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PhD THESIS

SKIN AUTOFLUORESCENCE IN THE ASSESSMENT OF CARDIOVASCULAR RISK AND CHRONIC COMPLICATIONS IN PATIENTS WITH TYPE 2 DIABETES

– A B S T R A C T –

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INTRODUCTION

In Romania, diabetes affects one in nine adults, with the PREDATORR study identifying 2.4% of these cases as undiagnosed. The prevalence of diabetes mellitus (DM) increases with age and is higher among males, highlighting its significant public health impact (1,2). Globally, the incidence of type 2 diabetes mellitus (T2DM) is on the rise, having doubled among adults over the past twenty years–a development recognized by the World Health Organization as a critical health concern(3,4).

Cardiovascular (CV) diseases continue to be the primary cause of mortality in T2DM patients, primarily due to coronary, cerebrovascular, or peripheral arterial complications (5–8). The atherosclerotic process, which is the main trigger of this, is aggravated by the adverse impacts of glycemic fluctuations on the endothelium, leading to inflammation. This results in a 2-3 fold increased risk of myocardial infarction and stroke in T2DM patients, underscoring the disease's extensive systemic effects (9,10).

The range of microvascular complications associated with DM is extensive and severe, affecting multiple body systems. Pathological entities such as neuropathic damage and peripheral vascular impairment greatly increase the risk of ulcerations, infections, and limb amputations, drastically affecting patient’s quality of life and raising mortality rates (11–13). Notably, cardiac autonomic neuropathy (CAN) in DM patients correlates with a fivefold increase in CV mortality risk (14). Diabetic retinopathy remains a leading cause of global blindness, and DM is a primary factor in end-stage renal disease (15).

Recognizing T2DM's complexity, a multifaceted approach to its management is universally endorsed. Current protocols advocate for cardiovascular risk assessment models to precisely evaluate individual risks and pinpoint the most effective therapeutic measures. These approaches aim to mitigate both micro- and macrovascular complications by focusing on specific therapeutic targets and medications (6,8,11,12,16,17). In this context, there is a growing emphasis on identifying non-invasive, quick, cost-effective, and easily implemented methods for enhanced risk quantification. Such techniques are crucial for tailoring multifactorial treatments and minimizing the risk of cardiovascular or cerebrovascular incidents. Skin autofluorescence (SAF) has been thoroughly investigated and identified as a non-invasive indicator of advanced glycation end product (AGE) buildup, which is linked to vascular complications in both diabetic and non-diabetic patients. AGE levels are associated with advanced atherosclerosis and early vascular damage (18,19). The accumulation of AGEs, exacerbated by factors like hyperglycemia, dyslipidemia, uremia, and oxidative stress, plays a pivotal role in DM's chronic complications. The concept of metabolic memory has evolved with AGEs at its core, marking them as a key pathological substrate (20). SAF's role in measuring AGE concentration has been corroborated through numerous studies,

proving its efficacy in predicting long-term DM complications and mortality risks over periods extending 5-10 years, compared to shorter-term evaluations like hemoglobin A1c, which spans 2-4 months (21).

This study aims to enhance cardiovascular and complication risk assessments by incorporating SAF into routine clinical practice within a contemporary Romanian setting. This approach is anticipated to enable early detection of high-risk patients, facilitating timely and tailored diagnostic and therapeutic interventions. The goal is to evaluate the clinical utility of a validated, reproductible, and cost-effective method that not only anticipates but may also prevent the progression of chronic complications related to diabetes mellitus (DM).

MATERIAL AND METHOD

STUDY DESIGN

This study was conducted as a single-center, cross-sectional, consecutive-case, population-based investigation. It adhered to the principles outlined in the Declaration of Helsinki. Approval was obtained from the Institutional Ethics Committee of the Consultmed Hospital in Iași County, Romania (protocol number CMD102018006, dated October 18, 2018). This healthcare institution provides services that are covered by the National Health Insurance Program of Romania. Patients attended scheduled appointments with a referral from their family physician for regular monitoring as part of their diabetes care process.

PATIENT ENROLMENT METHODS

SAMPLE SIZE

Using data from the Iași County Statistical Yearbook 2022, a sample population of 885 individuals was calculated from a total of 944074 residents in Iași County, considering a 3.29% sampling error.

Inclusion Criteria:

- Patients possessing cognitive capacity and providing signed informed consent;
- Age exceeding 18 years;
- Type 2 Diabetes Mellitus confirmed diagnosis according to the American Diabetes Association Guidelines (2019) (22).

Exclusion Criteria:

- Patients lacking cognitive capacity or who decline to provide signed informed consent;
- Individuals younger than 18 years;
- Pregnancy or breastfeeding;
- Presence of autoimmunity markers such as IA2A, ICA, GADA, ZnT8 AB; Diagnosis of Type 1 Diabetes, Latent Autoimmune Diabetes in Adults, or secondary diabetes forms.

Of the 896 participants who gave their consent to participate, 11 were eventually excluded due to abnormal laboratory results that could suggest either deteriorated blood samples or malfunctions in the laboratory equipment.

PATIENTS' CARDIOVASCULAR RISK CLASSIFICATION

The patients who participated in this study were allocated into moderate, high, or very high-risk groups according to their CV risk profile, as determined by the ESC/EAS guidelines at the time of the study (2019 ESC/EAS classification) (23).

SKIN AUTOFLUORESCENCE METHOD

The skin autofluorescence (SAF) reader represents a non-invasive technique for evaluating skin advanced glycation end products (AGEs). This device contains an illumination window through which ultraviolet (UV) light interacts with the exposed skin, a small scanner for placing the subject's forearm, a spectrometer for analyzing the light emitted from autofluorescent AGEs, a monitor screen for displaying results, and a fiber optic transducer for transferring light to the spectrometer (24). In our study, we used the AGE reader (Diagnoptics Technologies, Groningen, the Netherlands).

GENERAL CHARACTERISTICS OF THE PATIENTS

The demographic characteristics of the study group are shown in Table 1.

