in the course of the disease	(123, 124)
The influence of glucagon to glucose homeostasis	(125)

Secondly, a considerable number of patients with type 1 diabetes developed subclinical complications already in childhood and adolescence: signs of subclinical atherosclerosis, assessed by cIMT measurement, were observed in patients as young as 9-10 years; subclinical DPN was found to be present in patients with a median age of 13.92 years; and SAF elevation could be seen in the youngest age

category e.g. 11-12 years already. As SAF is assumed to be a surrogate marker for micro- and macrovascular complications (at least in adults with type 1 diabetes), this finding strengthens the idea that detectable (early signs of) complications may already develop at a young age. However, future longitudinal studies should assess if these parameters can indeed function as surrogate markers. These studies should also address if the parameters can identify a subgroup of patients that behaves differently compared with the type 1 diabetes group as a whole, i.e. reflect heterogeneity.

In the period 2007-2018 several studies investigating early signs of complications in children and adolescents with type 1 diabetes supported the EDDDY-S study findings. Clinical DPN and subclinical DPN, subclinical retinopathy, nephropathy and macrovascular complications were found in children and young adolescents with type 1 diabetes by several investigators (Table 2 and Appendix 2)(25, 26, 34, 45, 54, 55, 126) stressing the need for sensitive tests for the young age group.

Table 2: Data from EDDDY-S sub-studies and comparable studies about surrogate markers and subclinical complications in children and adolescents with type 1 diabetes

A: Compound muscle action potential (CMAP) scan (early sign of diabetic peripheral neuropathy)

	Van der Heyden 2013(127) (n=30)
Age (yrs)	Pt-group 1 (n=13): 13.9 (12.6-17.1) Pt-group 2 (n=17): 18.8 yr (15.9-22.4)
Diabetes duration (yrs)	Pt-group 1: 3.8 (3.5-4.5) Pt-group 2: 12.6 (11.4-16.5)
HbA1c (%)	Pt-group 1: 7.1 (6.9-7.6) Pt-group 2: 9.2 (8.4-10.4)
Outcome variables measured by means of CMAP scan in short	Decreased axonal excitability in patients compared with controls

B: Nerve conduction velocity and sensory action potential of sensory nerves (early sign of diabetic peripheral neuropathy)

	Van der Heyden, unpublished (n=20)	Hyllienmark 1995(128) (n=75)	Riihimaa 2001(100) (n=100)	Nelson 2006(27) (n=73)
Age (yrs)	Pt-group 1: 14.0 (13.7-17.3) Pt-group 2: 16.4 (13.7-18.5)	15.4 (3.6)	13.7 (2.0)	13.7 (2.6)
Diabetes duration (yrs)	Pt-group 1: 3.8 (3.4-4.4) Pt-group 2: 12.1 (10.6-13.7)	8.2 (3.5)	7.0 (3.5)	8.1 (2.6)
HbA1c (%)	Pt-group 1: 7.3 (7.0-7.7) Pt-group 2: 9.4 (8.9-11.2)	7.0 (1.1)	8.5 (1.7)	9.0 (1.0)
Outcome NCV, SNAP	No difference in NCV between groups. SNAP amplitude decreased in Pt-group 2 (poorly controlled patients) compared with controls	Decreased NCV and SNAP of sensory sural and median nerve in patients compared with controls	Decreased NCV and SNAP of sensory sural and median nerve in patients compared with controls	Considerable number of patients with decreased NCV of sural nerve and small number with decreased SNAP, compared with controls

C: Carotid intima media thickness (surrogate marker and/or early sign of macrovascular disease)

	Van der Heyden 2016 (63) n=135	Lilje 2018(45) n=38	Schwab 2007(38) n=94	Dalla Pozza 2007 (40) n=150	Margeirsdottir 2010 (42) n=314
Age (yrs)	15.3 (13.4-18.2)	13.4 (3.4)	12.3 (8.5-16.8)	13.9 (2.8)	13.8 (2.8)
Diabetes duration (yrs)	After stratification by age 15.3 or ≥15.3 years: 5.6 (3.8-8.3) and 10.0 (6.3-14.0) respectively	5.8 (4.3)	3.8 (1.8-9.8)	Not mentioned for the Pt-group as a whole	5.5 (3.4)
HbA1c (%)	After stratification by age 15.3 or ≥15.3 years: 8.0 (7.4-8.7) and 8.4 (7.5-9.2) respectively	9.7 (1.6)	7.7 (6.8-10.0)	Male 7.5 (0.9), Female 7.4 (0.9)	8.4 (1.3)
Outcome cIMT	cIMT increased in patients compared with controls	No significant difference between patients and controls in cIMT: only in aIMT and fIMT	cIMT increased in patients compared with controls	cIMT increased in patients compared with controls	No significant difference between patients and controls; higher percentage of abnormal cIMT in the Pt-group

D: Longitudinal tracking of lipids (risk factor for macrovascular disease)

	Van der Heyden, submitted 2018 (n=651)	Maahs 2007(65) (n=360)	Edge 2008(66) (n=229)	Reh 2011(67) (n=46)	Katz 2017(68) (n=572)	Shah 2017(69) (n=1478)
Age (yr) at baseline	12.63 (4.6)	13.6 (4.1)	12.4 (3.2)	14.3 (3.4)	11.9 (2.9)	10.8 (3.9)
Diabetes duration (yr)	4.05 (2.1-7.2)	4.5 (0.3)	5.5 (4.0)	6.4 (3.8)	4.8 (3.1)	0.75 (0.5)
HbA1c (%)	7.7 (7.0-8.6)	8.8 (1.6)	8.8 (1.4)	8.1 (1.0)	8.9 (1.5)	7.6 (1.5)
Outcome lipid tracking in short	A considerable number of patients loses track	A considerable number of patients loses track	A considerable number of patients loses track	A considerable number of patients loses track	Percentage of losing track not examined	A considerable number of patients loses track

E: Skin autofluorescence (SAF) or skin intrinsic fluorescence (SIF) (surrogate marker for micro- and macrovascular complications)

	Van der Heyden 2016(129) (n=77)	Cho 2017(26) (n=135)	Lilje 2018(45) (n=38)	Barat 2012(83) (n=52)	Shah 2013(81) (n=133)
Age (yrs)	15.3 (13.6-17.0)	15.6 (2.1)	13.4 (3.4)	12 (6)	13.0 (3.0)
Diabetes duration (yrs)	6.6 (3.5-9.5)	8.7 (3.5)	5.8 (4.3)	5.92 (3.8)	6.0 (3.6)
HbA1c (%)	8.5 (1.4)	8.7 (1.5)	9.7 (1.6)	8.0 (1.1)	9.3 (2.0)
Outcome SAF/ SIF in short	SAF increased in patients compared with controls	SAF increased in patients compared with controls	SIF increased in patients compared with controls	SAF increased in patients compared with controls	SIF increased in patients compared with controls

Data expressed in median and interquartile range or mean and standard deviation and in the study of Schwab 68.5% CI Abbreviations: aIMT= aortic intima media thickness, cIMT= carotid intima media thickness, CMAP= compound muscle action potential, fIMT=femoral intima media thickness, Pt=patient, SAF= skin autofluorescence, SIF= skin intrinsic fluorescence (SIF), NCV = nerve conduction velocity, SNAP = sensory nerve action potential

8.3 Methodological considerations of the EDDDY-S study

Evaluating the EDDDY-S study as a whole, a few methodological issues should be addressed:

- sample size and study duration,
- the terminology of the used terms 'risk factor', 'surrogate marker', 'early sign of complication', 'complication',
- the absence of reference tests,
- use of data extracted from electronic databases,
- possibilities and limitations to generalize results to the 'general type 1 diabetes population'.

Sample size and study duration

Whereas sample sizes in the cross-sectional SAF and cIMT studies (Chapter 5 and 7) were comparable to several published studies in children and adolescents with type 1 diabetes (26, 38-40, 78, 85), small sample sizes in the NCV/SNAP and CMAP scan studies (Chapter 2 and 3) were the result of the explorative nature of the study design, complexity of the techniques and strict inclusion criteria.
The sample size of the longitudinal lipid tracking study (Chapter 6) was large for a single-centred European study in children and adolescents with type 1 diabetes. However, despite the local protocol that measures lipids every 2 years instead of every 5 years (17), the sample size decreased with increasing follow up duration. Comparable studies with approximately a similar study design (65, 66) encountered the same problems. The follow up of Shah's study was longer but included patients with a shorter disease duration at baseline (69). We think the need for more longitudinal lipid studies is stressed by 1) recent growing awareness of the presence of modifiable risk factors that appear to be present already in childhood and adolescence, and 2) the possibility to prevent further deterioration if intervention is initiated (10, 14, 130, 131).

