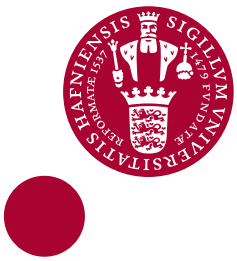

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Aspects of utility in type 1 diabetes

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Cardiac vagal tone as a novel screening tool to recognize asymptomatic cardiovascular autonomic neuropathy: Aspects of utility in type 1 diabetes

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ABSTRACT

Aims: To test the performance of the cardiac vagal tone (CVT) derived from a 5-minute ECG recording compared with the standardized cardiovascular autonomic reflex tests (CARTs).
Methods: Cross-sectional study included 56 well-phenotyped adults with type 1 diabetes (19–71 years, 2–54 years disease-duration). Autonomic testing included: standardized CARTs obtained with the VAGUS™, CVT, and indices of heart rate variability (HRV) obtained at 24- and 120-hour, and electrochemical skin conductance assessed with SUDOSCAN®. ROC AUC and cut-off values were calculated for CVT to recognize CAN based on ≥ 2 (*established CAN*, n = 7) or 1 (*borderline CAN*, n = 9) abnormal CARTs and compared to HRV indices and electrochemical skin conductance.
Results: Established CAN: The cut-off CVT value of 3.2LVS showed 67% sensitivity and 87% specificity (p = 0.01). Indices of HRV at either 24-hour (AUC > 0.90) and 120-hour (AUC > 0.88) performed better than CVT. *Borderline CAN:* The cut-off CVT value of 5.2LVS indicated 88% sensitivity and 63% specificity (p = 0.07). CVT performed better than HRV indices (AUC < 0.72). Electrochemical skin conductance (AUC:0.63–0.72) had lower sensitivity and specificity compared with CVT.
Conclusions: Implementation of CVT with a clinically applicable cut-off value may be considered a quicker and accessible screening tool which could ultimately decrease the number of unrecognized CAN and initiate earlier prevention initiatives.

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1. Introduction

The development of cardiovascular autonomic neuropathy (CAN) is a frequent and severe complication of type 1 diabetes [1,2]. The presence hereof independently predicts mortality and is strongly associated with cardiovascular diseases involving silent myocardial ischemia, coronary heart disease, arrhythmia, and other major cardiovascular events, [3,4] as well as hypoglycemic unawareness [5]. Pathologically, CAN encompasses disruption of coordinated heart control due to a diminution of sympathetic and parasympathetic neuronal function combined with secondary metabolic, oxidative, and inflammatory processes characteristic of long-term hyperglycemia [2,6]. However, asymptomatic CAN is often unrecognized and current routine testing is not recommended until five years after type 1 diabetes diagnosis [2,7].

Cardiovascular autonomic reflex tests (CARTs) remain the gold standard for CAN diagnosis, assessing the heart rate responses to deep breathing, postural change, the Valsalva maneuver, and orthostatic blood pressure alterations [1,8,9]; however, these are not readily accessible in the routine clinical practice. Current evidence shows that CAN, diagnosed based on CARTs may be present in up to 7% of people with type 1 diabetes at the time of diagnosis, and the prevalence increases hereafter with approximately 2% annually [10]. Thus, there is an unmet clinical need for early recognition of CAN [6]. Indices of heart rate variability (HRV) in time- and frequency-domains derived from 24-hour or more recently shorter electrocardiograms have been used to recognize early asymptomatic CAN[1,6,11]; however, differences in the techniques, length of recordings, and the software used raise challenges in correctly establishing cut-off values to compare different populations. More recently, the handheld Vagus™ was developed to perform three standardized CARTs based on heart rate during a 30-minute bedside procedure. Based on age-dependent cut-off values this procedure generates CAN scores used to differentiate between borderline and established CAN as previously reported [12,13]. In addition, assessment of sudomotor function through electrochemical skin conductance (ESC) during a 3-minute test with the SUDOSCAN® has been proposed as a rapid alternative; however, large variability in data generated by different groups, as well a recent *meta*-analysis, question the SUDOSCAN® ESC sensitivity as a reliable test for CAN [14–16]. All the above-mentioned methods are also either unavailable, resource-demanding, or expensive to perform in standard clinical practice [12,13]. Therefore, the implementation of simpler and readily available tests to document the presence of asymptomatic CAN is needed [17].

Cardiac vagal tone (CVT) is derived from a 5-minute resting electrocardiogram and is decreased in adults with type 1 diabetes and polyneuropathy [18]. In addition, it shows a good correlation to 24-hour HRV indices and thus, has been suggested as a novel marker of parasympathetic activity [18]. Furthermore, CVT does not require active participation or complicated instructions, making the case for ready applicability in standard clinical practice [18,19].

