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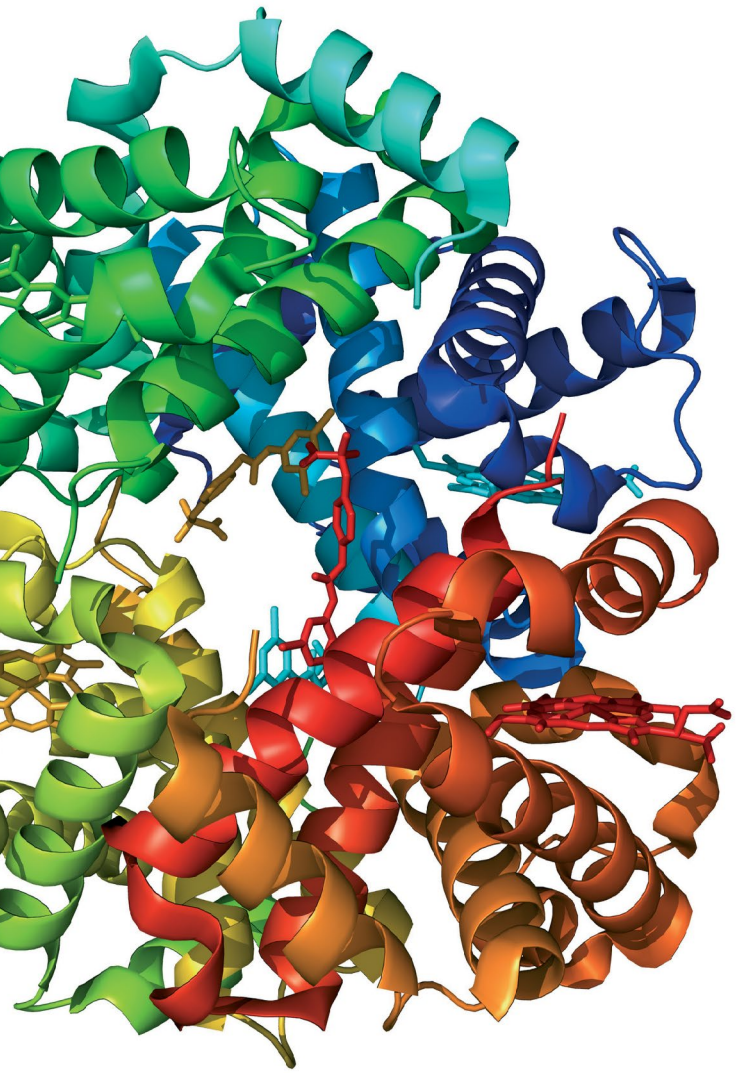

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Anna Adamska, Stanisław Piłaciński, Dorota Zozulińska-Ziółkiewicz, Agnieszka Gandecka, Agata Grzelka, Aneta Konwerska, Agnieszka Malińska, Michał Nowicki, Aleksandra Araszkiewicz

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Disturbances in angiogenesis and vascular maturation in the skin are associated with diabetic kidney disease in type 1 diabetes

ABSTRACT

Introduction. The skin, as one of the most accessible tissues, is frequently used for investigations of microcirculation and angiogenesis. The aim of this study was to assess the relationship between the dermal microvessel density (MVD) and maturity and the presence of diabetic kidney disease (DKD) in adults with type 1 diabetes (T1D). Skin as the most accessible organ served as a model for the study of angiogenesis.

Materials and methods. 148 consecutive T1D patients (87 men), median age of 41 [interquartile range (IQR): 31–49] years and diabetes duration of 21 (17–30) years, participated in the study. The patients were under the care of the Department of Internal Medicine and Diabetology, Poznan University of Medical Sciences. Diabetic kidney disease was diagnosed in patients with increased albuminuria and at least 10-year duration of diabetes or evidence of diabetic retinopathy. The skin biopsy was performed on distal part of lower leg, using a sterile, disposable 3 mm biopsy punch with plunger (Disposable Biopsy Punches, Integra™ Miltex®). In the immunohistochemical analyses, we used: anti-CD133, anti-CD34, anti-CD31, and anti-von Willebrand factor (vWF) autoantibodies. Microvessel density measurement in all specimens was performed using "hot spots technique". Slides were scanned using the MiraxMidi scanner (Carl Zeiss) and were viewed using CaseViewer (3DHISTECH Ltd. Budapest, Hungary). Data were analyzed using Statistica v. 13 software.

Results. In the study group 21 patients with diagnosis DKD+, as compared to 127 subjects withaout DKD–, had longer duration of diabetes [30 (IQR: 21–36) *vs.* 21 (16–28) years, $p = 0.002$], higher prevalence of hypertension [14 (67%) *vs.* 37 (29%), $p = 0.002$], lower estimated glomerular filtration rate (eGFR) [66 (55–88) *vs.* 94 (83–106) mL/min/1.73 m^2, $p < 0.001$]. Median MVD compared between groups with and without DKD, was similar for CD34+ vessels/1 mm^2 [123 (100–170) *vs.* 121 (100–170), $p = 0.775$], CD133+ vessels/1 mm^2 [79 (50–100) *vs.* 79 (63–93), $p = 0.823$], and for CD31+ vessels/1 mm^2 [29 (21–46) *vs.* 38 (17–58), $p = 0.454$]. Median MVD vWF+ vessels/1 mm^2 was lower in the group with than without DKD: 42 (25–54) *vs.* 54 (43–71), $p = 0.009$. The values given above were calculated for both layers of the dermis (papillary and reticular dermis). In multivariate logistic regression analysis presence of diabetic kidney disease was associated with lower median vWF+ MVD [odds ratio: 0.97 (95% confidence interval: 0.95–0.99), $p = 0.017$], with adjustment for age, gender, eGFR value, diabetes duration and presence of hypertension. MVD did not differ significantly between chronic kidney disease stages.

Conclusion. In patients with type 1 diabetes and diabetic kidney disease the disturbances in the angiogenesis and vascular maturation are present. The number of mature blood vessels (vWF+) in the skin is reduced. Disturbances in the angiogenesis occur at early stages of diabetic kidney disease. (Clin Diabetol 2019; 8, 5: 231–237)

Key words: type 1 diabetes, diabetes complications, diabetic kidney disease, microcirculation; microvessel density (MVD), von Willebrand factor (vWF)

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Introduction

Diabetic kidney disease (DKD) remains the most frequent cause of end-stage renal failure, despite progress in the treatment of diabetes. Traditionally, diagnosis of DKD is based on the presence of albuminuria and either at least 10-year duration of diabetes or evidence of retinopathy [1]. Hyperglycemia and glycemic fluctuations, hypertension, dyslipidemia, and smoking are modifiable and genetics, sex, age, age at onset, and duration of diabetes are non-modifiable risk factors of DKD [2]. According to previous studies, in some people with DKD unpredictable progressive increase of albuminuria and declining glomerular filtration rate (GFR) has been observed [3]. Due to small and insufficient number of early markers of diabetic nephropathy, the aim of this study was to search for new prognostic markers of renal injury [4].