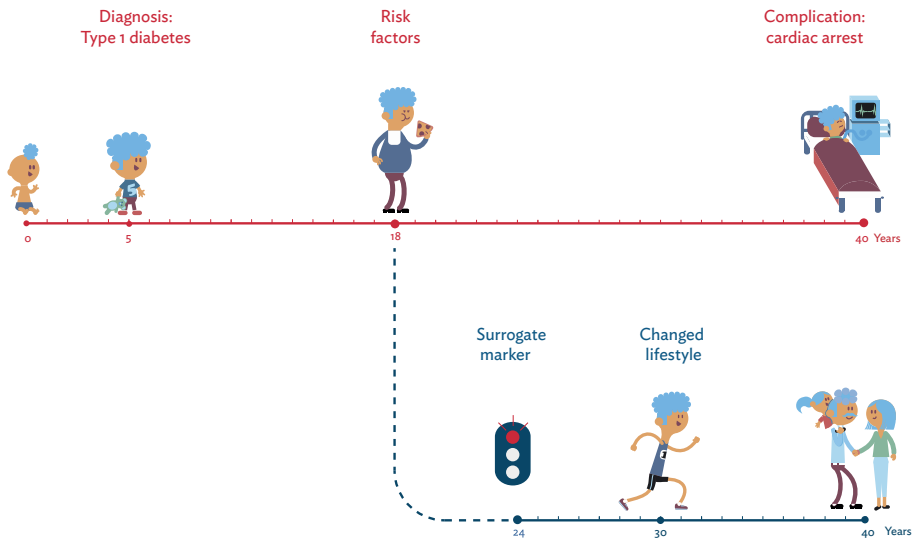
In general, small sample size poses two risks: potential lack of power and risk of selection bias. The first risk could not be mitigated in our studies. With regard to the second risk we compared our study population with study populations described in the literature, but could not completely rule out this risk. Overall, our study population compares relatively well with similar studies, although we report slightly better HbA1c levels and different ethnic composition (65, 66).

Terminology

In the literature we found that in the field of complication research many terms are used interchangeably. Especially with respect to AGEs, some express elevation of SAF as risk factor while others use it as surrogate marker for micro- and macrovascular disease. We advocate using these terms as suggested below and in Figure 2.

- Risk factor: each determinant in a determinant-endpoint relationship
- Surrogate marker (132): a substitute of a clinical endpoint which is associated with that endpoint or prognostic of that endpoint
- Early sign of complication: preclinical and/or clinical abnormality that may progress to a complication e.g. endpoint
- Complication: clinical condition with medical /personal consequences and requiring treatment and/or medication and/or intensive follow-up

Figure 2: Illustration of the current liftetime perspective of one patient in red. The expected change in lifetime perspective, based on an alarming result of a surrogate marker described in this thesis, is shown in blue.



Absence of reference tests

The absence of a good short term reference test (e.g. reduction in intra-epidermal nerve fiber density by means of skin biopsy in case of subclinical DPN) in many of the subjects that were studied in this thesis and previous studies complicates research in this field. In fact, in DPN, impaired NCV of the sensory nerve is frequently used as reference test (24, 27, 28). Determination of intra-epidermal fiber density by skin biopsy (133, 134) or myelinated fiber density examination by biopsy of the sural nerve may be a more appropriate reference test (135), but this method is too invasive to be used in a young age group (136). For skin AGEs, biopsies for studying collagen were also not easily to obtain. Meerwaldt et al had biopsies available and showed them to correlate with skin autofluoresence (137). In the field of vascular research, to the best of our knowledge the association between atherosclerosis and macrovascular disease is most convincingly supported by post mortem data combined with data on risk factors of macrovascular disease (35) and a long-term study showing thickening of the intima-media and plaque formation to associate with macrovascular disease (36).

Data extracted from electronic database

Our studies were based on patient data extracted from the electronic patient files. In a recent review, Farmer discussed a number of potential problems using data from patient files for research purposes (138). Three problem-categories are described; 1) omitted variables; 2) incomplete data, missing data or data with unclear validity; and 3) time-related biases in case of follow up studies.

For the EDDDY-S study examples of omitted variables (e.g. smoking and physical activity) were discussed in the lipid tracking and cIMT manuscripts (Chapter 5 and 6). However, it was also reasoned that a strong impact of these factors on outcome is not to be expected.

As an example of incomplete data, missing data or data with unclear validity, the incomplete data regarding positive family history for premature cardiovascular disease (CVD) can be considered. Although we did update family history for premature CVD in the cIMT study (Chapter 5), we did not do this for the tracking study (Chapter 6). As a result this possible determinant was excluded in the tracking study.

An example of time related biases includes the change and progression of the disease diabetes and its treatment. Factors such as insulin administration scheme, family history for premature CVD and smoking need regular reassessment, without deleting previous data. Except for the updated family history on premature CVD in the cIMT study, these factors were, partly for this reason, not included in the other studies of the EDDDY-S study.

Generalizing results to the 'general type 1 diabetes population'.
A potential problem with generalisation of the results is the difference in composition between European and American study groups. It is known that they differ in factors such as access to healthcare, lifestyle patterns and genetic background. Additionally, our well-regulated (in terms of HbA1c) study-population may potentially be not comparable with less optimally-regulated populations. Finally, as suggested in this thesis and by others, the heterogeneity in the disease diabetes itself seriously complicates generalisation of our study results.

8.4 Conclusion and further steps

Evaluating the results of the EDDDY-S study, taking into account scientific insights in the period 2007-2018, we propose setting up a screening protocol for detection of risk factors and surrogate markers for (early) complications in children and adolescents with type 1 diabetes. As evidence of definite better, alternative tests or screening intervals is still scarce, it is suggested to continue following the current ISPAD screening protocol (13) for risk factors for micro- and macrovascular complications and (early) signs of those complications for children and adolescents with type 1 diabetes.

However, the proposed screening protocol should additionally be based on scientific insights from 2007 to 2018 in the discussed complication fields and findings from the EDDDY-S study. It should include routine diagnostic tests but also tests which are still in the research setting (Figure 3). All tests should be selected on solid test characteristics and some practical considerations, e.g. they should be easily applicable in the outpatient clinic setting, patient-friendly and non-invasive. Other new tests were reported between 2007 and 2018. However, since these are outside of the scope of this thesis we will discuss these in Appendix 2.

Therefore the proposed screening protocol only includes the complication fields examined in the EDDDY-S study.

Applying the proposed screening protocol would also enable addressing a number of outstanding research questions, for example on spatial and temporal relationships of the occurrence and progression of complications. Whereas there is scarce information about the longitudinal course of subclinical DPN that can be detected in a proportion of patients 2-3 weeks after diagnosis of type 1 diabetes (139), there is even less information about the course of other measures which are also found to be abnormal after diagnosis, such as SAF (82). For this reason, we propose to initiate assessing several tests 3 months after the diagnosis of type 1 diabetes. Thereafter, testing should be performed simultaneously at a 2- year interval (except for SAF). After a period of 6 years (i.e. three 2-year intervals), we suggest to evaluate the findings of these simultaneous measurements, expecting to be able to draw conclusions about the validity of the tests that were performed in the research context and to limit the number of currently proposed tests to a subset(s) of tests able to identify patients at risk of developing one or more complications, regardless of their HbA1c. Results of longer longitudinal follow up of the patients and future information about the course of complication development in a lifetime perspective of the patients should emphasize the need for continuous evaluation of the screening protocol.

In conclusion, in 2007, the EDDDY-S study was designed with the aim to improve risk stratification in children and adolescents with type 1 diabetes. Tests for the detection of risk factors, surrogate markers and early signs of complications were studied for the underexposed complication fields in pediatrics: subclinical DPN, macrovascular complications and a surrogate marker for micro- and macrovascular complications (SAF). In 2018, after the EDDDY-S study and a review of the current literature, we have to conclude that neither we, nor others have yet succeeded in developing screening tests that could become internationally adapted appropriate screening tests in the nearby future.

However, based on the EDDDY-S study and the review of current literature, we propose that the following set of promising tests for these fields of complications are worthwhile studying in the near future and should be added to the current tests in the ISPAD consensus guideline (13)(Figure 3B):

- corneal nerve fiber density (CNFD) and corneal nerve fiber branch density (CNFBD) by corneal confocal microscopy (parameter category C)
- vibration perception threshold (VPT) by Biothesiometer/ Vibratory Sensory Analyser (parameter category B)
- stimulus intensities of the peroneal nerve by CMAP scan (parameter category C)
- carotid intima media thickness (cIMT) by ultrasound (parameter category B)
- pulse pressure by Dinamab (parameter category C)
- pulse-wave velocity by the SphygmoCor-Vx device (parameter category C)
- frequent lipid measurement; low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), non-HDL cholesterol (non HDL-C), triglycerides (TG) (parameter category B) and apolipoprotein B (apo B) (parameter category C)
- skin autofluorescence (SAF) by AGE-reader (parameter category C)

By proposing this diagnostic and research screening protocol, we think we took a step towards the ultimate aim of this thesis: appropriate early risk stratification in children and adolescents with type 1 diabetes. This may in time result in tailored screening and treatment, in turn, leading to an improved care process, increased health and quality of life and reduced long term health care costs.

Figure 3A: ISPAD Clinical Practice Consensus Guidelines 2014. Microvascular and macrovascular complications in children and adolescents. Pediatr Diabetes. 2014;15 Suppl 20:257-69.