We hypothesized that a 5-minute CVT recording could serve as a clinically applicable screening method for recognition of *established* and *borderline CAN*. The primary objective was to test the sensitivity and specificity of CVT for recognizing CAN compared with the standardized CARTs. Secondary objectives included comparing CVT with indices of HRV and explorative sudomotor function.

2. Subjects

This cross-sectional study included 56 adults with type 1 diabetes, of European descent, receiving stable antihyperglycemic medication (consisting of either long-/fast-acting insulin injections or insulin pump use with dosing adjusted according to standard regimens for at least 3 months). Exclusion criteria included established cardiovascular disease, current or previous alcohol consumption above the limits recommended by the Danish health authorities and/or recreational drugs use, current or prior chemotherapy, or any competing diagnoses reflecting neurological and/or psychiatric diseases. Participants were recruited from November 2017 to November 2018 from the outpatient clinic at the Department of Endocrinology, Aalborg University Hospital, Denmark, and from advertisement at the local diabetes association. The study was conducted in accordance with the Declaration of Helsinki and GCP guidelines, was approved by The North Denmark Region Committee on Health Research Ethics (N-20170045), and all research participants signed written consent.

3. Materials and methods

3.1. *Assessment of demographic and cardio-metabolic factors*

Demographic and clinical data including age, sex, diabetes duration, smoking status, presence of retinopathy, and use of insulin and other relevant pharmaceuticals were obtained from participants during the study visit and confirmed by the participants' medical record. Height was measured using a stadiometer (Seca GmbH & Co. KG., Hamburg, Deutschland) and weight was assessed with a standardized calibrated scale (BWB-800AS, Tanita, Arlington Heights, Illinois, USA). Heart rate, resting systolic and diastolic blood pressure were obtained after a 5-minute rest in a seated position using a blood pressure monitor (Intellisense®, Omron Healthcare, Inc., Bannockburn, Illinois, USA). Fasting blood samples were assessed for glucose, hemoglobin A1c, estimated glomeruli filtration rate, and lipid panel (total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol), while urine samples were obtained to estimate albumin-creatinine ratio. Both blood and urine samples were analyzed at the Department of Clinical Biochemistry, Aalborg University Hospital according to standard laboratory procedures.

3.2. *Assessment of cardiac vagal tone*

CVT is a validated cardiometrically derived index of parasympathetic efferent tone and thus, measures the brainstem efferent modulation of the heart [20]. Based on a reflex, the

cardiac heart rate is quickly adjusted through the interaction of baroreceptor stretching, parasympathetic vagal activation, and sinoatrial depolarization [7]. During rest, the relationship between the cardiac cycle, measured as R-R intervals, and the vagal efferent modulation resembles linearity and can be quantified on a linear vagal scale (LVS). On the LVS, zero represents atropinization, and measures above zero represent an efferent vagal tone, with higher measures indicating healthy parasympathetic function [19,21]. Participants were asked to refrain from coffee two hours prior to testing. Following a 5-minute relaxation period, a simple 3-lead electrocardiography (eMotion Faros 180 device (Bittium, Oulu, Finland)) was undertaken with electrodes (Ambu Blue Sensor P, Ballerup, Denmark) placed on the right and left subclavicular areas and at the cardiac apex. CVT was computed by the ProBioMetrics online app version 1.0 (ProBioMetrics, Kent, UK). Recording artifacts were defined as a change in two succeeding QRS complexes exceeding 15 beats per minute (e.g. coughing or movement), and consequently, files were inspected and cleaned by removing five heartbeats before and after in order to derive the true CVT. The file was discarded if the number of edited heartbeats exceeded 20%.

3.3. *Assessment of cardiovascular autonomic neuropathy*

To minimize the influence of circadian rhythm, all participants underwent cardiovascular autonomic reflex testing in the morning (between 8:00 and 10:00 a.m.) in a constantly lit, quiet laboratory. We used the Vagus™ device (Medicus Engineering ApS, Aarhus, Denmark), which has previously been validated with high diagnostic agreement against stationary equipment [13,22]. The testing includes electrocardiographic recordings at rest and during cardiovascular autonomic reflex tests comprised of expiration: inspiration ratio (deep breathing), Valsalva ratio, and 30:15 supine to standing ratio (postural change). The test was performed by a trained examiner (ALW), preceded by 10 min of rest. The CAN score was calculated based on these three examinations; however, if one cardiovascular autonomic reflex test was not completed, the CAN score was estimated from the remaining two procedures.