Endothelial cells (ECs) are constantly subjected to mechanical damage and chronic exposures to destructive factors, that lead to characteristic cell changes (morphology) and death (apoptosis) [5]. Terminally differentiated, mature ECs are characterized by a low proliferative potential, so endothelial progenitor cells (EPCs) derived from the bone marrow are involved in the creation and repair of blood vessels [6]. During new vessel formation, characteristic antigens are expressed on the surface of the endothelium, while some other antigens disappear. EPCs have properties of embryonal angioblast and are characterized by expression of CD133 (cluster of differentiation 133) and CD34 (cluster of differentiation 34) (late EPCs) [7]. ECs are characterized by an expression of von Willebrand factor (vWF), CD31, CD34, but do not express immature markers as CD133 [8]. In our study we used immunohistochemical markers [CD133, CD34, CD31 (cluster of differentiation 31) and vWF] to determine the morphological changes observed in dermal microangiopathy in diabetic patients.

The skin, as the most accessible organ, served as a model for the study of microcirculation [9, 10]. The objective of this study was to assess the dermal microvessel density (MVD) and maturity in relation to the presence of diabetic kidney disease in adults with type 1 diabetes (T1D).

Materials and methods

Patients

The study group consisted of 148 (87 men) consecutive patients with type 1 diabetes, median age (IQR) of 41 (31–49) years and diabetes duration of 21 (17–30) years. The patients were under the care of the Department of Internal Medicine and Diabetology, Poznan University of Medical Sciences.

Inclusion criteria were: age ≥ 18 years, type 1 diabetes of at least 10-year duration, written informed consent of the patient to participate in the study. Exclusion criteria were: activated partial thromboplastin time (APTT) > 37 s, international normalized ratio (INR) > 1.1, platelet count < 100 G/mm^3, anticoagulant or antiplatelet treatment, skin disorders.

The research protocol was approved by a local Bioethics Committee (No. 1064/15). The study was carried out in accordance with the World Medical Association Declaration of Helsinki.

Data collection procedures

All patients participating in the study completed the questionnaire containing demographic data, duration of diabetes and method of treatment, comorbidities, medication use and smoking-related data. Then, anthropometric measurements (body mass, height, waist and hip circumference, body mass index (BMI) = weight (kg)/squared height (m^2) and blood pressure measurement (twice using a sphygmomanometer in a sitting position after 10 minutes of rest) were performed.

Laboratory tests

Blood samples (10 milliliters) were taken after 10 hours of fasting, after a period of rest, with minimum occlusion of the vein using an S-Monovette blood collection system. The serum concentrations of creatinine, total cholesterol, high-density lipoproteins (HDL) cholesterol, low-density lipoproteins (LDL) cholesterol, and triglycerides (TG) were measured using standard methods. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) study equation. Glycated hemoglobin A_{1c} (HbA_{1c}) was measured in venous blood with competitive turbidimetric inhibition immunoassay method on Cobas analyzer (Roche Diagnostics, Basel, Switzerland) and expressed in % and IFCC (the International Federation of Clinical Chemistry and Laboratory Medicine) units (mmol/mol), the values were calibrated with respect to Diabetes Control and Complication Trial (DCCT)/National Glycohemoglobin Standardization Program (NGSP).

The urinary albumin excretion was assessed based on a 12 h urine collection, with the simultaneous determination of the albumin/creatinine ratio in the morning urine. Urinary albumin excretion from 30 to 300 mg per day in two of the three urine collections and albumin/creatinine ratio > 30 mg/g in the morning urine sample was considered to be increased albuminuria. Diabetic kidney disease was diagnosed in people with increased albuminuria and a 10-year

duration of diabetes or shorter duration of the disease with coexistence of retinopathy [1]. DKD was divided into stages based on the result of the eGFR: stage 1 (eGFR ≥ 90 mL/min/1.73 m^2), stage 2 (eGFR 60–89 mL/ /min/1.73 m^2), stage 3 (eGFR 30–59 mL/min/1.73 m^2). In the investigated group there were no patients with more advanced chronic kidney disease (CKD) stages.

Biopsy procedure

The skin biopsy was taken on the distal part of the lower limb (10 cm above the lateral malleolus) after the skin was anesthetized with 2% lidocaine injections in sterile conditions. We have used a sterile, disposable 3 mm biopsy punch with a plunger (Disposable Biopsy Punches, Integra™ Miltex®). The biopsy was minimally invasive and did not require suturing. The excised tissues were fixed in Bouin solution for 24 hours at room temperature, and then embedded in paraffin blocks.

Immunohistochemistry

Paraffin-embedded tissue blocks were cut into 3- to 4-μm-thick sections on a semi-automatic rotary microtome (Leica RM 2145, Leica Microsystems, Nussloch, Germany). All of the immunohistochemical (IHC) analyses employed the StreptABComplex/HRP method modified by the use of biotinylated tyramine (Dako Catalyzed Signal Amplification System, Peroxidase, K1500, DakoCytomation A/S, Glostrup, Denmark). The endogenous peroxidase activity was blocked with 10% hydrogen peroxide. The staining IHC protocol included the following steps: 1) preincubation with the appropriate normal goat serum in phosphate buffered saline for 30 minutes at room temperature, 2) incubation with the specific primary antibody overnight at 4°C in a hybridization chamber, 3) incubation with the secondary antibody for 60 minutes at room temperature, and finally 4) antigen-antibody complexes staining using 0.5% 3-3' diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO).

All of the sections from blood vessels samples from an individual patient were processed in the same IHC experiment. The specific primary antibodies were:

- anti-CD34 (Dako, Glostrup, Denmark; code M7165, diluted 1:30);
- anti-CD133 (Novus Biologicals, Littleton, CO, USA; code NB300–266, diluted 1:3000);
- anti-CD31 (Dako, Copenhagen, Denmark; code M0823, diluted 1:20);
- anti-vWF (Dako; code M0616, diluted 1:30).

All tissue sections were analyzed under an AxioImager Z.1 light microscope and selected pictures were taken with an attached AxioCam MRc5 digital camera (Carl Zeiss). The negative controls consisted of specimens incubated with non-immune IgG1 (X-0931, Dako, Gdynia, Poland) and sections for which the primary or secondary antibody was omitted.

Morphometric analyses

Microvessel density (MVD), defined as the mean number of blood vessels presented in 1 mm^2 of analyzed tissue, was calculated using the "hot spots technique". The histological preparation was viewed under a small magnification (20 ×) by selecting three areas with the highest number of blood vessels. Then, the vessel sections were counted under a magnification of 40× in a selected area. The arithmetic mean of the three "hot spots" was calculated for microvessel number and subsequently calculated to 1 mm^2. This procedure was applied separately for CD133, CD34, CD31, and vWF. Slides were scanned using the MiraxMidi scanner (Carl Zeiss) and were viewed using CaseViewer (3DHISTECH Ltd. Budapest, Hungary). All of the analyses were evaluated independently by two scientists on coded samples that included positive and negative controls.

Statistical analysis

Data were analyzed using Statistica v. 13 (StatSoft Inc., Tulsa, OK, USA), MedCalc v. 18.5 (MedCalc Software bvba, Ostend, Belgium). Patients with diagnosis of DKD (DKD+) and without DKD (DKD–) were compared using Mann-Whitney U test or Fisher exact test, as appropriate. Descriptive statistics and results of comparatory analyses are expressed as medians and IQR or numbers and percent. Kruskal-Wallis ANOVA was used to compare vWF+ MVD between stages of CKD (1, 2 and 3a). Multiple logistic regression was used to check the association between presence of CKD and vWF+ MVD, with adjustment for potential confounders (age, gender, diabetes duration and presence of hypertension). P value less than 0.05 was considered statistically significant.

Results

The study group there were 21 T1D patients (12 men) with DKD, median (IQR) age 44 (32–58) and 127 (75 men) individuals with T1D without DKD, median age 40 (30–49) years.