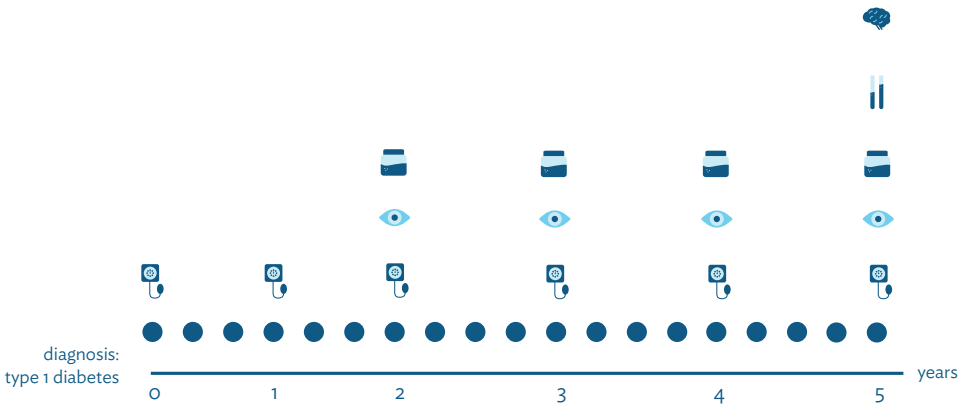


Figure 3B: Proposed screening protocol including tests which are currently still in the research setting

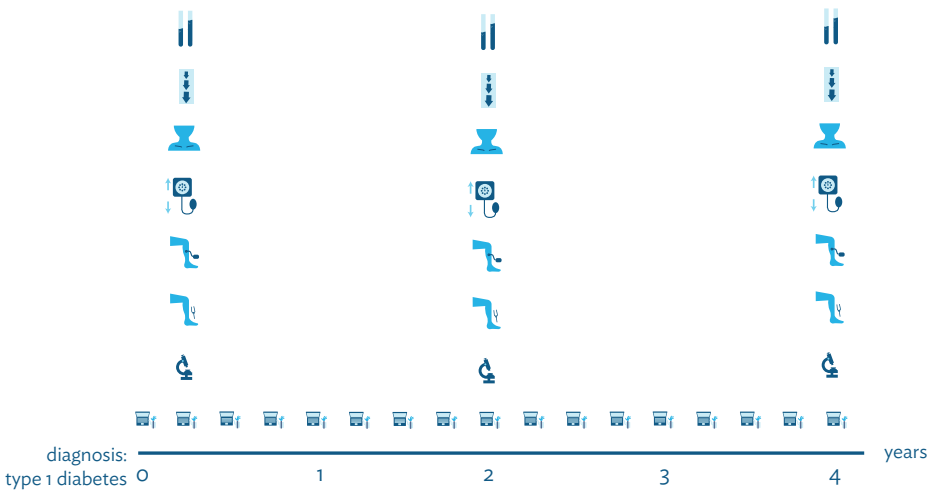


Figure 3A:


Neurological examination: history taking and physical examination.
When to commence screening is unclear.


Lipid profile: LDL-C, HDL-C, triglycerides after the age of 10 years.


AER/ACR or first morning albumin concentration from the age of 10 years (or earlier in case of onset of puberty before the age of 10) onwards with 2 to 5 years of diabetes duration


Fundal photography or mydriatic ophthalmoscopy from the age of 10 years (or earlier in case of onset of puberty before the age of 10) onwards with 2 to 5 years of diabetes duration


Blood pressure annually


Three monthly: HbA1c, BMI

Figure 3B:


Lipid profile PLUS apolipoprotein B and non-HDL-C

Pulse wave velocity


Carotid intima media thickness


Pulse pressure


Stimulus intensities by CMAP scan


Vibration perception threshold by Biothesiometer/Vibratory Sensory Analyser


Corneal confocal microscopy


Skin autofluorescence (SAF) measurement by AGE-reader

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Chapter 9

Summary / Nederlandse samenvatting

Summary

Chapter 1

In Chapter 1, we provide a short overview of type 1 diabetes and its complications. A considerable number of patients with type 1 diabetes still develops micro- and macrovascular complications despite having good glycaemic control (high HbA1c is the main risk factor for complications). We stress that early risk stratification, which was the aim of the Early Detection of Diabetes Damage in Youth and Search for early prevention (EDDDY-S) study presented in this thesis, will be an important step towards improvement of this burdensome course of the disease. A selection of diabetes-related complication fields (microvascular complication diabetic peripheral neuropathy, macrovascular complications and measurement of the surrogate marker advanced glycation end products [AGEs]) was made with the aim to cover a number of scientific gaps existing at the time the EDDDY-S study was conducted including the most relevant fields of complications. After a review of what was known about these fields, chapter 1 concludes with the rationale for the EDDDY-S study and the research questions. Appendix 1 provides a more extensive review about these fields and about the other complications in type 1 diabetes (that were not studied in this thesis).

Chapter 2

In Chapter 2, the usability of the compound muscle action potential (CMAP) scan was assessed in different groups of children and adolescents with type 1 diabetes. The CMAP scan provides a visual and quantitative impression of the build-up of a muscle in terms of motor unit size and motor unit number. A pilot study was conducted in children and adolescents with type 1 diabetes ('well-controlled' versus 'poorly controlled'), adult patients with type 1 diabetes and clinical neuropathy, and age matched healthy controls. Significantly increased stimulus intensities (SIs), reflecting reduced axonal excitability, were found in both young

patient groups compared with their controls. We postulated the reduced axonal excitability to be an (early) marker of subclinical diabetic peripheral neuropathy (DPN). Alternatively, it could be a stand-alone finding, not necessarily indicating that these patients will develop DPN earlier, but being an indicator of heterogeneity in patients with type 1 diabetes. Despite the lack of reference tests for subclinical DPN and the limited number of patients, we do think that the CMAP scan is a promising test.

Chapter 3

Chapter 4

In Chapter 4, we summarized our findings of Chapters 2 and 3 in response to a paper of Professor Malik in *Diabetes*, stating that screening for DPN by history taking, physical examination and electrophysiologic tests only assesses large myelinated nerve fiber function. This function is assumed to deteriorate late in the disease course of DPN. As smaller nerve fibers are thought to deteriorate earlier in the disease course of DPN, he advocates using screening tests that focus on the smaller nerve fibers, such as corneal confocal microscopy (CCM) or intra-epidermal nerve fiber density in skin biopsies. Professor Malik's suggestions were supported by two of our pilot studies (Chapter 2 and 3). The validity of assessing subclinical DPN was found to be low when measuring large myelinated sensory and motor nerve function using NCV and SNAP of the superficial peroneal and sural nerve and maximum CMAP amplitude of the peroneal nerve. Professor Malik stressed the need for more (and other) tests for detecting subclinical DPN. We proposed the observed decreased axonal excitability by means of CMAP scan as promising technique for future study.

Chapter 5

In Chapter 5, intima media thickness of the common carotid artery (cIMT) was studied in children and adolescents with type 1 diabetes and healthy controls. Previous studies suggested that cIMT is relevant as surrogate marker for macrovascular disease in type 1 diabetes in adulthood, but the use in childhood and adolescence is still being debated. In addition, we studied the impact of the risk factors on cIMT by regression analyses: gender, socio-economic status, ethnicity, and current and historical body mass index, blood pressure, HbA1c, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). We found that mean cIMT was increased in children and adoles-

cents with type 1 diabetes, compared with healthy controls, as was the median age-specific cIMT. Notably, median cIMT was equally greater across all age categories. The contribution of traditional macrovascular risk factors for increased cIMT was limited. Both findings suggest that considerable heterogeneity exists between patients. If so, there may be a subset of children and adolescents with type 1 diabetes that is prone to develop macrovascular complications, possibly already at a very young age. More research on variables allowing identification of these high risk patients is needed.

Chapter 6

Chapter 7

In Chapter 7, skin autofluorescence (SAF), a surrogate marker for both microvascular and macrovascular complications in adults with type 1 diabetes, was evaluated in children and adolescents with type 1 diabetes. SAF reflects advanced glycation end products (AGEs) in the long-lived protein collagen. AGEs are assumed to accumulate in both the collagen of the skin and in components like fibrinogen and myelin. In this cross-sectional study, SAF in children and adolescents with type 1 diabetes was compared with healthy controls across age categories. Associations with current and historical HbA1c, age, and duration of diabetes were evaluated in the patient group. In accordance with previous studies, SAF was elevated in patients with type 1 diabetes. Notably, in patients with type 1 diabetes, SAF was equally elevated across all age categories, but only weakly associated with duration of diabetes and age, and not correlated with the current and historical HbA1c values. Surprisingly, it was even found to be elevated in a subgroup of patients with an HbA1c ≤ 7.5% (58 mmol/mol), considered to be a 'HbA1c-within-target'. Although these findings were unexpected, the presence of a subgroup with elevated SAF despite having appropriate glycaemic control, suggests that measurement of SAF may have added value in risk-assessments for future diabetes related complications. The results also suggests that glycaemic control may not be the main or exclusive contributor in the development of complications in all patients. Consequently, determinants other than age, duration of diabetes and HbA1c, and/or heterogeneity within the patient group are likely to be involved.

Chapter 8

Chapter 8 briefly looks back at the general introduction and research questions, followed by a summary of the EDDDY-S study results. Then the two main general findings of the EDDDY-S study are presented: 1) in at least a subgroup of children and adolescents with type 1 diabetes in the EDDDY-S study glycaemic control did not or only moderately contribute to development of abnormal values of surrogate markers and/or early signs of micro- and macrovascular complications; 2) the EDDDY-S study showed already abnormality in surrogate markers and early signs of complications in a considerable number of the children and

adolescents with type 1 diabetes. Next, results are put in context of complications-related literature reported from 2007 to 2018 (while the EDDDY-S study was executed). For more extensive information about these fields and new scientific insights in the other complications of type 1 diabetes the reader is referred to Appendix 2. Finally, chapter 8 elaborates on EDDDY-S study related methodological considerations, integration of the EDDDY-S study results with the new scientific insights of 2007-2018 and future study implications.