Established CAN was defined as two or more abnormal tests, *borderline CAN* by one abnormal test, and *no CAN* when the obtained tests were within the normal range of the specific age-dependent cut-off values [9].

3.4. *Assessment of heart rate variability*

Time- and frequency-domain indices of HRV were derived from 24-hour or 120-hour electrocardiogram using the ePatch® (BioTelemetry Technology, Hørsholm, Denmark). The ePatch® system consists of the directly connected single-use, biocompatible, 3-lead electrode, and a rechargeable sensor. HRV indices were derived from R-R intervals using the CardiScope™ analysis software which prepared and filtered the recordings automatically (HASIBA Medical GmbH, Graz, Austria). Analyses were carried out at the Department of Cardiology at Aalborg University Hospital. Based on the recommendation from the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, [23] heart rate (HR) and the following four time-domain HRV indices were used: 1) SDNN: standard deviation of normal-to-normal intervals; 2) SDANN: standard deviation of the average normal-to-normal intervals for each 5-minute segment of the recording; 3) SDNNI: Mean of the standard deviation of all the normal-to-normal intervals for each 5-minute segment of the recording; 4) RMSSD: root mean square of successive RR interval differences. In addition, the following four frequency-domain HRV indices were used: 1) VLF: absolute power of the very-low-frequency band (0.0033–0.04 Hz); 2) LF: absolute power of the low-frequency band (0.04–0.15 Hz); 3) HF: absolute power of the high-frequency band (0.15–0.4 Hz); and 4) LF: HF: Ratio of LF-to-HF power. The content of VLF and LF are traditionally thought to represent the sympathetic activity, in contrast to HF and RMSSD which are considered to represent parasympathetic control. [24] LF:HF ratio is considered to reflect the sympato-vagal balance.

3.5. *Assessment of sudomotor function*

We used SUDOSCAN® (Impeto Medical, Paris, France) to assess the sudomotor function. This device measures the electrochemical reaction between the chloride ions in the sweat glands and the steel electrodes[15] and provides an ESC readout for all four extremities. Participants placed their hands and feet on the stainless-steel electrodes emitting a low current for 3 min. The average value for hands and feet was used in this study.

3.6. *Outcome measures:*

The primary outcome was to test the sensitivity and specificity of the 5-minute CVT compared with established CARTS. The secondary outcomes were to test the performance of the 5-minute CVT compared with indices of HRV and ESC.

3.7. *Statistical analysis*

CAN was stratified into three categories: *no CAN*, *borderline CAN*, and *established CAN*, and descriptive statistics for demographics and cardiometabolic factors for each stratification were reported depending on normality as mean and standard deviations or median and range for the continuous variables and number (percentage) for the categorical variables. Differences between CAN stratifications for demographics and cardiometabolic factors were performed depending on normality as a one-way ANOVA or Kruskal Wallis test, with Tukey's test for post hoc analysis of continuous variables and Fisher's exact test for categorical data.

Logistic regression receiver operating characteristic (ROC) curves were used to illustrate the sensitivity and specificity of CVT, HRV, and ECS for recognizing *established CAN* or *borderline CAN*. Further, the ROC provided areas under the curve (AUC) which calculates the performance of CVT, HRV, and ECS. Finally, based on Youden´s index, the optimal cut-off value for CVT to recognize *established CAN* and *borderline CAN* was suggested. All statistical analyses were performed using STATA software (StataCorp LLC, version 15.1).

4. Results

4.1. Diagnosis of cardiovascular autonomic neuropathy

The CAN score was unobtainable in six participants, as they had difficulties completing the active participation required for abnormal cardiovascular autonomic reflex tests, leaving 50 participants with valid tests. Participants were 19 to 71 years old (average 42 years), had type 1 diabetes between 2 and 54 years (average 22 years), with an average hemoglobin A1C of 62 mmol/mol, and a near equal distribution of gender (54% females). The presence of CAN was diagnosed in 32% (14% *established* CAN and 18% *borderline* CAN). Table 1 presents the clinical and cardiometabolic factors of the participants stratified by the presence of CAN, while the autonomic characteristics of the participants stratified by the presence of CAN are presented in the supplementary materials. There were no differences in age or disease duration between participants with *no* CAN, *borderline* CAN, and *established* CAN ($p > 0.05$). In participants with *established* CAN, proliferative retinopathy was more prevalent in comparison to *no* CAN (43% vs. 3%, $p = 0.01$).