DKD+ patients as compared to DKD– subjects had longer duration of diabetes [30 (21–36) *vs.* 21 (16–28) years, $p = 0.002$], more often had hypertension [14 (67%) *vs.* 37 (29%), $p = 0.002$ and lower eGFR [66.1 (54.7–87.0) *vs.* 93.5 (82.5–106.2) mL/min/1.73 m^2, $p < 0.001$].

The median MVD determined by CD34 blood vessels per 1 mm^2 dermal biopsies was 123 (100–170) in DKD+ group and 121 (100–154), $p = 0.775$ in

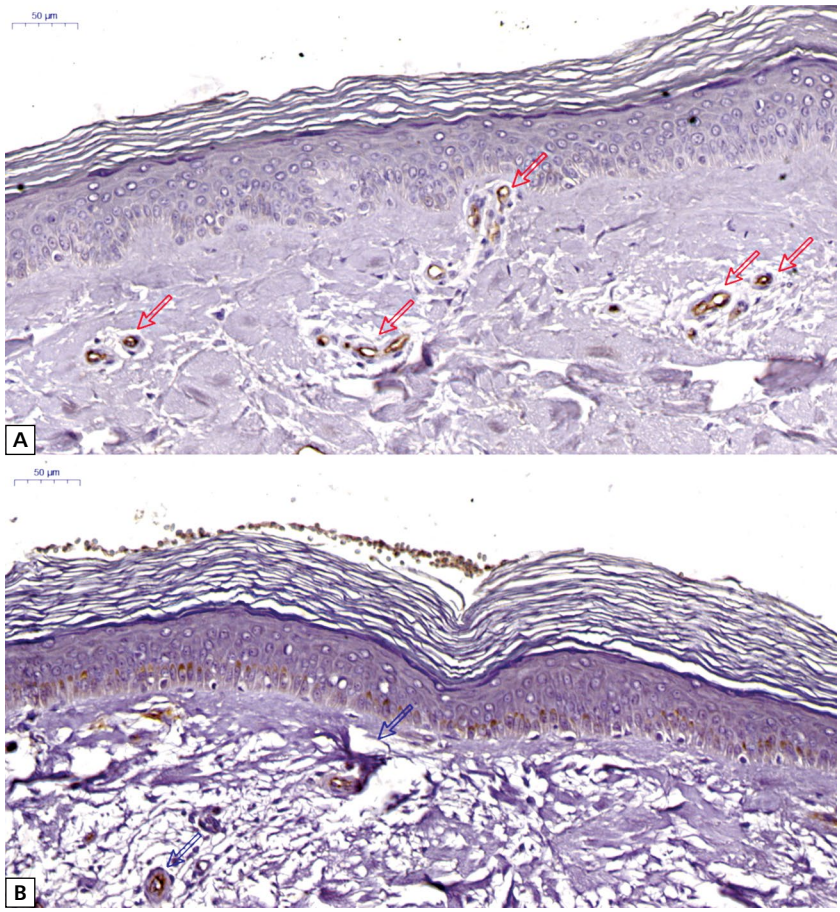

Figure 1A, B. Demonstration of microvessels density (MVD) in skin biopsies of adults with type 1 diabetes. Sections were stained by immunohistochemistry to show the MVD defined by von Willebrand factor (vWF) (**A**) were obtained from representative patient without diabetic kidney disease (DKD–) and (**B**) from patient with DKD+. Notice significantly lower microvessel density defined by vWF in patient with DKD+. Scale bar = 50 μm

DKD– group, defined by CD133 79 (50–100) *vs.* 79 (63–92), p = 0.823, by vWF 42 (25–54) *vs.* 54 (43–71), p = 0.009 and by CD31 29 (21–46) *vs.* 38 (17–58), p = 0.454 (Figure 1). The values given above were calculated for both layers of the dermis (papillary and reticular dermis). Comparison of patients DKD+ and DKD– is shown in Table 1.

The group contained 79 (52.7%) patients with CKD stage 1, 58 (39.2%) patients with CKD stage 2, 8 (5.4%) CKD patients with stage 3a and 3 (2%) patients with CKD stage 3b. There were no patients with CKD stages 4 and 5. Comparison of MVD defined by vWF+ in groups of patients depending on the CKD stage is shown in Table 2.

In multivariate logistic regression analysis presence of diabetic kidney disease was associated with lower median vWF+ MVD [odds ratio (OR): 0.97 (95% confidence interval {CI}: 0.95–0.99), p = 0.017]. In multivariate model the results were adjusted for age, gender, diabetes duration and presence of hypertension.

Discussion

Angiogenesis is involved in the pathogenesis of diabetic kidney disease. The abnormal new vessels present in glomerular capillary area (Bowman's capsule in the glomerular vascular pole) are dilated and the glomerular basement membrane is extremely thin. According to the study of Osterby et al. an "extra efferent arteriole" are detected in the early stages of diabetic nephropathy [11]. Hypertension may be another important driving factor in the progression of angiogenesis in diabetes [12]. Angiotensin-converting enzyme (ACE) inhibitors suppress angiogenesis in glomerulus [13]. One of the possible explanations is that these vessels can play the role as a "by-pass" to reduce intraglomerular pressure [11]. Morphological effect of neovascularization is glomerular hypertrophy while functional effect is temporarily excessive filtration (increased GFR).

New blood vessels are structurally and functionally immature, endothelial cells are swollen, the basement membrane is thin. All this leads to increased perme-

Table 1. Comparison of groups of patients with type 1 diabetes in relation to diabetic kidney disease (DKD). Patients with diagnosis of diabetic kidney disease (DKD+) and without diagnosis of diabetic kidney disease (DKD–)

Variables	DKD+ (N = 21)	DKD– (N = 127)	p value
Age (years)	44 (32–58)	40 (30–49)	0.226
Sex, female/male, N	9/12	52/75	1.0
Duration of diabetes (years)	**30 (21–36)**	**21 (16–28)**	**0.002**
Smoking, N (%)	7 (33)	36 (28)	0.61
Hypertension, N (%)	**14 (67)**	**37 (29)**	**0.002**
BMI [kg/m^2]	24.9 (22.8–29)	25 (22–29)	0.980
HbA_{1c} (%)	8.4 (7.0–9.3)	8.0 (7.3–8.9)	0.527
HbA_{1c} [mmol/mol]	68.3 (53–78.1)	63.9 (56.3–73.8)	
TG [mmol/l]	1.2 (1.0–1.7)	1.0 (0.8–1.4)	0.057
LDL-cholesterol [mmol/l]	2.6 (2.3–3.1)	2.6 (2.1–3.4)	0.709
HDL-cholesterol [mmol/l]	1.6 (1.3–1.9)	1.6 (1.4–2.2)	0.459
eGFR (MDRD) [ml/min./1.73 m^2]	**66.1 (54.7–87.9)**	**93.5 (82.5–106.2)**	**< 0.001**
MVD CD34+ [vessels/1 mm^2]	123 (100–170)	121 (100–154)	0.775
MVD CD133+ [vessels/1 mm^2]	79 (50–100)	79 (63–92)	0.823
MVD vWF+ [vessels/1 mm^2]	**42 (25–54)**	**54 (43–71)**	**0.009**
MVD CD31+ [vessels/1 mm^2]	29 (21–46)	38 (17–58)	0.454
CD34/CD31 ratio	4.1 (2.1–6.6)	2.7 (1.8–5.3)	0.148
CD133/CD31 ratio	3.0 (1.6–4.2)	1.7 (1.3–3.3)	0.093
vWF/CD31 ratio	1.4 (0.5–2.3)	1.3 (0.8–2.0)	0.583