Nederlandse samenvatting

Hoofdstuk 1

In hoofdstuk 1 wordt een kort overzicht gegeven over het ziektebeeld type 1 diabetes en de complicaties die hierbij kunnen optreden. Het beschrijft dat een aanzienlijk gedeelte van de patiënten micro- en macrovasculaire complicaties ontwikkelt en dat dit gebeurt ondanks een goede regulatie van de belangrijkste risicofactor; slechte glucosecontrole (ofwel een hoog HbA1c). In hoofdstuk 1 benadrukken we dat vroege risicostratificatie uiteindelijk moet leiden tot een verbetering van deze sombere prognose. Het onderzoek dat wordt gepresenteerd in dit proefschrift (EDDDY-S [Early Detection of Diabetes Damage in Youth and Search for early prevention] studie) heeft als doel deze risicostratificatie te verbeteren. Met focus op de onderbelichte onderdelen binnen het veld van complicaties beslaat de EDDDY-S studie maar een deel van het gehele veld van complicaties. Hoofdstuk 1 geeft een overzicht van wat er bekend was in 2007 over de onderzochte complicatievelden; de microvasculaire complicatie diabetesgerelateerde perifere neuropathie (DPN), macrovasculaire complicaties en meting van de surrogaatmarker 'advanced glycation end products' (AGEs). Hoofdstuk 1 eindigt met de rationale van de EDDDY-S studie en de onderzoeksvragen. Appendix 1 bevat een uitgebreider overzicht van de kennis in 2007 omtrent de complicatievelden die reeds besproken werden in hoofdstuk 1 alsmede een overzicht van kennis in 2007 omtrent de andere complicaties in type 1 diabetes.

Hoofdstuk 2

In hoofdstuk 2 wordt het gebruik van de 'compound muscle action potential' (CMAP) scan in verschillende groepen kinderen en adolescenten met type 1

diabetes onderzocht. De CMAP scan geeft visueel en quantitatief de opbouw van een spier weer, waarbij gekeken wordt naar motor unit grootte en aantal. Er werd een pilot studie opgezet met daarin kinderen en adolescenten met type 1 diabetes (goed gereguleerde patiënten vs slecht gereguleerde patiënten) en volwassen patiënten met type 1 diabetes en klinisch neuropathie. Voor alle patiëntengroepen waren er leeftijd-gematchte gezonde controles. Er werd een significant verschil gemeten in de CMAP parameter stimulusintensiteit waarmee een verminderde axonale prikkelbaarheid werd aangetoond in de jonge patiënten met type 1 diabetes in vergelijking tot de gezonde controlegroep. We hypothetiseren dat deze verminderde axonale prikkelbaarheid een (vroeg) teken is van subklinische DPN. Een alternatief voor deze hypothese is dat het een op zichzelf staande bevinding is, waarbij de bevinding er niet op wijst dat deze patiënten per definitie DPN ontwikkelen en dat het wellicht meer een bevinding is die duidt op heterogeniteit in de groep patiënten met type 1 diabetes. We denken dat de CMAP scan een veelbelovende test is ondanks de afwezigheid van een referentietest en een beperkt aantal patiënten in deze studie.

Hoofdstuk 3

Hoofdstuk 4

Hoofdstuk 4 bevat een reactie (letter to the editor) op een manuscript van Professor Malik in het tijdschrift *Diabetes* waarin de conclusies van hoofdstuk 2 en 3 kort zijn samengevat. Malik stelt dat het navragen van DPN-gerelateerde klachten, het doen van lichamelijk onderzoek en het verrichten van electrofysiologische studies (zoals de zenuwgeleidingssnelheid en de SNAP amplitude) schade detecteren aan de grote, gemyeliniseerde zenuwvezels waar de gedachte is dat kleine, niet- gemyeliniseerde zenuwvezels eerder beschadigd raken in het traject van DPN. Hij pleit daarom voor testen die focussen op het detecteren van schade aan deze kleine, niet-gemyeliniseerde zenuwvezels en noemt als mogelijke testen de 'corneal confocal microscopy' en de intra-epidermale zenuwvezeldichtheid gemeten in huidbiopten. We hebben gereageerd op zijn publicatie omdat de bevindingen van onze twee pilotstudies, beschreven in hoofdstuk 2 en 3, zijn idee over de volgorde van schade ondersteunen. Immers, de validiteit van de testen voor subklinische DPN, die de functie meten van de grote gemyeliniseerde zenuwvezels met behulp van de zenuwgeleidingssnelheid, SNAP

amplitude (sensorische zenuwen nervus peroneus superficialis en de nervus suralis) en de maximale CMAP amplitude (motore zenuw nervus peroneus) was laag. Professor Malik benadrukt de noodzaak tot meer (en andere) testen dan de bestaande testen om subklinische DPN beter te kunnen detecteren. Wij onderschrijven dit en hebben de geobserveerde verminderde axonale prikkelbaarheid, gemeten door middel van de CMAP scan, genoemd als veelbelovende test die nadere studie behoeft.

Hoofdstuk 5

In hoofdstuk 5 wordt de intima-media dikte van de gemeenschappelijke arterie carotis (cIMT) gemeten in kinderen en adolescenten met type 1 diabetes. Eerdere studies hadden in volwassenen met type 1 diabetes aangetoond dat de cIMT als surrogaat marker van macrovasculaire schade kan dienen. Of je de cIMT op die manier ook zou kunnen gebruiken in kinderen en adolescenten met type 1 diabetes werd in 2007 nog bediscussieërd. We hebben de cIMT in deze patiëntengroep gemeten en vergeleken met gezonde controles. Daarnaast hebben we regressieanalyses verricht om de impact van de volgende factoren op cIMT te beoordelen in de patiënten: geslacht, socio-economische status (SES), ethniciteit, BMI, HbA1c, bloeddruk, HDL-C (high-density lipoprotein cholesterol) en LDL-C (low-density lipoprotein cholesterol) ten tijde van de cIMT meting en BMI, HbA1c, bloeddruk, HDL-C en LDL-C uit het verleden. We vonden dat de cIMT verhoogd is in kinderen en adolescenten met type 1 diabetes vergeleken met gezonde controles. Dit is ook het geval als je de cIMT per leeftijdscategorie bekijkt. Hierbij valt tevens op dat a) het verschil in cIMT tussen patiënten en controles steeds ongeveer even veel is en b) de impact van de onderzochte factoren op de (verhoging) van de cIMT maar matig is. We vermoeden dat deze bevindingen een gevolg zijn van heterogeniteit in de patiëntengroep. Als dat inderdaad zo is, zou dit ook kunnen betekenen dat een subgroep van patiënten gedoemd is om macrovasculaire complicaties te krijgen en dat dit zelfs al kan plaatsvinden op een relatief jonge leeftijd. Meer onderzoek om deze subset van hoog risico patiënten te detecteren is nodig.

Hoofdstuk 6

Hoofdstuk 7

Hoofdstuk 7 evalueert de autofluorescentie van de huid (SAF) in kinderen en adolescenten met type 1 diabetes. SAF is een surrogaatmarker voor zowel micro- als macrovasculaire complicaties in volwassenen met type 1 diabetes. Het geeft de hoeveelheid advanced glycation end products (AGEs) weer van collageen, een eiwit met een lange halfwaarde tijd. AGEs stapelen zich op in het collageen van

de huid en in componenten zoals fibrinogeen en myeline. SAF werd gemeten bij kinderen en adolescenten met type 1 diabetes en vergeleken met SAF gemeten in gezonde controles in verschillende leeftijdscategorieën in deze cross-sectionele studie. In de patiëntengroep werd er tevens gekeken naar associaties tussen SAF en HbA1c ten tijde van de SAF meting en HbA1c uit het verleden en associaties tussen SAF en de determinanten leeftijd en duur van de ziekte diabetes.
Net als eerdere studies vonden wij dat SAF was verhoogd in patiënten met type 1 diabetes vergeleken met de gezonde controles. Opmerkelijk was de bevinding dat SAF gelijkmatig was verhoogd in patiënten van verschillende leeftijdscategorieën. De associatie met de duur van de ziekte diabetes en de leeftijd was matig en er werd geen associatie gevonden met SAF en actueel HbA1c of HbA1c waardes uit het verleden. Tenslotte viel op dat er een klein groepje patiënten was dat een verhoogde SAF had ondanks een goed HbAc (HbA1c ≤ 7.5% [58 mmol/mol]). Deze bevinding alsmede de bevinding dat er geen associatie werd gevonden tussen SAF en Hb1Ac uit het verleden hadden we niet verwacht. Desalniettemin leidt de aanwezigheid van een subgroep patiënten met een verhoogd SAF ondanks goed HbA1c tot de conclusie dat SAF meting mogelijk toegevoegde waarde heeft in de risico-inschatting voor toekomstige diabetesgerelateerde complicaties. Deze studieresultaten suggereren daarnaast dat niet slechts of vooral glucosecontrole bijdraagt aan de ontwikkeling van complicaties in alle patiënten. Andere factoren dan leeftijd, duur van de ziekte diabetes en HbA1c en/of heterogeniteit van patiënten spelen hierbij mogelijk een rol.