Table 1. Patients' demographics and anthropometrics data.

Characteristic	n=885
Demographics	
Urban, %, n	74.69% (661)
Age (years), mean (SD)	62.9±7.7
Women, %, (n)	53.7% (475)
Anthropometrics	
BMI (kg/m2), mean (SD)	32.3±5.3
Waist circumference (cm), mean (SD)	104.74±11.7
Hip circumference (cm), mean (SD)	109.34±9.94
Waist-hip ratio, mean (SD)	0.96±0.074

SD – standard deviation; BMI – body mass index.

The complete characteristics of the patients are outlined in Table 2.

Table 2. Patients' risk factors.

Characteristic	n=885
Risk factors	
Obesity, %, (n)	64.6% (572)
CVD, %, (n)	13.9% (123)
HBP, %, (n)	83% (737)
SBP (mm Hg), mean (SD)	132±16.2
DBP (mm Hg), mean (SD)	80±9.6
ESC (µS), mean (SD)	80.31±9.68
ABI left, mean (SD)	1.13±0.13
ABI right, Mean (SD)	1.11±0.13
Total-C (mg/dl), mean (SD)	185.1±43.3
HDL-C (mg/dl), mean (SD)	44.9±11.8
TG (mg/dl), median (interquartile range)	142 (104 to 197)
LDL-C (mg/dl), mean (SD)	107.7±36.0
Uric acid (mg/dl), mean (SD)	5.9±5.2
Creatinine (mg/dL), median (interquartile range)	0.83 (0.68 to 0.9)
eGFR (ml/min/1.73 m2), mean (SD)	87.5±20.6

CVD - cardiovascular disease; HBP – high blood pressure; SD – standard deviation; SBP – systolic blood pressure; DBP – diastolic blood pressure; ESC - electrochemical skin conductance; ABI- ankle-brachial index Total-C – total cholesterol; HDL-C – high density lipoprotein cholesterol; TG- triglycerides; LDL-C – low density lipoprotein cholesterol; eGFR – estimated glomerular filtration rate.

The characteristics of DM among the patients are shown in Table 3. Diabetes complications included: DSPN at 67.9%, DR at 4.29%, CKD at 8.7%, albuminuria at 6.21%.

Table 3. The characteristics of DM and complications.

DM Characteristics	n=885
DM mean duration, mean (SD)	9.0±4.4
HbA1c (%), mean (SD)	7.1±1.3
DSPN, %, n	67.9% (601)
DR, %, n	4.29% (38)
CKD, %, n	8.7% (76)
Stage V CKD, %, n	0
Stage IV CKD, %, n	3 (0.34%)
Stage IIIa CKD, %, n	13 (1.47%)
Stage IIIb CKD, %, n	60 (6.78%)
Albuminuria, %, n	6.21% (55)

DM – diabetes mellitus; SD –standard deviation; DSPN – diabetic sensory peripheral neuropathy; DR – diabetic retinopathy; CKD – chronic kidney disease.

Table 4 summarizes the T2DM agents, and the other pharmacological agents administered to the included patients.

Table 4. T2DM and other Pharmacological Agents.

Agent	n=885
Insulin, %, (n)	25.2% (223)
Metformin, %, (n)	87.0% (687)
DPP-4i, %, (n)	13.0% (115)
GLP-1 RAs, %, (n)	8.1% (71)
SGLT2i, %, (n)	3.9% (34)

Agent	n=885
Sulfonylurea, %, (n)	13.1% (116)
Thiazolidinediones, %, (n)	1.35% (12)
ACEi/ARBs, %, (n)	61.5% (544)
Calcium channel blockers, %, (n)	33.1% (293)
Beta-blockers, %, (n)	54.57% (483)
Antiplatelet drugs, %, (n)	44.85% (397)
Statins, %, (n)	67.0% (593)
Ezetimibe, %, (n)	4.5% (40)
Fibrate, %, (n)	8.7% (77)
Non-vitamin K antagonist oral anticoagulants, %, (n)	1.8% (16)
Vitamin K antagonists, %, (n)	1.12 % (10)

ACEI/ARBs – angiotensin converting enzyme inhibitors/angiotensin receptor blockers; DPP-4i – dipeptidyl peptidase 4 inhibitors; GLP-1 RAs – glucagon like peptide 1 receptor agonist; SGLT2i – sodium-glucose cotransporter-2 inhibitors.

Table 5 summarizes SAF, NEPHRO risk score, CAN risk score mean values for the population from the study cohort.

Table 5. Skin autofluorescence, NEPHRO risk score, CAN risk score in the study cohort.

Characteristic	n=885
SAF mean level, mean (SD)	2.6±0.5
NEPHRORS, mean (SD)	65.63 ± 14.5
CANRS, mean (SD)	33.46 ± 8.73

SAF – skin autofluorescence; SD – standard deviation; NEPHRORS - NEPHRO risk score; CANRS- cardiac autonomic neuropathy risk score

RESULTS

INTERRELATIONS BETWEEN DEMOGRAPHIC VARIABLES AND DIABETES MELLITUS CHARACTERISTICS

GENDER DISTRIBUTION

Of the total study group, 53.7% were female patients, with a sex ratio of F/M = 1.2/1.

BODY MASS INDEX (BMI)

The series of values for BMI was homogeneous, suggesting that statistical significance tests can be applied: variations in the range of 19.92-49.35 kg/m^2; group mean 32.28 kg/m^2 ±5.26; median 32.01 kg/m^2 close to the mean value; Skewness test result p =0.518.

BMI was weakly, reversely and statistically significantly correlated with age (r =-0.156; p =0.001). The mean BMI level was significantly lower in males (31.55 kg/m^2 ±4.99) versus females (32.90 kg/m^2 ±5.41), p=0.001.

The percentage distribution of cases with T2DM, according to epidemiological characteristics and BMI level, are shown in Table 6.