Note: data are presented as median (interquartile range) or N (%); BMI — body mass index; CD — cluster of differentiation; eGFR — estimated glomerular filtration rate; HbA_{1c} — glycated hemoglobin A_{1c}; HDL — high-density lipoproteins; LDL — low-density lipoproteins; MDRD — Modification of Diet in Renal Disease; MVD — microvessel density; N — number of patients; TG — triglycerides; vWF — von Willebrand factor

Table 2. Comparison of microvessel density (MVD) defined by von Willebrand factor (vWF) in groups of patients depending on the chronic kidney disease (CKD) stage

CKD stage	N (%)	eGFR MDRD	MVD vWF+ [vessels/1 mm^2]
1	79 (52.7)	103.6 (96.1–114.9)	54.2 (41.7–70.8)
2	58 (39.2)	55.9 (53.1–57.5)	52.1 (37.5–70.8)
3a	8 (5.4)	55.6 (53.1–57.5)	47.9 (12.5–56.3)
3b	3 (2)	37.1 (31.1–37.8)	20.8 (0–41.7)

Note: data are presented as median (interquartile range) or N (%); p = 0.33 for comparison vWF+ MVD between groups with CKD stages 1,2, and 3a; p = 0.056 for comparison vWF+ MVD between groups with CKD stages 1,2, and 3 (combined); Kruskal-Wallis test; N — number of patients; eGFR — estimated glomerular filtration rate; MDRD — Modification of Diet in Renal Disease

ability and finally results in the extravasation of plasma protein [11]. Vascular endothelial growth factor (VEGF) plays the main role in this process. Vascular endothelial growth factor A (VEGF-A) is derived from podocytes and tubular epithelial cells and vascular endothelial growth factor receptor 2 (VEGFR-2) is expressed in glomerular and peritubular capillaries. Another phenomenon called "uncoupling of VEGF-A with NO (nitric oxide)" (a low NO bioavailability along with high VEGF), observed in the diabetic mice could potentiate the vascular permeability in the glomerulus [14]. The final effect of unfavorable processes is progression to renal fibrosis. Due to advancing fibrotic changes and loss of endothelial cells, podocytes, and tubular epithelial cells, the production of VEGF is decreased and the process progresses to the advanced stages of chronic kidney disease [15].

VEGF reflects the pathology of neovascularization. The next step in understanding of the mechanisms of the pathology of neovascularization induced by metabolic hypoxia is the possibility of assessing the maturity of the vessels. We evaluated the following markers of angiogenesis in the material from skin biopsy: CD34, CD133, CD31 and vWF. Previously, we have observed that MVD, assessed by CD34 (a marker of "late" EPCs) and CD133 (a marker of early EPCs), were significantly higher in patients with cardiac autonomic neuropathy

(CAN). Also, CD34 MVD was higher in patients with diabetic peripheral neuropathy (DPN), as compared to subjects without complications [16]. These results support the concept that angiogenic processes are involved in the pathogenesis of neuropathy and confirm the neurovascular nature of chronic diabetes complications. Interestingly, we did not find any relationship between markers of early ECs and the presence of DKD. In the animal model of kidney disease, local quantity of CD34+ capillaries were decreased with increasing severity of glomerular and tubulointerstitial lesions [17]. We did not find any data that confirm this phenomenon in patients with DKD.

CD133+ progenitor cells were used for the treatment in an animal model of acute kidney injury (AKI). CD133+ cells promoted the restoration of the renal tissue, limiting the presence of markers of injury and pro-inflammatory molecules, promoted angiogenesis and protected against fibrosis [18]. Moreover, in the previous study we have found a negative correlation between CD31 MVD and skin auto fluorescence (AF) [19]. ECs are capable of undergoing endothelial to mesenchymal transition under the influence of transforming growth factor-β_1. They lose the expression of such antigens as CD31, von Willebrand factor, and vascular-endothelial cadherin and initiate the expression of mesenchymal cell antigens [α-smooth muscle actin (α-SMA), extra domain A fibronectin, N-cadherin, vimentin, fibroblast specific protein-1] [20]. According to previous studies, endothelial-to-mesenchymal transition is associated with albuminuria in diabetic nephropathy. In these studies, a decreased number of CD31+ endothelial cells and increased α-SMA expression in glomeruli of diabetic mice was observed [21, 22].

Von Willebrand factor (vWF) is a glycoprotein that is most researched in the context of its role in haemostasis and von Willebrand disease (vWD). Quantitative and qualitative vWF changes can lead to an increased risk of bleeding, on the other hand it is regarded as risk factor for cardiovascular disease or venous thromboembolism [23, 24]. The study of Lenting et al. [25] showed that vWF plays a role in the angiogenesis, cell proliferation and inflammation. The storage of von Willebrand factor is a function of Weibel Palade bodies that are ultrastructural organelles found only in mature vascular endothelial cells [26]. We found a decreased number of MVD determined by vWF blood vessels per 1 mm^2 in DKD+ group. We did not observe that correlation in relation to other chronic diabetic complications. Interestingly, we found a decreased number of mature vessels despite the very early stages of CKD present in the vast majority of patients. Lack of the association between vascular markers and eGFR suggests that altered expression of vascular markers denote earlier pathology than decreased glomerular filtration. The progression of chronic kidney disease is a very complex process and different factors may be dominant, depending on stage [27]. We can only speculate if this lower expression of mature vessels is caused by the altered angiogenesis and disturbances of vessel maturation as mechanisms involved in the development of diabetic nephropathy [28]. The process seems to be different than in other diabetic complications. In the course of neuropathy, excessive angiogenesis expressed by the increased number of MVD CD34+ and CD133+ plays a dominant role. The reduction of eGFR and positive albuminuria are already indicative of significant glomerular pathology, considered to be an important risk factor for ischemic heart disease. Perhaps at this stage, a simple marker that is vWF can be used to further determine the cardiovascular risk in patients, as it is directly related to the diagnosis of DKD. The observed phenomenon seems to be interesting but requires further research to explain.

Conclusions

In patients with type 1 diabetes and diabetic kidney disease the disturbances in the angiogenesis and vascular maturation are present. The number of mature blood vessels (vWF+) in the skin is reduced. Disturbances in the angiogenesis occur at early stages of diabetic kidney disease.

Statement of competing interests

The above-mentioned authors declare that there is no conflict of interest.