Hoofdstuk 8

Hoofdstuk 8 kijkt terug op de introductie van het proefschrift en de onderzoeksvragen, gevolgd door een samenvatting van de resultaten in de EDDDY-S studie. Hierna worden de twee overkoepelende bevindingen van de EDDDY-S studie gepresenteerd: 1) in tenminste een subgroep van de kinderen en adolescenten met type 1 diabetes uit de EDDDY-S studie droeg glucosecontrole niet of maar matig bij aan de ontwikkeling van abnormale waardes van de surrogaatmarkers en/of vroege tekenen van micro- en macrovasculaire complicaties; 2) in een vrij groot aantal van de kinderen en adolescenten met type 1 diabetes in de EDDDY-S studie waren afwijkende surrogaatmarkers of vroege tekenen van complicaties al aantoonbaar. Hoofdstuk 8 wordt vervolgd met een integratie van

de resultaten van de EDDDY-S studie en complicatiegerelateerde literatuur uit 2007-2018 (periode waarin de EDDDY-S studie werd uitgevoerd). Voor meer uitgebreide informatie omtrent deze onderwerpen en nieuwe wetenschappelijke inzichten in de andere complicatievelden van type 1 diabetes wordt verwezen naar appendix 2. Tenslotte bespreekt hoofdstuk 8 methodologische kwesties die de EDDDY-S studie aangaan. Hoofdstuk 8 eindigt met implicaties van de bevindingen voor toekomstige studies.

Nederlandse samenvatting

Appendix 1

Here we summarize the available data in 2007 on A) risk factors other than glycaemic control for development and/or progression of micro- and macrovascular complications, B) relevant and promising tests for detection of early signs of these micro- and macrovascular complications.

Microvascular complications

Nephropathy

Approximately 30% of the patients with type 1 diabetes develops diabetic nephropathy and about 10% develops end stage renal disease after 30 years of diagnosis (1-4). In 2007, screening for nephropathy was performed by the annual measurement of urinary albumin concentration or urinary albumin-to-creatinine-ratio in spot urine from the age of 11 years onwards with 2 years of diabetes duration and from the age of 9 years with 5 years of diabetes duration (5, 6). In adults, soluble intercellular adhesion molecule-1 (s-ICAM), an inflammatory related adhesion molecule, and increased levels of soluble CD40 (sCD40) ligand were shown to precede the development and worsening of microalbuminuria (7, 8).

Neuropathy

Clinical neuropathy (defined as clinical diabetic polyneuropathy (DPN) and/or autonomic neuropathy) develops in about 20% of the adult patients with type 1 diabetes (3, 4, 9, 10), whereas subclinical neuropathy (defined as abnormal nerve conduction velocity and/or autonomic neuropathy) was found in about 30 % (9). In adolescents with type 1 diabetes, clinical and subclinical neuropathy is already prevalent in a considerable part of the patients (11, 12). Its prevalence has been shown to increase whereas the prevalence of retinopathy and microalbuminuria declined over the years (11). In addition to DPN, autonomic neuropathy is

assumed to develop early in the disease process as well (13).

Table 1: Types of diabetic neuropathy (14-16)

Sensorimotor diabetic polyneuropathy
Dysfunction of the peripheral sensorimotor nerves
Focal neuropathy
Mononeuropathy, cranial neuropathy
Autonomic diabetic neuropathy
Cardiovascular, gastro-intestinal, bladder, erectile, and sudomotor dysfunction

Diabetic neuropathy is regarded to be a microvascular complication, related to damage of endothelial cells in vessels supplying peripheral nerves and ganglia. The pathophysiology is thought to be multifactorial (16, 17). Factors such as advanced glycation end products (AGEs), oxidative stress, glucotoxicity, certain genetic polymorphisms, vascular cell adhesion molecules, neurotrophine growth factors and inflammatory changes are reasoned to be involved in damaging not only the endothelial cells, but also other structures such as Schwann cells and neurofilaments (17, 18). As implicated by the different forms of neuropathy, large- and small-diameter, myelinated and unmyelinated, motor, sensory and autonomic nerve fibers can be affected (16, 18). The order of deterioration of these various nerve fibers is still an ongoing debate. In general it is assumed that sensory nerve fibers deteriorate earlier in the disease course of DPN than motor nerve fibers do (17, 19). Moreover, some postulate small myelinated and unmyelinated (sensory) nerve fibers to precede deterioration of the myelinated (sensory and motor) nerve fibers (12, 20, 21).

The screening guideline for diabetic neuropathy includes history taking, mainly covering complaints related to DPN (6). The more objective tests for (sub)clinical DPN in this guideline consist of examination of autonomic nerve function, testing the vibration perception threshold (VPT) (large myelinated nerve fibers test), testing the thermal perception thresholds (small myelinated and unmyelinated

nerve fiber test) and performance of NCV (large myelinated nerve fiber test). The guideline includes testing of large sensory and motor myelinated nerve fibers but underexposes the possible order of small nerve fiber deterioration preceding the large ones.
By 2007, more tests became available for earlier detection of (subclinical) DPN with more attention for small nerve fibers and were studied in addition to tests focussing on early detection of large nerve fiber damage.
These tests included examining the density of the smaller unmyelinated and myelinated nerve fibers by fascicular sural nerve biopsy (21, 22), examining the intra-epidermal nerve fiber density in skin biopsy (23, 24), looking at corneal nerve fiber density by corneal confocal microscopy (24), measuring the amplitude of motor peripheral nerves (20, 25, 26) and examining electrophysiological features of the dorsal sural nerve (27). In addition to various tests assessing small myelinated and unmyelinated nerve fibers and large myelinated nerve fibers, attention was also paid to the detection of early signs of autonomic neuropathy. Testing of sudomotor function using the Neuropad (23, 28) and the horizontal pupillary diameter (29) were described to be feasible as novel tests.

Retinopathy

Retinopathy and laser-treated retinopathy develops in approximately 10-50% and 20% respectively of the patients with type 1 diabetes (3, 4, 30, 31). In 2007, annual screening for diabetic retinopathy was advised to be performed by 7-field stereoscopic fundus photography, fluorescein angiography, indirect ophthalmoscopy or monochromatic single-field photography from the age of 11 years onwards in case of ≥ 2 years diabetes duration and from 9 years onwards in case of ≥ 5 years duration (6, 32). Tumor necrosis factor alfa (TNFalfa) serum levels (33, 34) and retinal arteriolar, venular diameter and the arteriolar/venular ratio by retinal photography (assessed with a computer-assisted programme) were suggested to predict development and progression of retinopathy (31, 35).

Macrovascular complications

Mortality due to the macrovascular complications ischaemic heart disease and cerebrovascular disease in childhood-onset type 1 diabetes is substantially increased in type 1 diabetes compared with the general population (36-38). The ISPAD guideline (6) recommends screening every year for the risk factor hypertension and every 5 years for the risk factor dyslipidemia from the age of 12 years onwards. It also presents target levels regarding body mass index (BMI), smoking behaviour and other known risk factors for macrovascular disease such as HbA1c. These recommendations lack assessment of any surrogate marker for macrovascular disease.

By 2007, measurement of carotid intima media thickness, endothelial dysfunction and arterial stiffness, were already suggested to be surrogate markers for macrovascular disease.

Atherosclerosis leads to premature cardiovascular disease (39) and has been shown to develop, among others, in the carotid intima (40). Therefore, measurement of the carotid intima media thickness (cIMT) was considered to be an appropriate surrogate marker of subclinical atherosclerosis. Indeed, increased cIMT was shown to associate with coronary heart disease in adults without type 1 diabetes (39, 41). In the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study (42) it was shown that risk factors associated with extended atherosclerosis (notably hyperglycaemia) coincide with risk factors for increased cIMT (43, 44) and are able to predict future cIMT increases (45). Moreover, increased cIMT has been found in adult patients with type 1 diabetes (46). In this research setting it was also found that atherosclerosis was already present in patients aged 15-34 years who died of external causes. Most, but not all, studies found an increased cIMT in the children and adolescents with type 1 diabetes as compared with healthy controls (47-51).

Endothelial dysfunction is associated with diminished nitric oxide levels and altered expression of cellular adhesion molecules which in turn result in enhanced leucocyte adhesion to the endothelium surface, decreased vasodilatation and increased thrombotic state. As a consequence of this disrupted vascular homeostasis, cardiovascular disease occurs (52-54). Endothelium dysfunction is reflect-

ed by: an impaired vasodilatory response of the coronary arteries to acetylcholine (examined by quantitative angiography); impaired flow-mediated dilatation (FMD) of the brachial artery using high-resolution ultrasound; and altered intra-arterial forearm blood flow responses to substances such as methacholine by means of using strain gauge plethysmography (52-56). These parameters have been found to be associated with type 1 diabetes, cardiovascular risk factors and cardiovascular disease itself (53-58).