Table 6. Descriptive data on epidemiological characteristics by weight status.

Characteristics	BMI						P (Chi² LR test)
	<25 kg/m² (n=53)		25-30 kg/m² (n=261)		>30 kg/m² (n=571)		
	n	%	n	%	n	%	
Female	29	54.7	114	43.7	332	58.1	**0.001**
≥64 years	30	56.6	156	59.8	279	48.9	**0.011**

BMI – body mass index; LR – likelihood ratio

Patients with obesity had the highest average duration of T2DM (9.05 years ±4.21), while those with normal weight had the lowest (8.42 years ±4.66), however, the difference was not statistically significant (p=0.587).

The anthropometric data consisted of homogeneous value series:

- **Abdominal/waist circumference (WC)** of 71-149 cm, with a mean of 104.74 cm ±11.71, and a median value of 104 cm.
- **Hip circumference (HC)** of 87-150 cm, with a mean of 109.34 cm ±9.95, and a median value of 108 cm.
- **The WHR** of 0.73-1.25, with a mean of 0.96±0.07, and a median of 0.96.

HbA1c

The value series for HbA1c was homogeneous, suggesting that statistical significance tests can be applied: variations in the range of 4.70-13.0%; group mean of 7.06% ±1.25; median of 6.8% close to the mean value; Skewness test result p=1.331.

HbA1c was weakly, reversely and statistically significantly correlated with age; (r =-0.122; p=0.001). The mean level of HbA1c did not significantly differ between genders (7.06% ±1.30 for males versus 7.06% ±1.20 for females; p=0.989). The percentage distribution of cases with T2DM, based on epidemiological characteristics and HbA1c level are shown in Table 7.

Table 7. Descriptive data on epidemiological characteristics by HbA1c level.

Characteristics	HbA1c ≤7% (n=518)		HbA1c >7% (n=367)		p (Chi² test)
	N	%	N	%	
Female	288	55.6	187	51.0	0.172
≥64 years	301	58.1	164	44.7	**0.001**

The correlation between HbA1c and anthropometric data was direct, but of low intensity; however, the results can be extrapolated to the general population:

- a higher HbA1c level was associated with higher BMI values (r=+0.196; p=0.001);
- a higher HbA1c level was associated with higher WC values (r=+0.217; p=0.001);
- a higher HbA1c level was associated with higher HC values (r= +0.165; p=0.001);
- a higher HbA1c level was associated with higher WHR values (r= +0.108; p=0.001).

The duration of T2DM was significantly reversely correlated with the level of HbA1c (r= -0.110; p=0.001). Yet, it is observed that patients with an HbA1c level >7% had, on average, a significantly longer duration of the disease compared to those with an HbA1c level below 7% (6.26 years ±0.48 versus 8.20 years ±1.11; p=0.001).

INTERRELATIONS BETWEEN DEMOGRAPHIC VARIABLES AND SAF, NEPHRO AND CAN RISK SCORES

SAF

The value series for the SAF level was homogeneous, suggesting that statistical significance tests can be applied: variations in the range of 1.30-6.50; group mean of 2.60±0.51; median of 2.60 close to the mean value; Skewness test result p =0.905.

SAF levels were weakly, positively an statistically significantly correlated with age (r =+0.294; p =0.001). The mean SAF score was significantly higher in males (2.63±0.57 AU) versus females (2.57±0.44 AU), p =0.04.

NEPHRORS

The value series for the NEPHRORS was homogeneous, suggesting that statistical significance tests can be applied: variations in the range of 14-142; group mean values of 65.63±14.47; median of 64 close to the mean value; Skewness test result p=0.637.

The NEPHRO score was moderately, positively and statistically significantly correlated with age (r =-0.575; p =0.001). The mean level of the NEPHRO score was significantly higher in males (67.46±15.77) versus females (64.05±13.07), p=0.001.

CANRS

The value series for the CANRS was homogeneous, suggesting that statistical significance tests can be applied: variations in the range of 0-64; group mean of 33.46±8.73; median of 35 close to the mean value; Skewness test result p =-0.573.

The CANRS score was moderately, positively and statistically significantly correlated with age (r =+0.250; p =0.001). The mean level of the CANRS score was significantly higher in females (31.93±9.40) versus males (34.78±7.88), p=0.001.

CORRELATIONS BETWEEN CLINICAL, PARACLINICAL, AND LABORATORY FINDINGS

LABORATORY PARAMETERS

The value series of total cholesterol (185.08±43.34 mg/dL), HDL-C (44.94±11.77 mg/dL), LDL-C (107.3±36.01 mg/dL), and eGFR (87.55±20.56 ml/min/1.73 m2) were

homogeneous, as the median values were close to the mean values, and the Skewness test results fell within the range of [-2, 2], suggesting that parametric statistical significance tests can be applied.

The ABI measurements in the cohort of 885 individuals showed a mean of 1.13±0.13 (left) and 1.11±0.13 (right), with corresponding medians of 1.13 and 1.12, both distributions having similar variance and skewness, indicating comparable spread and asymmetry in ABI values bilaterally. ABI did not exhibit a significant correlation with LDL-C for the left (r =-0.011; p =0.746) or right (r =0.032; p =0.348) sides. The mean LDL-C was slightly decreased in individuals with an ABI <0.9, both on the left side (103.67 mg/dL versus 107.88 mg/dL; p = 0.523) and on the right side (103.59 mg/dL versus 107.95 mg/dL; p =0.429).

The vast majority (95.9%) of ESC measurements are >60 µS, with only a small fraction falling between 40-60 µS (3.3%) or <40 µS (0.8%), indicating predominantly normal sudomotor function in the patient cohort.

Despite most CANRS occurring in the >60 µS ESC group, the high p-value of 0.957 suggests no statistically significant association between ESC levels and the presence of CAN. The data show no significant association (p=0.108) between ESC levels and PDSN across a sample of 821 subjects. Our crosstabulation analysis was specifically conducted on patients categorized within the very high-risk group, in light of the relatively small number of patients in the high (10 patients) and moderate (54 patients) risk categories.