4.2. Cardiac vagal tone

All participants with a valid CART test completed the CVT recording, however, 6 files were discarded due to poor data quality leaving 44 participants with the results from both tests. The performance of CVT for recognizing *established* CAN and *borderline* CAN is shown in Table 2. A CVT cut-off value of 3.2 LVS showed 67% sensitivity and 87% specificity for recognition of *established* CAN at an AUC of 0.80 ($p = 0.01$). Similarly, a CVT cut-off value of 5.2 LVS had 88% sensitivity and 63% specificity for recognition of *borderline* CAN, at an AUC of 0.72 ($p = 0.07$).

4.3. Heart rate variability indices

24-hour HRV analyses: Of participants with valid CART and CVT tests, 7 of the 24-hour HRV recordings were discarded due to poor data quality, leaving 37 participants for analysis. The performance of CVT in comparison to 24-hour HRV indices for recognition of *established* and *borderline* CAN is shown in Table 3 and depicted in as a figure in the Supplementary material. For *established* CAN, the ROC AUC for single

Table 1 – Demography, Diabetes risk factors and Cadiac risk factors for participants with no-, bordeline-, and established cardiovascular autonomic neruopathy (CAN).

	Variable	No CAN (n = 34)	Borderline CAN (n = 9)	Established CAN (n = 7)	p-value	
Demography	Age (years)	35 (19–71)	49 (27–65)	45 (24–67)	0.35	
	Male gender	17 (50%)	5 (56%)	1 (14%)	0.18	
	Body mass index	26 ± 4.1	25 (22–35)	25 ± 2.9	0.90	
Diabetes risk factors	Disease duration (years)	18 (2–54)	23 (3–48)	29 (9–44)	0.56	
	HbA1C (mmol/mol)	60 ± 11	63 ± 7	74 ± 32	0.11	
	Glucose (mg/dl)	188 ± 80	176 ± 49	176 ± 65	0.86	
	Insulin dose (IU)	42.5 ± 15.9	40.3 ± 19.4	44.8 ± 28	0.89	
	Retinopathy					
	Simplex	4 (12%)	2 (22%)	1 (14%)	0.72	
	Proliferative	1 (3%)	0 (0%)	3 (43%)	<0.01	‡
	Maculopathy	4 (12%)	0 (0%)	1 (14%)	0.53	
	eGFR (ml/min)	90 (75–90)	90 (63–90)	90 (33–90)	0.87	
	Albumin/creatinine ratio	9 (3–106)	11 (4–110)	33 (3–280)	0.20	
Cardiac risk factors	Cardiac vagal tone	6.6 (3.7–8.4)	3.8 (2.7–4.8)	2.6 (1.3–6.8)	0.02	‡
	Systolic blood pressure (mmHg)	133 ± 15	136 ± 13	132 ± 19	0.82	
	Diastolic blood pressure (mmHg)	74 ± 8	73 ± 9	74 ± 10	0.99	
	Heart rate (beats/min)	69 ± 9	70 ± 9	75 ± 7	0.19	
	Smoking					
	Current smoker	5 (15%)	2 (22%)	0 (0%)	0.33	
	Previous smoker	4 (12%)	3 (33%)	3 (43%)	0.15	
	Social smoker	5 (15%)	1 (11%)	0 (0%)	0.59	
	Cholesterol (mmol/l)	4.4 ± 1.0	4.5 ± 0.7	4.3 ± 0.5	0.94	
	Triglyceride (mmol/l)	0.8 (0.4–2)	0.9 ± 0.4	1.0 ± 0.4	0.79	
	HDL cholesterol (mmol/l)	1.6 (0.8–3.2)	1.7 ± 0.5	1.8 ± 0.2	0.21	
	LDL cholesterol (mmol/l)	2.3 (0.7–5.3)	2.3 ± 0.5	2.0 ± 0.5	0.48	
	Use of antihypertensive	8 (24%)	3 (33%)	4 (57%)	0.20	
	Use of statins	8 (24%)	4 (44%)	4 (57%)	0.15	
	Use of aspirin	2 (6%)	2 (22%)	1 (14%)	0.32	