Arterial stiffness is a consequence of changes of the predominant components of the intracellular and extracellular matrix of the vascular wall: collagen and elastin. These changes are due to factors such as elevated luminal pressure, inflammation, altered metalloproteinases and the amount of advanced glycation end products, and result in impaired cross-links and an altered proportion of functioning collagen and elastin in the vascular wall (59, 60). Increased vascular stiffness has been shown to associate with cardiovascular disease (61-63), the cardiovascular risk factor hypertension (64) and type 1 diabetes (61, 64, 65). It can be measured by various methods, e.g. calculating the augmentation index from radial tonometry values (66), calculating pulse pressure from systolic and diastolic blood pressure measured by a random sphygmomanometer (61), and determine pulse-wave velocity by an automatic device (64).

In addition to these surrogate markers for macrovascular complications, attention has increased for improving macrovascular risk stratification by looking into the atherogenicity of the lipid profile and inflammatory markers. The lipid profile got extended with the determination of apolipoprotein B (apoB) levels (67, 68), apoB/apolipoprotein A-I (apo A-I) levels, and low-density lipoprotein cholesterol (LDL-C) density markers (67) and inflammatory markers such as C-Reactive protein (CRP) (69, 70). Finally, two lipid studies in children and adolescents with type 1 diabetes in respectively 2007 and 2008 showed the lipid level to change from low-risk into borderline-high-risk or high-risk lipids levels or from borderline-high-risk into high-risk lipid levels ('lose track of lipids') in a considerable number of patients within a shorter interval of time than the suggested 5 year interval of the guideline (6, 71, 72). This warrants earlier detection of and consequently intervention for the risk factor dyslipidemia.

Additional risk factors and surrogate markers

Advanced glycation end products (AGEs) as surrogate marker for complications

AGEs are products of reactive oxygen species and non-enzymatic reactions between sugars and amino groups of proteins ('Maillard reaction') (73, 74). Its production is enhanced in patients with higher glucose levels and other saccharide derivatives (75-77). Local accumulation of AGEs causes structural alteration of long-lived proteins such as collagen, fibrinogen and myelin through the formation of intermolecular and intramolecular cross-links (75-77). AGEs also affect the formation of free radicals and nitric oxide and trigger receptor dependent (e.g. RAGE) and receptor independent pro-inflammatory pathways (75-77). These processes result in, among others, increased stiffness of blood vessels, decreased arterial and myocardial compliance, glomerular basement membrane thickening, basement membrane thickening of the retinal blood vessels, reduction of LDL-C uptake and decreased blood flow to nerves due to micro-angiopathy (75-77). Associations between AGEs and micro- and macrovascular complications in type 1 and type 2 diabetes were described in adults (76-79). Quantification of AGEs has been studied by: measurement of serum levels of certain AGEs (80, 81); measurement of collagen glycation by determining the plantar fascia thickness in children and adolescents (82); and skin autofluorescence (SAF) in adults only (79, 83).

Inflammatory markers as surrogate marker for complications

Several studies showed that the serum levels of inflammatory markers such as soluble tumor necrosis alfa receptor (sTNF-R), tumor necrosis alfa factor (TNF alfa), C-Reactive Protein (CRP) and interleukine 6 (IL-6) associate with the concomitant presence of (early signs) of complications (34, 69, 70, 84, 85). In type 1 diabetes related nephropathy, sICAM-1 and soluble CD40 levels have been suggested to be useful as prediction markers of vascular endothelial damage and thus complications (7, 8, 86).

Insulin resistance as additional factor for the development of complications

Yip et al showed by euglycaemic hyperinsulinaemic clamp method that adult patients with type 1 diabetes and microalbuminuria had a decreased glucose disposal rate (measure of insulin sensitivity) when compared with patients with normoalbuminuria (87). Orchard et al confirmed this finding by their study in

which they found insulin resistance, measured by an estimated glucose disposal rate (eGDR), to associate with coronary artery disease independent of diabetes duration and age in a subgroup of childhood-onset type 1 diabetes patients of the Pittsburgh Epidemiology of Diabetes Complication Study (88).

Genetic susceptibility as additional risk factor for the development of complications

Only a few studies highlighted the importance of polymorphisms in genes involved in the process of arterial stiffness and nephropathy in type 1 diabetes (8, 88, 89).

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Appendix 1

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Appendix 2

New scientific insights in the period 2007-2018 while the EDDDY-S study was performed.

Microvascular complications

Nephropathy

Both the approaches by the research group of Donaghue (1, 2) and by the Oxford Regional Prospective Study of Childhood Diabetes (3) are worthwhile to consider for the identification of the adolescent patients with type 1 diabetes at risk for developing nephropathy. The former approach used a cut-off limit of the albumin excretion rate (AER) lower than the 20 microgr/min recommended in the guideline (1, 2, 4, 5), because a previous study of Chase showed this lower cut-off limit to associate with future abnormal AER (5). In the latter approach ACRs (Albumin to Creatinin Ratio) in the highest tertile of the normal range were found to predict the development of microalbuminuria (3, 6). Alternative ways for the detection of early signs of nephropathy, were regarded to be tests such as retinal vascular geometry and measurement of Cystatine C. Associations between several abnormal retinal vascular geometry measures and baseline abnormal ACR, short-term future abnormal ACR and long-term micro- and macroalbuminuria were shown (7-10). In a 10 year follow-up study of adults with type 1 diabetes, Premaratne et al. showed serum Cystatine C to be more sensitive in predicting a declining renal function than creatinine-based measures (11). Few studies in adolescents with type 1 diabetes were performed. These studies showed the same might be true for this age-group, but the follow up duration in the studies was only 1.5-2 years, addressing the need for long-term data in pediatric patients with type 1 diabetes (12, 13).

Neuropathy

In the period 2007-2018, the literature increasingly focused on small nerve fiber dysfunction preceding large nerve fiber dysfunction in the development of DPN. Additionally, more sensitive tests in both detecting large nerve fiber dysfunction and autonomic nerve fiber dysfunction were evaluated.

Promising tests being indicative for small nerve fiber function were developed. In adult patients with type 1 diabetes, corneal nerve fiber density (CNFD) and corneal nerve fiber branch density (CNFBD) measured by means of corneal confocal microscopy (CCM) were shown to correlate with intra-epidermal nerve fiber (branch) density in skin biopsies (14). Additionally, the significance of associations between several corneal nerve measurements and severity of clinical DPN (14-16), small nerve fiber function tests (14, 17) and the development of future DPN (18) was investigated and confirmed. Contradicting results were found regarding the benefits of corneal sensation threshold measurements by noncontact corneal esthesiometry (NCCE) (15, 18).

Potentially more sensitive screening tests detecting large nerve fiber dysfunction were also evaluated (19). Regarding the determination of the vibration perception threshold (VPT), the Biothesiometer or Vibratory Sensory Analyser appeared to have a better diagnostic utility than the tuning fork or the Vibratron II (1, 2, 19)(20) in detecting subclinical DPN in children and adolescents with type 1 diabetes. Besides, testing the tactile function with smaller monofilaments (1 mN) than the thicker ones used in adult screening was evaluated and appeared promising (as also suggested by Vinik (21)). However, sensitivity appeared low in a study in 88 children with type 1 diabetes (22). A follow-up study of Hyllienmark showed that the 9 patients (15% of the total group) having clinical DPN at young adult age had an impaired peroneal and median motor nerve conduction velocity and/or sural sensory nerve action potential at baseline (mean age at baseline 15.5 years [3.22 SD])(23). Monlun reported hyperglycaemie to affect nerve conduction velocity but not sensory nerve action potential when present during neurophysiologic testing (24).

Recent research regarding autonomic neuropathy paid more attention to early warning signs of cardiac autonomic neuropathy (CAN) and sudomotor function. CAN appeared to be detectable in the first years after diabetes onset by means of validated cardiovascular reflex tests supported by newer procedures (25). Such

warning signs include reduced heart rate variability during deep breath, prolongation of QT interval (determined by ECG), decreased baroreflex sensitivity with consequent abnormal blood pressure regulation, and orthostatic hypotension (26). The applicability of testing the sudomotor function by the Neuropad test and Electrochemical skin conductance (e.g. sudoscan) was examined in several studies. Abnormal tests were associated with impaired small nerve fiber function tests and neuropathic severity (27-30), although the results were contradicting (27, 28, 31, 32).

Despite the potential of these novel tests, recent studies in both adults and in children and adolescents, do not clarify if small nerve fiber testing alone is sufficient for detecting subclinical DPN (which would be in line with the hypothesis that small nerve fiber dysfunction precedes large nerve fiber dysfunction (21, 33, 34)). In adults with type 1 diabetes, Breiner showed ≥ 1 of the 4 small nerve fiber tests to be abnormal in 97% of the patients with clinical large nerve fiber dysfunction (defined by abnormal electrophysiologic studies and/or clinical DPN)(34). However, this study lacks longitudinal data on future large nerve fiber impairment in the patients with ≥ 1 abnormal small nerve fiber test and no clinical signs of large nerve fiber function at baseline. Pritchard performed a (short) longitudinal study and found both baseline small and large nerve fiber dysfunction to be associated with clinical DPN after a mean follow up period of 47 months (+/- 3 standard deviation) (18). In children, adolescents and young adults with type 1 diabetes, only cross-sectional studies were performed. These studies tested small nerve fiber function only or performed a combination of large- and small nerve fiber function tests (1, 2, 35-37). In a pilot study in adolescents with type 1 diabetes (n=6) Sellers et al. did not find a difference in corneal confocal microscopy (CCM) measures when compared with healthy age-matched controls (37), perhaps due to small sample size. Cho et al. tested VPTs (large nerve fiber) by means of Biothesiometer/ Vibratory Sensory Analyser in combination with thermal thresholds (small nerve fibers) by means of Thermal Threshold Tester/ Neurosensory TSA-II and found subclinical DPN in 14-28% of the children and adolescents with type 1 diabetes (1, 2). Regrettably, it was not specified which of the two performed tests were abnormal in their patient group. Weintrob and Blankenburg investigated large nerve fiber dysfunction and small nerve fiber dysfunction in type 1 diabetes in young adults and adolescents respectively (35, 36). There were more patients showing small nerve fiber dysfunction than patients showing large

nerve fiber dysfunction. However, neither study could show if patients with small nerve fiber dysfunction concomitantly had large nerve fiber dysfunction. Moreover, longitudinal data are lacking.