EVALUATION OF CARDIOVASCULAR RISK CATEGORIES AND ATTAINMENT OF TREATMENT GOALS WITHIN THE STUDY GROUP

We aimed to investigate the distribution of CV risk categories according to the 2019 ESC/EAS guidelines(23) in our cohort of Romanian T2DM patients and determine the proportion achieving LDL-C, HbA1C, and blood pressure targets. Additionally, we analyzed the use of new T2DM treatments with cardiorenal benefits and statins in this group.

Therapeutic goals for each CV risk category were determined based on the 2019 ESC/EAS guidelines (23) for LDL-C and the 2019 American Diabetes Association (ADA) guidelines for HbA1c and BP (25,26):

- Moderate-risk category: LDL-C <100 mg/dL, HbA1c <7%, BP <130/80 mmHg.
- High-risk category: LDL-C <70 mg/dL, HbA1c <7%, BP <130/80 mmHg.
- Very high-risk category: LDL-C <55 mg/dL, HbA1c <7%, BP <130/80 mmHg.

The risk distribution among the cohort shows 821 patients (92.8%) categorized as Very High risk, 10 patients (1.1%) as High risk, and 54 patients (6.1%) as Moderate risk, highlighting a high prevalence of severe risk factors or conditions in the majority of the patients.

Gender and age distributions reveal significant differences across risk categories: 445 females (54.2%) in Very High, 8 (80.0%) in High, and 22 (40.7%) in Moderate risk groups with a p-value of 0.034, and 443 individuals aged ≥64 years (54.0%) in Very High, 4 (40.0%) in High, and 18 (33.3%) in Moderate risk groups, showing significant age-related variations with a p-value of 0.009.

Anthropometric data shows statistically significant differences across risk categories for BMI (Very High: 32.44±5.25, High: 31.08±4.23, Moderate: 30.01±5.16; p=0.003), WC (Very High: 105.05±11.69, High: 101.40±9.35, Moderate: 100.59±11.61; p=0.017), and HC (Very High: 109.58±9.99, High: 106.90±8.65, Moderate: 106.24±9.09; p=0.043), with no significant difference in WHR (Very High: 0.96±0.08, High: 0.96±0.08, Moderate: 0.95±0.07; p=0.458).

Laboratory parameters reveal significant variations among the risk categories, particularly in LDL-C (p=0.002) and total-C (p=0.003), along with slight but not statistically significant differences in systolic and diastolic BP.

T2DM duration varies significantly across risk categories, with the Very High group averaging 9.26±4.25 years, the High group at 14.20±1.40 years, and the Moderate group at 3.50±2.47 years, demonstrating a statistically significant correlation (p=0.001) between longer DM duration and higher risk levels.

Figure 1 present very high, high and respectively moderate CV risk category's attainment of treatment targets according to 2019 ESC/EAS Guidelines for LDL-C(23), 2019 ADA Guidelines for HbA1c and BP(25,26) and prescription rates of innovative antidiabetic and statin medications.

	Patients Achieving Target	Patients with SGLT2i Prescription	Patients with GLP-1 RAs Prescription	Patients with Statin Prescription
Treatment Target for Patients with Very High CV Risk Category (n = 821)				
LDL-C < 55 mg/dL, %, (*n*)	5.0% (41)	0.3% (3)	0.8% (7)	2.7% (22)
HbA1c < 7%, %, (*n*)	50.4% (446)	1.4% (12)	2.7% (24)	35.0% (310)
BP < 130/80 mmHg, %, (*n*)	27.5% (243)	0.8% (7)	2.4% (21)	20.3% (180)
LDL-C < 55 mg/dL + HbA1c < 7%, %, (*n*)	2.3% (20)	0.2% (2)	0.5% (4)	1.2% (11)
HbA1c < 7% + BP < 130/80 mmHg, %, (*n*)	15.5% (137)	0.5% (4)	0.6% (5)	11.3% (100)
LDL-C < 55 mg/dL+ BP < 130/80 mmHg, %, (*n*)	1.5% (13)	0.1% (1)	0.3% (3)	0.6% (5)
LDL-C < 55 mg/dL + HbA1c < 7% + BP < 130/80 mmHg, %, (*n*)	0.7% (6)	0.1% (1)	0.1% (1)	0.2% (2)
Treatment Target for Patients with High CV Risk Category (n = 10)				
LDL-C < 70 mg/dL, %, (n)	0.2% (2)	0	0	0
HbA1c < 7%, %, (n)	0.9% (8)	0	0	0.5% (4)
BP < 130/80 mmHg, %, (n)	0.7% (6)	0	0	0.5% (4)
LDL-C < 70 mg/dL + HbA1c< 7%, %, (n)	0.2% (2)	0	0	0
HbA1c < 7% + BP < 130/80 mmHg, %, (n)	0.6% (5)	0	0	0.3% (3)
LDL-C < 70 mg/dL+ BP <130/80 mmHg, %, (n)	0.2% (2)	0	0	0
LDL-C < 70 mg/dL + HbA1c < 7% + BP < 130/80 mmHg, %, (n)	0.2% (2)	0	0	0
Treatment Target for Patients with Moderate CV Risk Category (n = 54)				

	Patients Achieving Target	Patients with SGLT2i Prescription	Patients with GLP-1 RAs Prescription	Patients with Statin Prescription
LDL-C < 100 mg/dL, %, (n)	1.6% (14)	0.1% (1)	0% (0)	0.7% (6)
HbA1c < 7%, %, (n)	3.6% (32)	0	0.1% (1)	1.9% (17)
BP < 130/80 mmHg, %, (n)	2.0% (18)	0	0.2% (2)	1.0% (9)
LDL-C < 100 mg/dL + HbA1c < 7%, %, (n)	0.8% (7)	0	0	0.3% (3)
HbA1c < 7% + BP < 130/80 mmHg, %, (n)	1.2% (11)	0)	0.1% (1)	0.6% (5)
LDL-C < 100 mg/dL+ BP < 130/80 mmHg, %, (n)	0.8% (7)	0	0	0.3% (3)
LDL-C < 100 mg/dL + HbA1c < 7% + BP < 130/80 mmHg, %, (n)	0.6% (5)	0	0	0.2% (2)

GLP-1 RAs – glucagon like peptide 1 receptor agonist; SGLT2i – sodium-glucose cotransporter-2 inhibitors ;CV – cardiovascular; BP –blood pressure; LDL-C – low density lipoprotein cholesterol.