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REFERENCES

1. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease. Am J Kidney Dis. 2007; 49(2 Suppl 2): S12–154, doi: 10.1053/j.ajkd.2006.12.005, indexed in Pubmed: 17276798.
2. Harjutsalo V, Groop PH. Epidemiology and risk factors for diabetic kidney disease. Adv Chronic Kidney Dis. 2014; 21(3): 260–266, doi: 10.1053/j.ackd.2014.03.009, indexed in Pubmed: 24780453.
3. Macisaac RJ, Jerums G. Diabetic kidney disease with and without albuminuria. Curr Opin Nephrol Hypertens. 2011; 20(3): 246–257, doi: 10.1097/MNH.0b013e3283456546, indexed in Pubmed: 21422923.
4. Fowler MJ. Microvascular and macrovascular complications of diabetes. Clin Diab. 2008; 26(2): 77–82, doi: 10.2337/diaclin.26.2.77.
5. Sabatier F, Camoin-Jau L, Anfosso F, et al. Circulating endothelial cells, microparticles and progenitors: key players towards the definition of vascular competence. J Cell Mol Med. 2009; 13(3):

454–471, doi: 10.1111/j.1582-4934.2008.00639.x, indexed in Pubmed: 19379144.
6. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997; 275(5302): 964–967, doi: 10.1126/science.275.5302.964, indexed in Pubmed: 9020076.
7. Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood. 2000; 95(3): 952–958, indexed in Pubmed: 10648408.
8. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998; 67: 395–424, doi: 10.1146/annurev.biochem.67.1.395, indexed in Pubmed: 9759493.
9. Sandeman DD, Shore AC, Tooke JE. Relation of skin capillary pressure in patients with insulin-dependent diabetes mellitus to complications and metabolic control. N Engl J Med. 1992; 327(11): 760–764, doi: 10.1056/NEJM199209103271103, indexed in Pubmed: 1501652.
10. Khan F, Elhadd TA, Greene SA, et al. Impaired skin microvascular function in children, adolescents, and young adults with type 1 diabetes. Diabetes Care. 2000; 23(2): 215–220, doi: 10.2337/diacare.23.2.215, indexed in Pubmed: 10868834.
11. Østerby R, Hartmann A, Bangstad HJ. Structural changes in renal arterioles in type I diabetic patients. Diabetologia. 2002; 45(4): 542–549, doi: 10.1007/s00125-002-0780-2, indexed in Pubmed: 12032631.
12. Nakagawa T, Sato W, Kosugi T, et al. Abnormal angiogenesis in diabetic nephropathy. Diabetes. 2009; 58(7): 1471–1478, doi: 10.2337/db09-0119, indexed in Pubmed: 19564458.
13. Joles JA, Braam B, Verhaar MC. ACE inhibition and glomerular repair: restructuring or regeneration? Kidney Int. 2006; 69(7): 1105–1107, doi: 10.1038/sj.ki.5000237, indexed in Pubmed: 16609678.
14. Nakagawa T. Uncoupling of the VEGF-endothelial nitric oxide axis in diabetic nephropathy: an explanation for the paradoxical effects of VEGF in renal disease. Am J Physiol Renal Physiol. 2007; 292(6): F1665–F1672, doi: 10.1152/ajprenal.00495.2006, indexed in Pubmed: 17545302.
15. Kim BS, Goligorsky MS. Role of VEGF in kidney development, microvascular maintenance and pathophysiology of renal disease. Korean J Intern Med. 2003; 18(2): 65–75, doi: 10.3904/kjim.2003.18.2.65, indexed in Pubmed: 12872442.
16. Adamska A, Pilacinski S, Zozulinska-Ziolkiewicz D, et al. An increased skin microvessel density is associated with neurovascular complications in type 1 diabetes mellitus. Diab Vasc Dis Res. 2019 [Epub ahead of print]: 1479164119850831, doi: 10.1177/1479164119850831, indexed in Pubmed: 31144511.
17. Masum MdA, Ichii O, Elewa YH, et al. Local CD34-positive capillaries decrease in mouse models of kidney disease associating with the severity of glomerular and tubulointerstitial lesions. BMC Nephrol. 2017; 18(1): 280, doi: 10.1186/s12882-017-0694-3, indexed in Pubmed: 28870174.
18. Aggarwal S, Grange C, Iampietro C, et al. Human CD133 renal progenitor cells induce erythropoietin production and limit fibrosis after acute tubular injury. Sci Rep. 2016; 6: 37270, doi: 10.1038/srep37270, indexed in Pubmed: 27853265.
19. Adamska A, Araszkiewicz A, Pilacinski S, et al. Dermal microvessel density and maturity is closely associated with atherogenic dyslipidemia and accumulation of advanced glycation end products in adult patients with type 1 diabetes. Microvasc Res. 2019; 121: 46–51, doi: 10.1016/j.mvr.2018.10.002, indexed in Pubmed: 30312628.
20. Piera-Velazquez S, Jimenez SA. Endothelial to mesenchymal transition: role in physiology and in the pathogenesis of human diseases. Physiol Rev. 2019; 99(2): 1281–1324, doi: 10.1152/physrev.00021.2018, indexed in Pubmed: 30864875.
21. Peng H, Li Y, Wang C, et al. ROCK1 induces endothelial-to-mesenchymal transition in glomeruli to aggravate albuminuria in diabetic nephropathy. Sci Rep. 2016; 6: 20304, doi: 10.1038/srep20304, indexed in Pubmed: 26842599.
22. Liu F, Zhang S, Xu R, et al. Melatonin attenuates endothelial-to-mesenchymal transition of glomerular endothelial cells via regulating miR-497/ROCK in diabetic nephropathy. Kidney Blood Press Res. 2018; 43(5): 1425–1436, doi: 10.1159/000493380, indexed in Pubmed: 30212830.
23. van Schie MC, van Loon JE, de Maat MPM, et al. Genetic determinants of von Willebrand factor levels and activity in relation to the risk of cardiovascular disease: a review. J Thromb Haemost. 2011; 9(5): 899–908, doi: 10.1111/j.1538-7836.2011.04243.x, indexed in Pubmed: 21342431.
24. Smith NL, Rice KM, Bovill EG, et al. Genetic variation associated with plasma von Willebrand factor levels and the risk of incident venous thrombosis. Blood. 2011; 117(22): 6007–6011, doi: 10.1182/blood-2010-10-315473, indexed in Pubmed: 21163921.
25. Lenting PJ, Casari C, Christophe OD, et al. von Willebrand factor: the old, the new and the unknown. J Thromb Haemost. 2012; 10(12): 2428–2437, doi: 10.1111/jth.12008, indexed in Pubmed: 23020315.
26. Edgell CJ, Haizlip JE, Bagnell CR, et al. Endothelium specific Weibel-Palade bodies in a continuous human cell line, EA.hy926. In Vitro Cell Dev Biol. 1990; 26(12): 1167–1172, indexed in Pubmed: 2079463.
27. Tsai WC, Wu HY, Peng YS, et al. Risk factors for development and progression of chronic kidney disease: a systematic review and exploratory meta-analysis. Medicine (Baltimore). 2016; 95(11): e3013, doi: 10.1097/MD.0000000000003013, indexed in Pubmed: 26986114.
28. Fogo AB. Mechanisms of progression of chronic kidney disease. Pediatr Nephrol. 2007; 22(12): 2011–2022, doi: 10.1007/s00467-007-0524-0, indexed in Pubmed: 17647026.

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Anthropometric, metabolic and clinical factors associated with diabetes and prediabetes prevalence in women aged 65–74 living in central Poland

ABSTRACT

Background. Prevalence of type 2 diabetes mellitus is rising worldwide. Similar trend is also observed in Poland, especially in elderly population. The aim of this cross-sectional study was to assess prevalence and to identify anthropometric, metabolic and clinical factors associated with diabetes and prediabetes among women at early elderliness living in central Poland.

Methods. 364 women aged 65–74 years, were included into the study. In all patients a history of diabetes and cardiovascular disease was obtained, blood pressure and anthropometric measurements were performed, blood samples for laboratory tests (fasting plasma glucose, lipid metabolism and creatinine) were drawn, ankle/brachial index was calculated, abdominal ultrasound with abdominal aorta diameter was performed and carotid intima/media thickness was measured. Data were collected during March and April 2012 in Gniewkowo, the rural-urban municipality in central Poland.