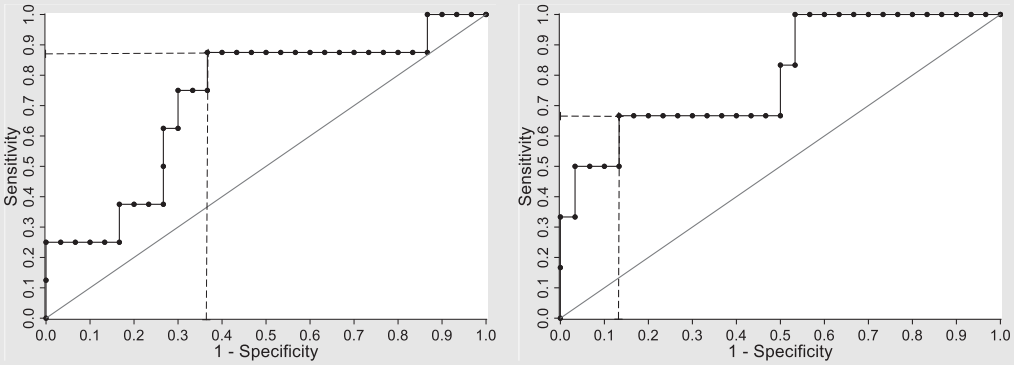
Descriptive data stratified on no, borderline, and established cardiovascular autonomic neuropathy (CAN). Data presented as mean ± standard deviations, median (range), and number (%). Stratifications of no, borderline, and established CAN was compared using a one-way ANOVA or Kruskal Wallis test, with Turkeys test as post hoc, or a $\chi 2$-test.

‡ A significant difference was found between no CAN and established CAN

Abbreviations: eGFR – estimated glomerular filtration rate; HDL – high-density lipoprotein; LDL – low-density lipoprotein.

Table 2 – Receiver operator curve for cardiac vagal tone (CVT) for determining borderline cardiovascular autonomic neuropathy (CAN) and established CAN.

	Borderline	Established
Area under the curve	0.72 (0.51;0.93)	0.80 (0.58;1.00)
Sensitivity	0.88 (0.53;0.98)	0.67 (0.30;0.90)
Specificity	0.63 (0.46;0.78)	0.87 (0.70;0.95)
Positive predictive value	0.39	0.50
Negative predictive value	0.95	0.93
Cut-off value	5.24	3.18
P-value	0.07	0.01



Data is shown with 95% confidence intervals
The x-axis shows 1-specificity while the y-axis shows the sensitivity of the receiver operator characteristics curve as a black line with dots. The dashed lines show the intersection of the sensitivity and specificity at the optimal cut point.

HRV indices were larger (0.9–0.99) than CVT ROC AUC, demonstrating the superior performance of HRV. However, the LF:HF ratio ROC AUC was smaller (0.64) than CVT ROC AUC, which indicates the superior performance of CVT for this measure. In *borderline CAN*, the ROC AUC for all HRV indices were smaller (0.41–0.7) than CVT ROC AUC, indicating the superior performance of CVT to recognize this entity.

120-hour HRV analyses: Of participants with valid CART and CVT tests, 12 of the 120-hour HRV recordings were discarded due to poor data quality, leaving 32 participants for analysis. The duration of the recordings was on average 118 ± 9 h. The performance of CVT in comparison to 120-hour HRV indices for recognition of *established* and *borderline* CAN is shown in Table 3 and depicted in as a figure in the Supplementary material. In *established CAN*, the ROC AUC for single HRV indices were larger (0.88–0.99) than CVT ROC AUC, demonstrating the superior performance of HRV. However, the LF:HF ratio ROC AUC was smaller (0.6) than CVT ROC AUC, indicating the superior performance of CVT for this measure. In *borderline CAN*, the ROC AUC for all HRV indices (0.6–0.72) were smaller than CVT ROC AUC, indicating the superior performance of CVT to recognize this entity.

4.4. *Sudomotor function*

Fig. 1A-B shows ROC generated to plot the performance of CVT compared to ECS of hands and feet for recognition of *borderline CAN* and *established CAN*. In *established CAN*, the ROC AUC for ESC of the hands (0.70) and feet (0.72) were smaller than CVT ROC AUC. In *borderline CAN*, the ROC AUC for ESC of the hands (0.63) and feet (0.67) were smaller than CVT ROC AUC.

Table 3 – Area under the receiver operator curve for 24-hour and 5 day heart rate variability measures to recognize established or borderline CAN.

	Established CAN		Borderline CAN	
	24-hours	5-day	24-hours	5-day
HR (bpm)	0.90	0.93	0.42	0.58
SDNN (ms)	0.97	0.99	0.63	0.65
SDANN (ms)	0.92	0.98	0.57	0.66
SDNNi (ms)	0.94	0.96	0.64	0.64
RMSSD (ms)	0.94	0.92	0.74	0.76
VLF (ms^2)	0.94	0.95	0.65	0.63
LF (ms^2)	0.96	0.95	0.66	0.68
HF (ms^2)	0.93	0.93	0.78	0.80
LF:HF	0.67	0.69	0.62	0.63