SAF, CV RISK AND DM COMPLICATIONS, NEPHRO AND CAN RISK SCORES IN THE STUDY COHORT

The mean SAF level (2.61 versus 2.22; p=0.003) was significantly higher in patients with very high CV risk, while the mean NERPHRORS was significantly higher in patients with moderate CV risk (70.11 versus 63.30; p=0.05); and the mean CANRS (34.90 versus 27.50; p=0.001) was significantly higher in patients with high CV risk.

In the univariate analysis, several significant correlations were observed:

- SAF demonstrated a positive, moderate and statistically significant correlation with age, Pearson coefficient of 0.294, p<0.001.
- CANRS was also found to be positive, weakly and significantly correlated with SAF, with a Pearson coefficient of 0.136 and a p <0.001.
- Moreover, a positive, very weak but statistically significant correlation was observed between SAF and HbA1c, Pearson's rho value of 0.091 and a p=0.007 Additionally, SAF displayed a negative, moderate and statistically significant correlation with NEPHRORS, Pearson coefficient of -0.230, p <0.001.

Moreover, after adjusting for age and eGFR in the multivariate regression model, HbA1c values proved to be correlated with SAF levels. Specifically, for each increase of 1 SD in HbA1c value, there was an observed increase of 0.105 SDs in SAF levels (Nagelkerke R^2=0.110; p<0.001).

The mean SAF level was significantly higher in individuals with an HbA1c >7% (2.65±0.53) compared to those with an HbA1c <7% (2.56±0.53), p=0.018. Additionally, SAF levels and the duration of DM were found to be independent (r= -0.028, p=0.401).

When assessing SAF levels among various groups of DM complications - including DSPN, DR, CKD, and albuminuria - we found no significant differences, except in respect to the presence of CKD (2.76 vs. 2.58; p=0.003).

In determining the cut-off value for SAF levels to predict the presence of a very high CV risk (2.35), we achieved a sensitivity of 67.7% and a specificity of 56.2%. The area under the curve (AUC) value was 0.634 (95% CI: 0.560-0.709), p=0.001.

In the subgroup analysis, significant differences were only evident in age and HbA1c levels when compared to the SAF cut-off level of 2.35, p=0.001. No noteworthy differences were observed concerning gender, duration of DM, and DM complications.

Logistic regression models examining predictors of SAF>2.35 have revealed a notable correlation between elevated SAF levels and an increased CV risk, especially following adjustments for age, gender, and HbA1c levels.

Age demonstrated a significant association with increased risk (OR: 1.072; 95% CI: 1.048-1.096; p=0.001), indicating that older patients are more likely to be classified into the very high CV risk group with elevated SAF levels.

Additionally, gender emerged as a significant factor (OR: 1.426; CI: 1.057-1.923; p=0.02), alongside higher HbA1c levels, which further heightened this risk (OR: 1.171; CI: 1.039-1.321; p=0.01).

Between DM complications and SAF levels, the associations are β=0.09, p=0.004 for DSPN, β=0.08, p=0.533 for DR, β=0.01, p=0.299 for CKD. Furthermore, between antidiabetic treatment and SAF levels, correlations are β=0.07, p=0.015 for insulin, β=-0.022 p=0.323 for metformin, β=0.072, p=0.007 for GLP-1 RAs, β=0.014, p=0.258 for iSGLT2 and β=0.007, p=0.751 for iDPP4. For other treatment and SAF levels, correlations are β=0.083, p=0.01 for SRAA, β=0.025 p=0.458 for beta-blockers, β=0.081, p=0.009 for calcium channel blockers, β=0.04, p=0.205 for statin. For eGFR and microalbuminuria, the correlation with SAF levels are β=-0.127, p<0.001 and β=0.067, p=0.017, respectively.

Multiple linear regression in Model 6 highlighted that 12.1% of the SAF score can be explained by gender, patient age, DM duration, BMI, HbA1c, and eGFR (R_adjusted =0.121; p=0.001): YAGE =1.611 -0.134 sex +0.018 Age +0.003 DM duration +0.001 BMI +0.045 HbA1c -0.003 eGFR.

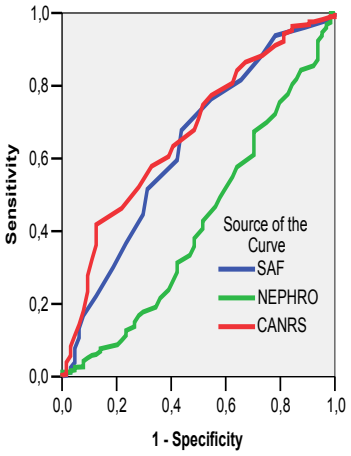
For the NEPHRO score, applying multiple linear regression in Model 2 revealed that only gender and age significantly explain 33% of the score (R_adjusted = 0.330; p=0.001): YAGE = 134.729 –1.092 sex –1.073 Age.

Multiple linear regression in Model 4 demonstrated that 38.6% of the CANSC score can be explained by gender, patient age, DM duration, and BMI (R_adjusted = 0.386; p=0.001): YAGE = 21.942 +0.738 sex +0.377 Age +0.004 DM duration +0.946 BMI.