Results. 98 women had diabetes (25 *de novo*) and 94 ones had prediabetes (81 *de novo*). Waist circumference, BMI, lipid abnormalities as well as anthropometric and metabolic indices: waist-to-height ratio (WHtR), triglycerides/HDL cholesterol ratio and visceral adiposity index (VAI) were significantly associated with abnormal glucose metabolism. Backward stepwise logistic regression analysis identified WHtR as the best single indicator of patients with diabetes, while again WHtR and VAI were the only independent indicators of any type of impaired glucose metabolism.

Conclusions. Abnormal glucose metabolism is highly prevalent among women at early elderliness, especially in those with visceral obesity and abnormal lipid metabolism. Anthropometric and metabolic indices (WHtR and VAI) were better indicators of impaired glucose metabolism compared to separate measurements of single parameters. (Clin Diabetol 2019; 8, 5: 238–247)

Key words: diabetes, prediabetes, obesity, anthropometric parameters, metabolic parameters

Introduction

Prevalence of diabetes mellitus (DM), predominantly type 2, reached an epidemic range with 425 million people suffering from DM worldwide in 2017 [1]. In Poland, according to National Health Fund data, almost 2.34 million people (6.08% of the whole population) were using antidiabetic medications in 2014 [2]. Type 2 DM is especially highly prevalent among elderly [3]. In South-Eastern Poland its prevalence in people

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aged > 65 exceeded 18% of population at that age range (the National Health Fund data, unpublished). However, known DM is only a part of the problem. The second part is a large number of people not aware of having DM, and this number reaches in Europe 37.9% of all cases of DM [1]. Moreover, the number of people having impaired fasting glucose (IFG), and/or impaired glucose tolerance (IGT) is similar to that with DM [1]. It is well known that DM can lead to several negative long-term health consequences including increased risk of cardiovascular diseases, chronic kidney disease, visual impairment, diabetic neuropathy and cancer [4]. Thus, early diagnosis of DM or prediabetes (IFG and/or IGT) is of utmost importance for the patients' prognosis.

The primary objective of this cross-sectional study was to identify the prevalence of known and undiagnosed glucose metabolism abnormalities: DM and prediabetes among women at early elderliness, living in a rural-urban community in central Poland. The secondary objective was to identify among analyzed variables the best indicators and predictors of overt DM as well as prediabetes.

Material and methods

Study participants

All women aged 65–74 years, living in Gniewkowo, the rural-urban community in central Poland, and being under care of a primary care clinics, were invited to participate in the study. We decided to choose females in such age range, due to high expected prevalence of glucose metabolism abnormalities in this population, and expected survival time long enough to develop chronic complications of diabetes — in the year 2016 life expectancy in Poland reached 20.4 years for women aged 65, and 13.5 years for women aged 74 [5]. Population of women living in this community is homogenous, and all invited females were of Caucasian ethnicity. In response to the invitation 364 women agreed to participate in the study, which accounted for about 60% of all invited ones.

Data collection

Data were collected in March and April 2012. All women were interviewed for DM and cardiovascular diseases (CVD) history and also demographic data were collected. Then weight, height, waist and hip circumference were measured and upon these data body mass index (BMI), waist/hip ratio (WHR) and waist-to-height ratio (WHtR)were calculated. Women with BMI ≥ 25.0 kg/m^2 were considered overweight and with BMI ≥ 30.0 kg/m^2 obese. Blood pressure was measured by trained nursing staff with the use of standardized sphygmomanometer validated by the appropriate authorities. Measurement on the ankles was performed with the use of a Doppler probe and the ankle/brachial index (ABI) was calculated. To assess the cardio-metabolic risk the lowest ABI score was taken into analysis. Fasting blood samples were collected for the assessment of plasma glucose concentration, serum lipid profile and creatinine level, and they were analyzed in a certified laboratory. Estimated glomerular filtration rate (eGFR) was calculated using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI$_{cr}$) equation, currently recommended by Diabetes Poland [6, 7]:

$$GFR = 141 \times \min(Scr/\kappa, 1)^{\alpha} \times \max(Scr/\kappa, 1)^{-1.209} \times \times 0.993^{Age} \times 1.018\ [\text{if female}] \times 1.159\ [\text{if black}]$$

Scr is serum creatinine [mg/dL], κ is 0.7 for females and 0.9 for males, α is –0,329 for females and –0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1

In women with fasting plasma glucose ≥ 126 mg/dL measurement was repeated, while in females with fasting glycaemia within IFG range (100–129 mg/dL) oral glucose tolerance test (OGTT) was performed. DM, IFG or IGF were diagnosed in accordance with Diabetes Poland criteria from 2012 (which were identical to current ones) [7]. Metabolic syndrome (MS) was diagnosed according to the most current consensus definition [8]. Upon obtained data also two metabolic indices were calculated: Triglycerides (TG) to HDL cholesterol (TG/HDL) ratio (expressed in mg/dL) and visceral adiposity index (VAI). VAI was calculated using formula developed by Amato et al. [9] for women:

$$VAI = \left[\frac{WC}{36.58 + (1.89 \times BMI)}\right] \times \left[\frac{TG}{0.81}\right] \times \left[\frac{1.52}{HDL}\right]$$

TG and HDL cholesterol expressed in mg/dL.

Also, all patients underwent abdominal ultrasound examination with the assessment of abdominal aorta diameter, and carotid intima/media thickness (CIMT) measurement to assess the relationship between these results and status of the glucose metabolism in the study participants. Abdominal ultrasound examination was performed using a convex transducer and the diameter measurement was made on the abdominal aorta (from renal arteries to the bifurcation). Carotid intima/media thickness measurement was performed using a linear transducer. The ultrasound measurements were performed by a trained radiologists with the required certification for ultrasound examination. The highest CIMT measurement outcome was used in the analysis.

To avoid bias associated with failure to report of a 40% of the primarily invited women, 40 randomly selected women from that group were re-invited. They

reported to the clinic, where they underwent anthropometric and blood pressure measurements. Their results were not significantly different from the first group of women. They were not included in the analysis, because they did not undergo laboratory tests, ABI measurement, abdominal ultrasound and CIMT measurement.

Ethical approval

The study was approved by the Bioethics Committee at the Collegium Medicum in Bydgoszcz of the Nicolaus Copernicus University in Toruń and it was conducted in accordance with ethical standards laid down in an appropriate version of the Declaration of Helsinki and in Polish national regulations. All study participants signed informed consent form before beginning of the study procedures.

Statistical analysis

Statistical analysis of the data was performed using SigmaPlot for Windows, version 12.5 (Systat Software Inc., San Jose, CA, USA). The nominal variables are presented as numbers and percentage. The continuous data are presented as mean and standard deviation (SD) in parentheses. The normality of data distribution was checked using the Shapiro-Wilk test. Differences between groups (diabetes, prediabetes and normal glucose tolerance) were analyzed using an unpaired two-tailed Student's t-test or by a Mann-Whitney rank sum test where appropriate. The categorical data were compared using χ^2 test. We also calculated odds ratios (OR) and area under curve (AUC) in receiver operating characteristics (ROC) curve for significant associations between impaired glucose metabolism and analyzed variables. Linear correlation between continuous variables was analyzed with the use of Spearman Rank Order Correlation test. To identify predictive variables for glucose metabolism abnormalities we used backward stepwise regression analysis. We assumed a P value of < 0.05 as statistically significant.

Results

Impaired glucose metabolism (IGM = DM + prediabetes) was found in more than half of 364 included women. DM was found in 98 of them and 94 females had prediabetes. In this number there were 25 cases of newly diagnosed DM and 81 new cases of prediabetes revealed in OGTT. Overall, 55.2% of women with IGM were unaware of having abnormal glucose metabolism. MS was present in 60.7% of cases. Impaired kidney function with eGFR < 60 mL/min/1.73 m^2 was found in 37 cases (10.2% of the study participants).