5. Discussion

In this study, we demonstrate that the CVT, a simple test derived from a 5-minute electrocardiogram recording, has high sensitivity to recognize *borderline CAN* and a high specificity to recognize *established CAN* as diagnosed by standardized CARTs. Thus, implementing the CVT with the described cut-off values is clinically applicable and suitable as a quick screening tool, which could ultimately decrease the number of unrecognized CAN and initiate earlier prevention initia-

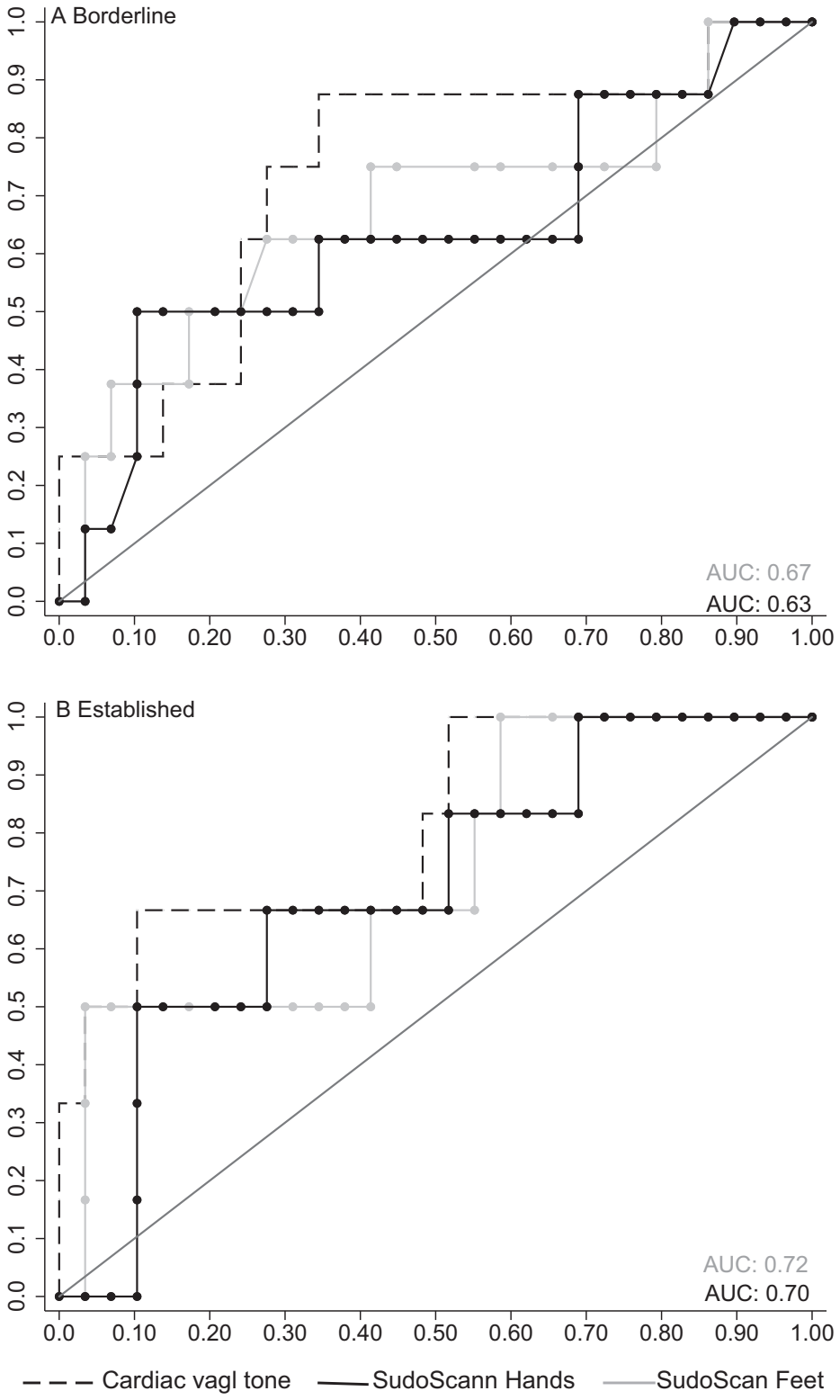


Fig. 1 – A-B: Receiver operator curve for cardiac vagal tone (dashed line), ESC feet (grey line) and ESC hands (black line) for A) borderline cardiovascular autonomic neuropathy (CAN) and B) established CAN. The x-axis shows 1-specificity while the y-axis shows sensitivity.

tives. The prevalence of CAN in this cohort of adults with type 1 diabetes, when assessed with cardiovascular autonomic reflex testing, was 32%.