ROC curve analysis confirms that age, BMI, and WC are significant predictors of very high CV risk. Specifically, age with an AUC of 0.668 (95% CI 0.589-0.748; p<0.001), BMI with an AUC of 0.631 (95% CI 0.556-0.706; p<0.001), and waist circumference with an AUC of 0.613 (95% CI 0.542-0.684; p=0.003) demonstrate good predictive abilities.

Some biological parameters, including HbA1c and eGFR, displayed AUC values slightly above 0.500, indicating a moderate predictive ability for very high CV risk. LDL cholesterol, with an AUC of 0.380 and a statistically significant p-value, along with SBP, just under the threshold (AUC<0.600) with an AUC of 0.599, suggest potential as relevant factors in CV risk assessment.

The ROC curve analysis confirms SAF (AUC=0.634; 95% CI 0.560-0.709; p=0.001) as a reliable predictor of very high CV risk. When using a cut-off of 2.35 for the SAF score, the sensitivity is 67.7% and the specificity is 56.2%. Similarly, the CANRS (AUC=0.671; 95% CI 0.604-0.738; p=0.001) is a strong predictor. With a cut-off of 27.5, the CANRS score reveals a sensitivity of 77.3% and a specificity of 45.3%, establishing it as a valuable tool for cardiovascular risk assessment in high-risk patients (Figure 2).



Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
SAF	,634	,038	,000	,560	,709
NEPHRO	,423	,039	,039	,346	,500
CANRS	,671	,034	,000	,604	,738

The test result variable(s): SAF, NEPHRO, CANRS has at least one tie between the positive actua state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figure 2. ROC Curve with Sensitivity/Specificity Balance of Analyzed Scores as Predictors of Very High Cardiovascular Risk

Logistic regression models emphasized for SAF>2.35 had a 1.43-time higher probability for very high CV risk patients depending on older age p=0.001, female gender, p=0.02 and a higher HbA1c level, p=0.01.

In the logistic regression models, SAF levels greater than 2.35 were significantly associated with very high CV risk in T2DM patients, as evidenced by the odds ratios (ORs) for age at 1.072 (95% CI: 1.048-1.096, p=0.001), gender at 1.426 (95% CI: 1.057-1.923, p=0.020), and elevated HbA1c levels at 1.171 (95% CI: 1.039-1.321, p=0.010), confirming these factors as predictors for CV risk assessment.

DISCUSSIONS

Demographic Data and Diabetes Metrics

Excess weight increases the risk of developing T2DM threefold (27,28). Despite the modifiable nature of this risk factor, our cohort showed high obesity prevalence, with females constituting nearly two-thirds of those affected and 60% of overweight patients being > 65 years of age. Moreover, those with obesity had the longest average duration of T2DM. A steady, albeit moderate, positive correlation was observed between HbA1c levels and anthropometric measures, with a significant proportion of patients displaying increased body mass and adiposity corresponding to elevated HbA1c values. Similar relationships have also been reported by Kotwas et al. and Ye et al.(29,30). Patients with HbA1c levels above 7% typically had a significantly longer disease duration than those with HbA1c levels below 7%. Kawa

Skin autofluorescence, NEPHRO and CAN Risk Scores in the Cohort

The aging process is linked to AGEs accumulation (31,32). Additionally, metabolic syndrome, CV disease, CKD, and neurodegenerative disorders can further raise AGE levels due to impaired glucose metabolism and reduced clearance, leading to oxidative stress and inflammation (18,19,33–36). Our study found that SAF levels rose with age, especially in males compared to females. Using the Sudoscan® device, we evaluated nephropathy and CAN risk scores. The NEPHRO score declined with age, reflecting the natural renal function decline (37), and was notably higher in males, indicating gender differences. Similarly, CANRS increased with age, with females showing significantly higher mean scores than males, highlighting gender disparities.

In assessing feet ESC, most patients had values above 60 µS, indicating normal sudomotor function. Despite nearly 70% of the cohort having DSPN, there was no significant correlation between ESC levels and polyneuropathy. This contradicts with previous evidence supporting Sudoscan®'s effectiveness in detecting early neuropathic signs (38). Similar studies attributed the lack of significant findings to short diabetes duration or well-controlled HbA1c levels (39). Another study suggested 54 µS as the optimal threshold for detecting neuropathy using feet ESC (40). Given our cohort's average ESC of 80 µS, this may explain the absence of significant correlations.

Analyzing Achievement of Cardiovascular Risk Management Goals in the Study Cohort

We used the 2019 ESC/EAS guidelines (23) to classify CV risk categories in our study cohort and examined the attainment of therapeutic goals and prescription patterns.

The majority of our cohort was included in the very high CV risk group, similar to findings in the Santorini study where over 90% fell into this category (41). Significant demographic variations were observed in the CV risk groups, with a higher percentage of females and older individuals in the very high-risk category, highlighting the necessity for tailored risk management approaches for these groups. Anthropometric measurements like BMI, waist, and hip circumference indicate differences in CV risk groups, emphasizing their importance in risk assessment. Longer T2DM durations associate with higher CV risk, suggesting more intensive intervention for longer durations. Variations in lipid profiles, especially LDL and total cholesterol, were noted, with lower levels in the highest CV risk category. Only a small percentage (5.0%) of those in the highest CV risk category managed to achieve their LDL-C target of < 55 mg/dl. Despite this, the majority of patients were able to meet their HbA1c (50.6%) and BP (31.8%) goals. Very few patients (0.7%) were able to meet all three objectives simultaneously. Furthermore, among those who did reach all three targets, the use of new antidiabetic drugs such as SGLT2 inhibitors and GLP-1 receptor agonists was limited. Our results are consistent with other studies, such as the Da Vinci study, which had a 4% success rate in achieving the 2019 ESC/EAS LDL-C goal for the same CV risk category. The Da Vinci study suggested that secondary prevention patients may have lower LDL-C levels, indicating under-assessment of CV risk and clinical inertia in treating primary prevention patients (42). In our study, over two-thirds of patients with very high CV risk were treated with statins, exceeding the proportion of similar patients treated in the Santorini study. A study by Ray et al., involving high- and very high-risk European patients, revealed that over 70% failed to achieve their 2019 ESC/EAS LDL-C risk-based target (41). This highlights areas for improvement, as the AACE recommends a target of less than 55 mg/dL since 2017 (43). Several other studies have found poor achievement rates for lipid targets, despite existing guidelines (41,42,44,45). Recent research suggests that reducing LDL-C levels by 40 mg through statin use can result in a 10% decrease in overall mortality and a one-fifth reduction in major cardiovascular events (46,47). This calls for better management and increased awareness to improve lipid target achievement rates in high-risk populations. In our study we observed a tendency to prescribe antidiabetic agents with cardio-renal protection more often to patients classified as very high risk. Our data also showed that 3.9% of the entire population used SGLT2 inhibitors, 8.1% used GLP-1 RAs, and 13% used DPP-4i. The limited use of GLP-1 RAs and SGLT2is in our group may be due to specific local policies in Romania in 2019. National prescription protocols required in 2019 that these newer agents be prescribed only when HbA1c exceeded 7% and there were restrictions on drug combinations and their administration sequence. In our study, HbA1c values were below 7.1% for the high CV risk group, and below 7% for the high and moderate-risk groups (48).