We found significant differences between women with diabetes, prediabetes, IGM and normal glucose tolerance (NGT) in anthropometric parameters. BMI, prevalence of obesity, waist and hip circumference, WHR and WHtR were significantly lower in women with NGT, and they were increasing along with with the degree of glucose metabolism impairment. Women with DM compared to females with prediabetes had significantly higher BMI, WC and WHtR (Table 1).

The number of females with the history of myocardial infarction (MI) was insignificantly different between DM and NGT groups, P = 0.051 (Table 2). However, women with the history of MI had over 3-fold higher probability of having diabetes compared to the rest of study participants, odds ratio (OR) 3.26, and 95% confidence interval (CI, 1.22–8.71), P = 0.028. Hypertension was significantly less frequent among women with NGT compared to IGM and prediabetes groups. Women with NGT had also significantly lower systolic blood pressure (SBP) compared to females with DM, and significantly lower pulse pressure compared to women with DM and IGM. Diastolic blood pressure (DBP) was not significantly different between the study groups (Table 2). Although no significant differences were found between the groups with regards to vascular parameters, we revealed, which is interesting finding, significantly lower abdominal aorta diameter in 25 women with newly diagnosed DM (upon fasting plasma glucose level or in OGTT) compared to females with known DM, prediabetes and NGT: 17.0 ± 2.5 cm *vs.* 18.6 ± 2.9 cm, 18.1 ± 2.2 cm and 18.6 ± 3.4 cm respectively. P values for comparisons between new DM *vs.* known DM, prediabetes and NGT were P = 0.005, P = 0.011 and P = 0.005 respectively. Neither ABI nor CIMT was significantly different between DM, prediabetes and NGT groups (Table 2). However, we found borderline significant linear correlation between CIMT and fasting plasma glucose, R = 0.105, P = 0.046. Kidney function was significantly worse in women with prediabetes and IGM compared to NGT group. However, number of females with eGFR $\geq$ 90, 60–89 and < 60 mL/min/1.73 m^2 was not significantly different between the groups (Table 2).

All metabolic parameters and indices were significantly different between NGT and DM or IGM groups (Table 3). However, females with DM were significantly more frequently using statins compared to women with prediabetes and NGT (61.2%, 37.2% and 38,4% respectively), and after adjustment to statin use, differences between DM or IGM and NGT groups regarding total, non-HDL and LDL cholesterol became insignificant. Prevalence of MS was significantly higher in women with DM, prediabetes and IGM compared to NGT group, 90,8%, 92,6%, 91.7% and 26,2% respectively. Presence of MS was associated with 10-fold higher

Table 1. Demographic and anthropometric characteristics of the study population divided into four subgroups. The results are presented as mean and standard deviation (SD) or number and percentage. Significant differences in bold italic

Variable	Diabetes (A) n = 98	IFG/IGT (B) n = 94	IGM (C) n = 192	NGT (D) n = 172	P value			
	Mean ± SD or n (%)	Mean ± SD or n (%)	Mean ± SD or n (%)	Mean ± SD or n (%)	A *vs.* B	A *vs.* D	B *vs.* D	C *vs.* D
Age (years)	69.3 ± 3.2	69.5 ± 3.4	69.4 ± 3.3	69.0 ± 3.3	0.779	0.290	0.181	0.147
Smoking (n)	31 (31.6)	24 (25.5)	55 (28.6)	61 (35.5)	0.438	0.613	0.128	0.200
BMI [kg/m^2]	32.8 ± 5.3	31.0 ± 5.0	31.9 ± 5.2	29.2 ± 5.0	***0.022***	***< 0.001***	***0.004***	***< 0.001***
Normal weight (n)	6 (6.1)	11 (11.7)	17 (8.9)	34 (19.8)				
Overweight (n)	24 (24.5)	32 (34.0)	56 (29.2)	68 (39.5)	p_{trend} 0.084	***p_{trend} < 0.001***	p_{trend} 0.071	***p_{trend} < 0.001***
Obesity (n)	68 (69.4)	51 (54.3)	119 (62.0)	70 (40.7)				
WC [cm]	103.5 ± 11.6	100.2 ± 11.1	101.8 ± 11.4	93.7 ± 12.1	***0.046***	***< 0.001***	***< 0.001***	***< 0.001***
Hip [cm]	116.0 ± 9.9	113.7 ± 10.5	114.8 ± 10.2	109.3 ± 11.1	0.120	***< 0.001***	***0.002***	***< 0.001***
WHR	0.89 ± 0.08	0.88 ± 0.06	0.89 ± 0.07	0.86 ± 0.08	0.102	***< 0.001***	***< 0.001***	***< 0.001***
WHtR	0.66 ± 0.08	0.64 ± 0.07	0.65 ± 0.08	0.60 ± 0.08	***0.014***	***< 0.001***	***< 0.001***	***< 0.001***

IGM — impaired glucose metabolism; NGT — normal glucose tolerance; BMI — body mass index; WC — waist circumference; WHR — waist/hip ratio; WHtR — waist-to-height ratio

Table 2. Clinical and vascular parameters of the study population divided into four subgroups. The results are presented as mean and standard deviation (SD) or number and percentage. Significant differences in bold italic

Variable	Diabetes (A) n = 98	Prediabetes (B) n = 94	IGM (C) n = 192	NGT (D) n = 172	P value			
	Mean ± SD or n (%)	Mean ± SD or n (%)	Mean ± SD or n (%)	Mean ± SD or n (%)	A *vs.* B	A *vs.* D	B *vs.* D	C *vs.* D
Comorbidities								
History of MI (n)	9 (9.2)	3 (3.2)	12 (6.3)	5 (2.9)	0.157	0.051	0.806	0.208
History of stroke (n)	5 (5.1)	1 (1.1)	6 (3.1)	5 (2.9)	0.233	0.560	0.592	0.853
Hypertension (n)	84 (85.7)	82 (87.2)	166 (86.5)	131 (76.2)	0.923	0.086	***0.045***	***0.017***
Blood pressure								
SBP [mm Hg]	154.7 ± 22.6	149.1 ± 20.2	152.0 ± 21.6	148.3 ± 22.6	0.069	***0.020***	0.553	0.074
DBP [mm Hg]	84.8 ± 10.2	82.3 ± 9.9	83.6 ± 10.1	83.9 ± 10.9	0.086	0.547	0.223	0.794
Pulse pressure [mm Hg]	69.9 ± 17.9	66.8 ± 16.2	68.4 ± 17.1	64.3 ± 16.2	0.204	***0.011***	0.156	***0.015***
Vascular parameters								
ABI	0.97 ± 0.18	0.99 ± 0.15	0.98 ± 0.17	0.98 ± 0.15	0.484	0.983	0.430	0.647
CIMT [mm]	1.07 ± 0.19	1.08 ± 0.20	1.07 ± 0.19	1.06 ± 0.23	0.865	0.231	0.190	0.128
AAD [mm]	18.2 ± 2.9	18.1 ± 2.2	18.1 ± 2.6	18.6 ± 3.4	0.852	0.426	0.532	0.388
Kidney function								
Creatinine [μmol/L]	66.3 ± 21.2	66.3 ± 15.0	66.3 ± 18.6	63.6 ± 15.0	0.173	0.611	***0.037***	0.118
eGFR (CKD-EPI) [mL/min/1.73 m^2]	81.1 ± 16.6	79.1 ± 14.3	80.1 ± 15.5	83.5 ± 17.4	0.138	0.357	***0.009***	***0.034***
≥ 90 (stage G1) (n)	35 (35.7)	25 (26.6)	60 (31.3)	69 (40.8)				
60–89 (stage G2) (n)	51 (52.0)	59 (62.8)	110 (57.3)	85 (50.3)	0.309	0.563	0.096	0.157
< 60 (stage G3–G4) (n)	12 (12.2)	10 (10.6)	22 (11.5)	15 (8.9)				