The present study revealed that the average CVT values were decreased in participants with the presence of CAN in comparison to *no CAN*. However, CVT in the group with *established CAN* and *borderline CAN* were comparable with previous findings in adults with type 1 diabetes, distal symmetrical polyneuropathy, and concomitant orthostatic hypotension [18,25]. Additionally, CVT values in the three CAN strata were lower than CVT values reported in healthy subjects, [18,21] indicating lower parasympathetic tone. However, it is worth noting that in comparison to normative data obtained from 200 healthy humans (95% CI: 1.9–17.8 LVS), [21] we found that only three out of 50 participants had CVT values below the 95th percentile, which may challenge the existing lower limit of the confidence interval of normal cardiac vagal tone.

One-third of the participants in our cohort had a classification of CAN. There were no differences in age and disease duration between the three CAN strata, which may indicate that the current standard of testing for CAN every year after five years of a diagnosis of type 1 diabetes seems sensible. Furthermore, as proliferative retinopathy was exclusively reported in the group with *established CAN*, this relatively common eye diagnosis should raise clinical awareness of the need to test the autonomic nervous system in order to recognize asymptomatic CAN.

Cardiovascular autonomic reflex testing is the gold standard for autonomic testing recommended by the Toronto consensus [8]. However, the comprehensive and time-consuming test paradigm of the cardiovascular reflex tests are not feasible in a clinical setting, where time is essential. Therefore, the use of Vagus^{TM} is an acceptable clinical solution, but it needs thorough instruction and active patient participation. In contrast, the less technical demanding CVT provides a quick measure computed from a 5-minute electrocardiographic recording. This was underscored by an excellent specificity (87%) of CVT for recognizing *established CAN* and with the introduction of a cut-off value of 3.2 LVS. The method provides an easily performed and readily applicable test, which could not only aid healthcare professionals in recognizing CAN in clinical settings but also provide a much-needed endpoint in clinical trials testing novel treatment approaches for CAN. Thus, earlier recognition of CAN is pivotal as optimal glycemic control may halt the progression of the entity.

In advanced autonomic testing, HRV should be considered an addition to cardiovascular reflex testing, allowing supplemental prognostic information. However, cardiovascular autonomic reflex testing and HRV are notorious for disagreement when applied as diagnostic tools. Therefore, in *established CAN,* we tested the performance of CVT against 24-hour and 120-hour HRV indices. Our results indicate that all single HRV indices performed equally or were better at recognizing *established CAN* in contrast to CVT. This is not a surprise, since both CVT and HRV are based on electrocardiographic measurements and computed from changes in R-R waves, so the HRV is affected by multiple physiological factors with direct influence on heart rhythms such as sleep, ingested meals, and physical activity. In contrast, CVT performed better than the LF:HF ratio reflecting sympato-vagal balance. This result is plausibly caused by the fact that LF:HF ratio consists of two derived HRV indices and thus, contains variability in both the nominator and denominator, rather than CVT providing a better proxy of sympato-vagal balance. Furthermore, crude comparison of 24-hour and 120-hour HRV time- and frequency-domain indices within the CAN strata showed that all single HRV indices were decreased in the *established CAN* groups compared to *no CAN,* further supporting our results.

In the search for quick, clinically applicable autonomic methods, a short recording of sudomotor function has surfaced as a potential tool [16]. However, recent controversy has arisen regarding the biological plausibility of this measure [14]. Therefore, we explored the performance of ESC compared to CVT to recognize established CAN and found that ESC underperformed. ESC is believed to peripheral sympathetic unmyelinated thin C-fibers, and is in the literature associated with tests reflecting sympathetic function like orthostatic hypotension and low-frequency power components of HRV, rather than those reflecting parasympathetic function such as the cardiovascular autonomic reflex test used conducted by the Vagus device [26,27]. These parasympathetic test are generally chosen as parasympathetic denervation often is the first sign of cardiovascular autonomic neuropathy. However, a study by Jacob et al. [28] did suggest that hyperadrenergic orthostatic hypotension could an early form of autonomic impairment, though this needs further investigation.

Due to its asymptomatic nature, CAN is unfortunately often well established and irreversible when recognized [29]. Therefore, early recognition should be the focus of clinical progression as it will enable earlier prevention initiatives and investigation of treatment possibilities [1,7,30]. A reasonable approach might consist of reducing universal testing by implementing a clinically applicable screening tool. As one single abnormal result of three cardiovascular autonomic reflex tests is considered sufficient for recognition of early CAN, we investigated the performance of CVT as a screening tool to recognize *borderline CAN*. By the use of a CVT cut-off value of 5.2 LVS, our results indicated a good performance with high sensitivity (88%), moderate specificity (63%), though borderline significant. However, the moderate specificity is caused by a single data point where one person had CVT values (12 LVS) considerably above the average of healthy participants (9 LVS) [21]. Therefore, the suggested cut-off value for screening purposes seems robust even though it did not reach significance.