Skin autofluorescence, Cardiovascular risk, and Diabetic Complications in the Examined Group SAF and Cardiovascular Implications

In our study, we aimed to test the hypothesis that SAF levels would be higher in T2DM individuals in higher CV risk categories, as supported by previous research on SAF as a predictive tool for CV risk (49–51). Our goal was to establish a cut-off value for our demographics.

In our cohort, patients with very high CV risk had the highest average SAF measurements (2.61 AU vs. 2.22 AU vs. 2.44 AU; p=0.003), confirming the link between higher SAF measurements and increased CV risk. This aligns with prior studies that suggest SAF, reflecting AGEs, contributes to advancing CV disease in individuals with T2DM (52). Notably, a SAF measurement above 2.35 in our high-risk group predicted CV risk with 68% sensitivity and 56% specificity (AUC=0.634; 95% CI between 0.560-0.709; p=0.001). Previous studies have also supported the use of SAF to identify patients needing immediate or specific interventions to mitigate CVD risk. Research spanning over 4.5 years found that patients with past CVEs had higher SAF levels, linked to fewer years lived without further CVEs and lower complication-free survival stating that SAF can identify highly vulnerable patients early as it remained significant after adjusting traditional risk factors (53). The Rotterdam study showed strong correlations between subclinical CVD and SAF values, particularly in individuals with diabetes (54). Alkhami et al. found that patients with SAF levels above the median value for their cohort had a higher rate of revascularizations, even after adjusting for other risk factors, suggesting that SAF could be used to improve screening and risk assessment processes for T2DM patients, potentially leading to life-saving interventions (55). In the Lifelines Cohort Study, including 2,349 T2DM individuals, the authors examined SAF's predictive value for CVD and mortality. Recently diagnosed T2DM individuals had lower SAF values than those with a long-standing diagnosis, suggesting that high blood sugar levels over time elevate SAF measurements in more chronic cases. Over 3.7 years, higher SAF measurements were significantly linked to a nearly threefold increased risk of CVD or death (56). This underscores SAF's significance as an independent predictor of cardiovascular events and mortality in T2DM patients, emphasizing its importance in effectively monitoring and managing these risks over time.

Our study's logistic regression models show a strong relationship between high SAF levels and increased CV risk category, especially after adjusting for age, gender, and HbA1c levels. This finding aligns with previous research, demonstrating SAF's value as a CV risk marker in individuals with T2DM (52). Older age was a significant predictor, with individuals over 60 more likely to have higher SAF levels and be in the very high CV risk category (OR=1.072, $p < 0.05$). Gender also plays a role, with females being more likely to have

elevated SAF levels, potentially due to biological or behavioral factors that influence CV risk differently for men and women. Furthermore, poorer glycemic control may significantly contribute to increased CV risk. SAF can effectively identify T2DM patients at high risk for CV events, helping clinicians customize interventions for preventive care. Further research is needed to investigate gender differences in SAF levels and how to clinically address them.

SAF and diabetes complications, NEPHRO and CAN Risk Scores

In the initial analyses, SAF levels were significantly linked to CKD, but this association disappeared in multivariate models that controlled for other variables, showing no independent link between CKD and SAF. However, as significant associations were observed with DSPN (positive), eGFR (negative), and microalbuminuria (positive), this suggests that SAF's remains a valuable marker for assessing renal function (via eGFR and microalbuminuria links) and neuropathy risk (DSPN). Insulin and GLP-1 receptor agonists correlated positively with SAF levels, possibly because they are prescribed later in diabetes progression, after prolonged poor glycemic control, thus higher SAF levels. Conversely, treatments like metformin, iSGLT2, and iDPP4 inhibitors showed no significant association.

SAF's non-invasive tool potential for microvascular complications screening has been explored in Hosseini et al.'s meta-analysis which sustained SAF's effectiveness in predicting both micro and macrovascular complications in diabetes, despite variations among studies (57). Other studies have shown the utility of SAF in diagnosing various diabetic neuropathic conditions, with moderate to low specificity. It is suggested to use SAF in conjunction with other tests for increased accuracy. A DSPN cutoff of ≥2.95, higher than our cohort's mean SAF score of 2.6±0.5, indicates that SAF levels and screening thresholds may vary based on patient demographics and disease duration (58). A study by Borderie et al. analyzed T2DM patients admitted to the hospital between 2009 and 2017 and found a significant association between higher SAF levels and the development of diabetic foot ulcers (DFUs). SAF demonstrated its potential as a non-invasive predictor of foot ulcer risk (cutoff of 2.65 AU), even after accounting for other risk factors and complications (59). In contrast, our study's T2DM patients had better glycemic control, fewer chronic diabetes complications, and were of younger age. The differences between the two studies suggest that our cohort may be at an earlier stage of diabetes progression, which could influence the outcomes and applicability of our findings.