Retinopathy

Retinal photography continues to be the predominantly performed test for screening for retinopathy. The DCCT/EDIC Research group re-evaluated the intervals of fundus photography screening schedule. Based on standardized stereoscopic seven-field fundus photographs of the DCCT/EDIC population and a probability model, they concluded that the screening schedule can be individually adapted to the state of retinopathy and HbA1c value (38).

Retinal vascular geometry in children and adolescents with type 1 diabetes gained more attention. Retinal arteriolar and venular calibre, retinal vascular fractal dimension and arteriolar and venular tortuosity at baseline were shown to associate with (different states of) retinopathy after a follow up period of up to 16 years (9, 39-41). Besides, baseline abnormalities of retinal vascular geometry are found to predict other microvascular complications than retinopathy (7-9, 39). In addition to retinal vascular geometry, the handheld fundus camera may be of interest as well because of its ease in use. Despite its shown accuracy and efficiency in vision-threatening diabetic retinopathy (42), to our knowledge no research has been done with regard to the detection of the early signs of retinopathy.

Macrovascular complications

Between 2007 and 2018, the 'state of the art' consisted predominantly of extending the studies in the field of surrogate markers of macrovascular complications that were already investigated in 2007: carotid intima media thickness (cIMT), arterial endothelial function and arterial stiffness. The number of studies in children and adolescents with type 1 diabetes for these markers increased considerably. The same applied to 'losing track of lipids' e.g. the change of a low-risk or borderline-high-risk lipid level into a borderline-high-risk/high-risk lipid level or a high-risk level respectively.

Several studies showed cIMT to be increased in young study population with type 1 diabetes as compared with healthy peers (43-45). In addition, cIMT progres-

sion was detected in high risk patients within only a short follow up period (43, 46). However, a review of observational studies showed that cIMT is not always increased in pediatric patients with type 1 diabetes when compared with healthy controls (47), possibly due to small study populations, young age, a short duration of diabetes (and thus follow-up), wide reference ranges for cIMT in children and adolescents and heterogeneity in cIMT study protocols (47). Several studies addressed the question if measurement of the aortic IMT (aIMT), instead of cIMT, should be the main marker of interest since atherosclerosis is thought to start in the intima of the distal abdominal aorta (48). Indeed, aIMT was found to be increased in the children and adolescents with type 1 diabetes as compared with their peers, whereas cIMT was not or to a lesser degree (49-52). However, this parameter is more difficult to measure in case of increased tissue penetrance. Additionally, longer measurement duration (15 minutes) has been mentioned as another disadvantage of this method (50-52).

Markers reflecting endothelial dysfunction and measuring endothelial dysfunction itself gained more attention. Marcovecchio showed the intercellular adhesion molecule-1 (ICAM-1) and myeloperoxidase (MPO), both biomarkers of endothelial dysfunction in the blood, to be increased to a similar extent in a small study group of adolescents with obesity or type 1 diabetes compared with healthy adolescents (53). Flow-mediated dilation (FMD) of the brachial artery as a result of reactive hyperaemia after shear stress was measured by several investigators using different techniques in children and adolescents with type 1 diabetes. Scaramuzza measured FMD through a fingertip plethysmograph (54), whereas most others (52, 55-57) performed high resolution ultrasound of the brachial artery. Despite FMD being a non-invasive objective measure of endothelial function, the required conditions of measurement in a regulated room temperature (52, 54, 55), the possible influence of acute hyperglycaemia on FMD (55, 58) and the absence of pediatric reference ranges, make its implementation in routine outpatient care challenging.

Arterial stiffness in type 1 diabetes has been extensively studied in the period 2007-2018. Several measures for arterial stiffness, such as small artery elasticity (SAE), pulse pressure (PP), pulse wave velocity (PWV), augmentation index (A175) and brachial distensibility (BrachD) have been studied.
Benitez-Aquirre evaluated SAE and PP in young adults (27.5 yrs +/- 14.5 SD) with

type 1 diabetes with microvascular complications and without microvascular complications and healthy controls (59). The patients with complications showed an earlier SAE peak in life and a diminished mean SAE in (young) adulthood compared with the patients with type 1 diabetes without complications and the healthy controls (59). PP, in this study measured by diastolic pulse-wave analysis (PulseWave) and an automated Sphygmomanometer, showed a slight decrease with increasing age in all three groups (healthy controls, patients with type 1 diabetes with and without complications) until the age of approximately 30-40 years old and showed a slight increase beyond this age. This increase was found to be more pronounced in the patients with type 1 diabetes and complications compared with the other two groups. In children with type 1 diabetes, PP (measured by sphygmomanometer or Dinamab) was increased compared with pediatric reference values of two large studies (60). Notably, in type 1 diabetes, PP was already higher than the reference range in infancy. Moreover, PP particularly increased during the first 4 years and then stabilized (60). Dabelea evaluated the course of PWV in adolescents with type 1 diabetes, starting with a measurement in adolescence and repeating the measurement approximately 5 years later (61); PWV had significantly increased, but comparison with reference values was absent. Obermannova and Shah did compare results with reference ranges (62, 63). Obermannova found no abnormal PWV in the adolescents with type 1 diabetes (median age 16 yr (IQR 14-17), median diabetes duration 9 yr (IQR 6-16)(62). In contrast, the SEARCH study (Shah) did find an elevated PWV and augmentation index (AI75) and a decreased brachial distensibility in patients with type 1 diabetes when compared with healthy controls (63, 64). These patients had approximately the same age and diabetes duration as the Obermannova study-group or were even younger with a shorter diabetes duration (63, 64). Reference values for European children and adolescents have been determined for PWV (65), PP (66) and SAE (67). To our knowledge, reference values of augmentation index (A175) and a diminished brachial distensibility have not been established yet. Less accurate measurement of PP and PWC have been suggested in case of a very young age and an increased waist circumference respectively (61, 62).

In addition to the early studies of Maahs (68) and Edge (69), more recent studies support the concept of 'losing track of lipids' among children and adolescents with type 1 diabetes (70-72). Associations between predominantly the determinants HbA1c and BMI and lipid dynamics were shown (68, 70, 71). Also, several

cross-sectional studies in this age-group with type 1 diabetes showed added value in risk stratification by extending the lipid profile (low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), non-HDL-C, total cholesterol and triglycerides) with lipids such as apolipoprotein B and LDL-C particle size that are supposed to inform better about the atherogenicity (73-75).

Additional risk factors and surrogate markers

Advanced glycation end products (AGEs) as surrogate marker for complications

AGE levels were examined non-invasively in the skin in several studies in the period 2007-2018. In most studies, AGE levels were determined by skin autofluorescence (SAF) or skin intrinsic fluorescence (SIF) (76-79). In adults with type 1 diabetes, several studies confirmed the associations between increased SAF/SIF and the prevalence of micro- and macrovascular complications that were already shown in earlier studies (76-78, 80, 81). In children and adolescents with type 1 diabetes, SAF/SIF were shown to be increased in this age-group when compared with healthy controls (49, 82-84). However, only a few studies examined SAF/SIF with regard to early signs of micro- and macrovascular complications in this age-group. Cho showed SAF to associate with retinopathy and a measure of heart rate variability, but not with peripheral nerve abnormalities and albumin excretion rate or glomerular filtration rate (2). Lilje showed SIF to associate with aortic intima media thickness (49). In both adults and children and adolescents with type 1 diabetes, correlations between SAF/SIF and HbA1c were examined. Despite the assumption that SAF/SIF provides information about glycaemic control of the preceding years (85), conflicting results on the association between past HbA1c and SAF/SIF were shown (2, 76, 78, 79, 81, 86, 87). Moreover, increased SAF/SIF was already shown in children that were just recently (< 1 week) diagnosed with type 1 diabetes (83) and was shown to be present in a subgroup of patients with normal HbA1c levels (2, 79).

C-peptide as additional risk factor for the development of complications

Baseline and stimulated c-peptide levels, reflecting residual beta cell activity, are shown to protect against development of microvascular complications and certain risk factors of macrovascular disease in the course of type 1 diabetes (88, 89).

Steffes and Panero not only showed higher c-peptide levels to be more protective, but also showed that the presence of even minimal c-peptide levels was more beneficial when compared with undetectable levels (depending on the assay used defined by 0.03 and 0.06 nmol/l respectively)(88, 89). With the study of Kuhtreiber that shows c-peptide levels as low as 0.011 nmol/l to associate with a reduced complication risk (90), and the study of Wang that presents an even more sensitive assay for c-peptide detection (2.5 pmol/l)(91), c-peptide measurement for risk-stratification in patients with type 1 diabetes becomes a promising subject for future research.