It is generally accepted that time-consuming HRV recordings (24-hours or more) are reproducible, stable over time, and have increased sensitivity compared to short-term recordings (5 to 30-minute) [23]. To our surprise, our results indicated that a 5-minute recording of CVT performed slightly better at recognizing *borderline CAN* than HRV indices derived from 24-hour and 120-hour HRV indices. This finding challenges the general acceptance of the diagnostic applicability of HRV indices in *borderline CAN*, which hitherto, has been considered a sensitive test for early recognition of CAN in asymptomatic patients [23]. The differences could be

grounded in our use of the cardiovascular autonomic reflex test as reference. Curiously, HF, the spectral HRV components assumed to be most affected by efferent vagal activity and circadian activity, [23] was superior to CVT for recognizing *borderline CAN*.

For recognition of early CAN in adults with type 1 diabetes, Selvarajah et al. [16] have previously reported ESC to be lower compared to those without CAN. Interestingly, Ang et al. [15] showed that ESC values decreased in type 1 diabetes after 12 months, though this was not the case for other autonomic tests including the Valsalva maneuver. Though our results showed no crude difference in ESC between the CAN strata, the AUC results indicated poor accuracy for recognizing *borderline CAN,* with an AUC of 0.67 for the feet and 0.63 for the hands; both measures are inferior to CVT. This is the first time that ECS has been compared with CVT, and our results are the first to show that CVT performs better than sudomotor function for classifying CAN. Our results, however, are comparable to those reported by Selvarajah et al., [16] although these authors are the only ones to report better performance of ESC in hands for recognizing CAN in comparison to ESC in feet. Taken together, CVT could ease the testing practice for health care professionals and decrease the number of unrecognized cases.

This study is not without limitations. Firstly, this is a cross-sectional pilot study of a relatively small cohort, and thus, generalizing the results should be done with caution. Secondly, the proportion of participants stratified to the presence of CAN may be underestimated due to selection bias, as our exclusion criteria hamper the true prevalence by excluding potential participants with established cardiovascular disease from the study. Thirdly, in contrast to suggestions by the Toronto consensus, [1] we did not measure orthostatic hypotension as part of the CAN formula, primarily because it is not part of the Vagus™ procedures. Fourthly, the mean CVT measure has been validated in the healthy, [31] and not in those with diabetes; however, we standardized the recording procedures in order to avoid potential fluctuations caused by physiological and environmental factors. For example, the circadian rhythm is known to influence CVT, CARTs, and ESC, and therefore, all experimental procedures were recorded in the mornings between 8.00 and 11:00. Additionally, though CVT has been shown to decrease with age[21], we did not use age-related normative values, which may impact or findings. Fifthly, HRV was measured for 24-hours and 120-hours and is therefore affected by multiple physiological factors with direct influence on heart rhythms such as sleep, ingested meals, and physical activity. However, we minimized noise and artifacts by automatic filtering and processing in order to avoid manual editing which could induce subjective bias [32]. Finally, it would have benefited the study if we had had measures of continuous glucose monitoring in order to investigate differences in "time in range" in the three CAN strata and we recommend that this be implemented in future prospective longitudinal studies.

5.1. *Conclusion*

We suggest the implementation of a reliable, low cost, and simple CVT measure with a clinically applicable cut-off value that may meet the unmet need for a suitable and quick screening test for recognition of asymptomatic established and borderline CAN. The 5-minute CVT measure performs with acceptable sensitivity and specificity for recognition of asymptomatic CAN. Although screening for CAN is clinically recommended 5 years after diagnosis of type 1 diabetes and yearly thereafter, this is rarely carried out in the clinical setting due to resource-demanding procedures. Ultimately, a quick screening test could decrease the number of asymptomatic and undiagnosed CAN cases and initiate earlier prevention initiatives, thereby effectively improving glycaemic control, increased quality of life, and survival time.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The study was conducted in accordance with the Declaration of Helsinki and GCP guidelines, was approved by The North Denmark Region Committee on Health Research Ethics (N-20170045), and all research participants signed written consent.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions: ALW, CB, AMD, RPB, and BB conceived and designed the analysis. ALW collected the data and EDL, SR, and NE contributed data or analysis tools. ALW and RPB performed analysis. ALW wrote the first draft, but all authors contributed to the final manuscript and critically reviewed it for intellectual content. CB is the guarantor of the work, with full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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