We did not find a significant correlation between SAF levels and DR in our study, possibly due to the low prevalence of DR and satisfactory metabolic control in our cohort (HbA1c=7.1%). However, other studies have consistently demonstrated a connection between elevated AGEs levels and DR, and SAF has been validated as an independent

predictor of proliferative DR. Although not substituting for eye fundus exams, SAF could be a valuable initial screening tool for DR, especially in resource-limited settings (60–67).

Diabetes-related CKD progression can be significantly impacted by AGEs (68). Our study found a strong correlation between increased SAF levels and decreased eGFR as well as increased microalbuminuria. This suggests that SAF could serve as an early indicator of renal damage in diabetic patients. By incorporating SAF monitoring into traditional renal function assessments, healthcare providers can gain deeper insights into kidney health and make more informed treatment decisions. Early detection and management of CKD in T2DM patients may be facilitated, potentially slowing disease progression. SAF independently predicts renal insufficiency and macroangiopathy in T2DM patients (69). It can forecast the risk of end-stage kidney disease and significant eGFR decline over 1.8 years, with approximately 38.7% of its effect being independent of traditional markers like eGFR and UACR as per Jin et al. (70).

Our multiple linear regression analysis revealed that age and HbA1c significantly impact SAF, explaining 12.1% of its variability; each additional year of age and each percentage increase in HbA1c raise SAF by 0.02 and 0.05 units, respectively. Age and gender account for 33% of the variability in NEPHRO scores, decreasing by about 1.07 units per year. The CANRS, affected by gender, age, diabetes duration, and BMI, accounts for 38.6% of cardiovascular risk variability, with each BMI unit increasing CANRS by nearly 1 unit. These insights can help clinicians tailor management strategies for diabetic patients based on specific risk profiles.

In our study, we observed a significant correlation between CANRS and SAF, indicating a potential link between higher SAF levels and the risk of CAN, consistent with findings by Papachristou et al. (71). The authors found higher AGEs in T2DM subjects with CAN, assessed using cardiovascular reflex tests (CARTs), with AGEs rising with abnormalities in CARTs. This approach, though different from ours, underscores the need for further research and demonstrates how various diagnostic tools can reflect AGEs' impact on autonomic function in diabetes.

Strengths and Limitations:

This study, while comprehensive, was conducted at a single center, which could limit the applicability of its findings to broader populations. As a cross-sectional analysis, it provides a snapshot of a specific point in time, restricting the ability to infer temporal sequences or causality from the observed associations. However, it effectively captured the current status of a representative subset of Romanian T2DM patients, with a cohort size of 885 patients being a major strength that enhances statistical power and allows for a detailed characterization of the group. This cohort has provided valuable insights into cardiovascular

risk categorization, diabetes-related complications, medication prescriptions, and their relationship with SAF levels. Importantly, the cohort's achievement of blood pressure and metabolic management targets provides valuable information and has the potential to guide appropriate directions for interventions aimed at optimizing the care of individuals with T2DM in Romania.

To assess AGE accumulation, we utilized SAF, which has limitations. Devices using autofluorescence might miss non-fluorescent AGEs and could be influenced by substances with natural fluorescence, potentially impacting the results. Factors like skin pigmentation, product use, and blood flow variations can compromise SAF's accuracy. Despite these limitations, SAF remains a valuable, non-invasive, reproductible, and cost-effective tool in research. However, inadequate patient reporting hindered our study's documentation of risk factors such as smoking, which should be included in future research to better assess SAF's predictive accuracy for cardiovascular complications.

Novelty and future directions:

This study is the first known to establish a SAF threshold correlated with cardiovascular risk and diabetes complications in Romanian T2DM patients, filling a gap for specific demographic cut-offs as highlighted by Stirban et al. (72). It's also the first study to describe the link between SAF with the CAN risk score using the Sudoscan device, notable because only one other study has shown a similar correlation albeit through different methods. Our research aims to longitudinally expand and include more participants to further validate SAF's utility in estimating CVD risk and managing complications within our demographic. SAF's non-invasive, accessible nature holds significant potential for aiding clinicians in identifying high-risk patients and optimizing treatment strategies. Future directions include a longitudinal follow-up to monitor micro- and macrovascular complications and evaluate CAN and NEPHRO scores over time, enabling targeted interventions tailored to the Romanian T2DM population, thus improving disease understanding and management.

FINAL CONCLUSIONS

- The vast majority of the study group (93%) were classified as having very high CV risk, indicating a need for targeted risk management strategies, particularly for females and older individuals.

- The highest average SAF measurements were found in very high CV risk patients, indicating its use as a predictive tool for CV risk. A SAF value above 2.35 predicted very high CV risk, establishing a cut-off for our demographic.

- Real-world scenarios pose challenges in achieving diabetes treatment targets, especially for lipid levels. Only 5% of patients in the very high CV risk category met LDL-C targets, in stark contrast to the higher attainment rates for HbA1c (50.4%) and blood pressure (27.5%) in the same risk category.

- Additionally, there is a substantial need to enhance the utilization of diabetes treatments that provide additional cardio-renal benefits, especially among high or very high CV risk patients. The usage of newer antidiabetic drug classes, such as SGLT2 inhibitors (3.9%) and GLP-1 receptor agonists (8.1%), were notably reduced.

- In the analyzed cohort, we found a high prevalence of obesity at 64.6%, particularly among females and individuals over 65 years old, and this was linked to longer durations of T2DM.

- Despite a high prevalence (70%) of DSPN in the cohort, most patients (96%) exhibited normal sudomotor function, with feet ESC values above 60 μS. However, there were no significant associations observed between ESC levels and polyneuropathy diagnosed using clinical methods in our cohort.

- Significant associations between SAF levels and DSPN, as well as eGFR and microalbuminuria, underscore SAF's potential as a non-invasive predictive tool for monitoring neuropathy and kidney health in diabetic patients.