HbA1c- and glucose-variability as additional risk factor for the development of complications

HbA1c variability has been found to be a risk factor for the development of micro- and macrovascular complications in several studies (92-94). Nevertheless, not all studies find a clear association between HbA1c variability and complications (95), possibly due to different measures of HbA1c variability between studies (95). Alternatively, the measure HbA1c variability may not be sensitive enough for looking into glycaemic fluctuations. Consequently, glucose variability has been proposed to be a better predictor. Derr and McCarter showed HbA1c to reflect predominantly the mean glucose levels and not glucose variability (96, 97). In addition, it has been argued that glucose variability and not mean glucose is more detrimental with regard to the development of complications (98-102). Enhanced oxygen stress as a result of glucose variability, has been one of the suggested causative factors in the pathogenesis of these complications. However, the existence of a (clear) link between glucose variability, micro- and macrovascular complications and/or measures of oxygen stress has not been demonstrated yet (103-107). The use of different measuring methods for glucose variability was put forward as an explanation (108, 109). Continuous glucose monitoring might lead to diagnostic opportunities, however consensus about which method to use for the expression of glucose variability has still to be determined (110-112).

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List of abbreviations

A175	augmentation index
ACR	albumin to creatin ratio
AER	albumin excretion rate
AGE	advanced glycation end products
aIMT	aortic intima media thickness
apo A-I	apolipoprotein A-I
apoB	apolipoprotein B
AUROC	area under the ROC-curve
BMI	body mass index
BrachD	brachial densibility
CAN	cardiac autonomic
CCA	common carotid artery
CCM	corneal confocal microscopy
C1-controls	age-matched healthy controls of the P1-patients
C2-controls	age-matched healthy controls of the P2-patients
C3-controls	age-matched healthy controls of the P3-patients
cIMT	carotid intima media thickness
CMAP	compound muscle action potential
CNFD	corneal nerve fiber density
CNFBD	corneal nerve fiber branch density
CRP	C-Reactive Protein
CV	coefficient of variation
CVD	cardiovascular disease
D	diagnosed neuropathy: adult patients with type 1 diabetes and clinical diabetic peripheral neuropathy
DBP	diastolic blood pressure
DPN	diabetic peripheral neuropathy
EDB	extensor digitorum brevis
EDDDY-S	Early Detection of Diabetes Damage in Youth and Search for early prevention

FMD	flow-mediated dilation
HbA1c	glycated hemoglobin
HDL-C	high-density lipoprotein cholesterol
ICAM-1	intercellular adhesion molecule-1
IQR	Interquartile range
LDL-C	low-density lipoprotein cholesterol
MPO	myeloperoxidase
NCCE	noncontact corneal esthesiometry
NCV	nerve conduction velocity
PC	poorly controlled: patients, 12.5-19.9 years old, with a disease duration of type 1 diabetes of more than 10 years and an HbA1c at least three times above 8.5% (69 mmol/mol) between 2006 and 2009 and/or (early) signs of microvas cular complications)
PC-co	age-matched healthy controls of the PC patients
PP	pulse pressure
P1-patients	young patients with a disease duration of type 1 diabetes between 2.5 and 5 years and an HbA1c below 64 mmol/mol (8%) since the time of diagnosis, who are considered to have no subclinical neuropathy
P2-patients	young patients with a disease duration of type 1 diabetes of at least 10 years, an HbA1c that was at least three times above 69 mmol/mol (8.5%) between 2006 and 2009 and/or (early) signs of microvascular complications, who are consid ered to have subclinical neuropathy
P3-patients	adult patients with type 1 diabetes and established clinical diabetic peripheral neuropathy
PWC	pulse wave velocity
RAG	receptor for AGE
ROC	receiver operating characteristic
SAE	small arterial elasticity
SAF	skin autofluorescence
sAG	skin advanced glycation end products

List of abbreviations

SBP	systolic blood pressure
SD	standard deviation
sCD40	soluble CD40
SDS	standard deviation scores
SI	stimulus intensity
SIF	skin intrinsic fluorescence
SNAP	sensory nerve action potential
sTNF-R	soluble tumor necrosis alfa receptor
TC	total cholesterol
TG	triglycerides
T1DM	type 1 diabetes
TNFalfa	tumor necrosis alfa factor
VPT	vibration perception threshold
WC	well-controlled: patients, 12.5-19.9 years old, with a dis ease duration of type 1 diabetes between 2.5 and 5 years and an HbA1c below 8% (64 mmol/mol) since the time of diagnosis
WC-co	age-matched healthy controls of the WC patients

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van der Heyden JC, Birnie E, Bovenberg SA, Veeze HJ, Mul D, Aanstoot HJ. Losing track of lipids in children and adolescents with type 1 diabetes; towards individualized patient care. Submitted.

Thus KA, den Boogert WJ, **van der Heyden JC**. Thrombocytopenia: a serious side effect of diazoxide. Submitted.

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PhD portfolio

Summary of PhD training and teaching activities

Name PhD student	Josefine Catherine van der Heyden
PhD period	2007-2018
Erasmus MC Department	Pediatrics, subdivision Pediatric Endocrinology
Promotor	Prof.dr. E.H.H.M. Rings
Co-promotores	Dr. E. Birnie, Dr. D. Mul

General and specific courses	Year	ECTS
Evidence Based Medicine, Utrecht	2006	1.0
Evidence Based Searching, Amsterdam	2007	0.3
ISPAD Science School, Copenhagen	2007	1.2
Classical Methods for Data-analysis (NIHES), Rotterdam	2008	5.7
ESPE Science School, Berlin	2011	1.0
Training Medical Research involving WMO & GCP, Rotterdam	2017	3.0

Seminars and workshops		
Course Pediatric Diabetes, Hoevelaken	2007	0.5
Reflectorium Pediatric Endocinology, Holten	2010	0.8

Reflectorium Pediatric Endocinology, Holten	2013	0.8
Teach the teacher III cursus, Rotterdam	2014	0.6
41st Erasmus Endocrinology Course, Noordwijkerhout	2015	1.0
42st Erasmus Endocrinology Course, Noordwijkerhout	2016	1.0
GOSH course Pediatric Endocrinology, Londen	2016	0.4
Radboud Adrenal Masterclass, Amsterdam	2017	1.0
44th Erasmus Endocrinology Course, Noordwijkerhout	2018	0,5
Lean course, Rotterdam	2018	0.2
Masterclass Pediatric Endocrinology, Utrecht, 2-yearly	2007-2018	0.2
Dutch Society Pediatric Endocrinology (SEK) meeting, Utrecht, 1-2 yearly	2007-2018	0.1
Growth Hormone advisory board meeting, Utrecht, 1-2 yearly	2007-2018	0.1

Presentations

Poster presentation 48th annual Meeting of ESPE	2009
Poster presentation 36th Annual Meeting of ISPAD, Buenos Aires	2010
Oral presentation Annual Meeting for Diabetes Research, Oosterbeek	2010
Poster presentation M. van Vliet, 70st Annual Meeting of ADA, Orlando	2010
Oral presentation SEK, Utrecht	2011

International conferences

5th Meeting International Pediatric Endocrinology Congres, Baveno	2008	1.0
68th Annual Meeting of ADA, San Francisco	2008	2.0
47th Annual Meeting ESPE, Istanbul	2008	2.0

48th Annual Meeting ESPE, New York	2009	2.0
6th International Pediatric Endocrinology Congres, Cologne	2010	1.0
49th Annual Meeting ESPE, Prague	2010	2.0
36th Annual Meeting of ISPAD, Buenos Aires	2010	2.0
Annual Meeting ENDO2014, Endocrine Society, Chicago	2014	2.0
53st Annual Meeting ESPE, Dublin, Ierland	2014	2.0
54th Annual Meeting ESPE, Barcelona	2015	2.0
56th Annual Meeting ESPE, Washington	2017	2.0

Teaching and other activities

Organizing committee of the regional meetings of Pediatric Endocrinology for pediatricians, Rotterdam	2012-2018
Lecturing Diabetic Ketoacidosis first year Dutch residents of Pediatrics, Lunteren	2015- 2018
Lecturing Hypoglycaemia first year Dutch residents of Pediatrics, Lunteren	2018
Lecturing "XXL-management" (obesity) medical students Erasmus University, Rotterdam	2013-2018
Consultant Cyberpoli (subject thyroid dysfunction)	2015-2018

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About the author

Josine van der Heyden was born in Breda, the Netherlands, on May 19th 1976. She lived in Etten-Leur and completed her pre-university education in 1994. She started her medical training at the Faculty of Medicine of the Radboud University after completing her first-year diploma Biomedical Sciences at the same faculty. In 2001, she qualified as a medical doctor and in 2008 as a pediatrician.
In the autumn of 2007, she started working as fellow Pediatric Endocrinology at Diabeter and the Sophia's Children Hospital in Rotterdam, the Netherlands. She completed this fellowship in 2011. From 2011 to 2013 she was working at Diabeter, Rotterdam, the Netherlands. In 2013, she started working in the Franciscus Gasthuis & Vlietland, Rotterdam, The Netherlands.

From 2007 to 2018, she was working on the current thesis.