IGM — impaired glucose metabolism; NGT — normal glucose tolerance; MI — myocardial infarction; SBP — systolic blood pressure; DBP — diastolic blood pressure; ABI — ankle/brachial index; CIMT — carotid intima/media thickness; AAD — abdominal aorta diameter

Table 3. Metabolic parameters of the study population divided into four groups. The results are presented as mean and standard deviation (SD) or number and percentage. Significant differences in bold italic

Variable	Diabetes (A) n = 98	Prediabetes (B) n = 94	IGM (C) n = 192	NGT (D) n = 172	P value			
	Mean ± SD or n (%)	Mean ± SD or n (%)	Mean ± SD or n (%)	Mean ± SD or n (%)	A *vs.* B	A *vs.* D	B *vs.* D	C *vs.* D
Total cholesterol [mmol/L]	5.00 ± 1.17	5.85 ± 1.20	5.41 ± 1.25	5.73 ± 1.28	***< 0.001***	***< 0.001***	0.572	***0.003***
HDL cholesterol [mmol/L]	1.64 ± 0.52	1.65 ± 0.37	1.64 ± 0.45	1.78 ± 0.45	0.390	***0.003***	***0.020***	***0.001***
Non-HDL cholesterol [mmol/L]	3.36 ± 1.20	4.20 ± 1.23	3.77 ± 1.28	3.98 ± 1.27	***< 0.001***	***< 0.001***	0.181	***0.033***
LDL cholesterol [mmol/L]	2.64 ± 1.07	3.46 ± 1.10	3.05 ± 1.16	3.42 ± 1.15	***< 0.001***	***< 0.001***	0.636	***< 0.001***
Triglycerides [mmol/L]	1.63 ± 1.00	1.54 ± 0.61	1.58 ± 0.83	1.28 ± 0.58	0.940	***< 0.001***	***< 0.001***	***< 0.001***
Dyslipidemia (TG >1.7 mmol/L and/or HDL < 1.3 mmol/L) (n)	39 (39.8)	40 (42.6)	79 (41.1)	41 (24.0)	0.809	***0.010***	***0.003***	***< 0.001***
Glucose [mmol/L]	7.81 ± 1.95	6.18 ± 0.61	7.00 ± 1.67	5.09 ± 0.42	***< 0.001***	***< 0.001***	***< 0.001***	***< 0.001***
TG/HDL ratio	2.72 ± 2.54	2.33 ± 1.27	2.53 ± 2.02	1.86 ± 1.26	0.855	***< 0.001***	***< 0.001***	***< 0.001***
VAI	5.42 ± 5.05	4.65 ± 2.77	5.04 ± 4.11	3.59 ± 2.55	0.838	***< 0.001***	***< 0.001***	***< 0.001***

IGM — impaired glucose metabolism; NGT — normal glucose tolerance; TG — triglycerides; VAI — visceral adiposity index

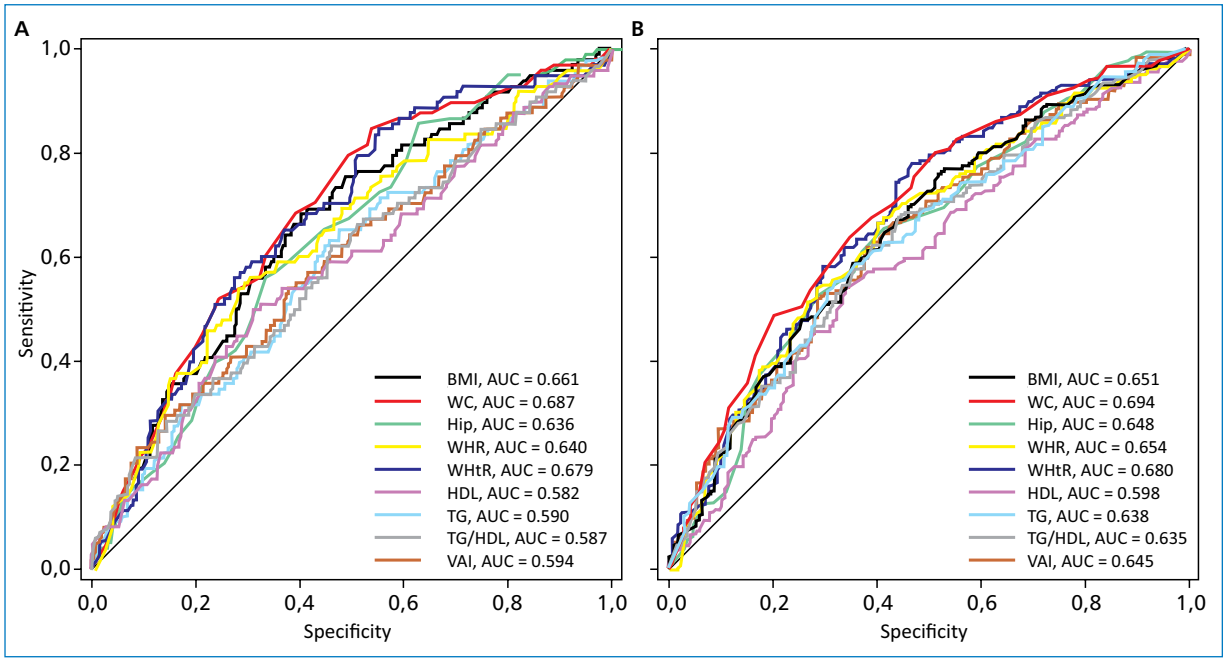

Figure 1. Receiver operating characteristic (ROC) curves and area under the curve (AUC) of anthropometric and metabolic parameters and indices associated with the prevalence of diabetes (**A**) and impaired glucose metabolism (**B**). BMI — body mass index; AUC — area under curve; WC — waist circumference; WHR — waist-to-hip-ratio; WHtR — waist-to-height ratio; HDL — high density lipoproteins; TG — triglycerides; VAI — visceral adiposity index

probability of having DM and over 30-fold higher probability of having IGM compared to NGT group, OR 10.04 (4.86–20.76), $P < 0.001$ and OR 31.04 (16.80––57.39), $P < 0.001$ respectively. However, glucose level > 5.5 mmol/L is a both MS component and a benchmark of IGM, and after adjustment to this variable these odds ratios decreased to OR 1.76 (1.07–2.89), $P = 0.034$ for DM and OR 2.10 (1.30–3.38), $P = 0.003$ for IGM.

All anthropometric and metabolic continuous variables significantly associated with abnormal glucose metabolism were then included in ROC analysis, separately for DM and IGM. The highest area under curve (AUC) for DM was found for WC, WHtR and BMI, $P < 0.001$ in all cases (Figure 1A), while for IGM there were WC, WHtR and WHR, $P < 0.001$ also in all cases (Figure 1B). Glucose level, as a diagnostic criterion for DM and prediabetes was excluded from these analyses.

We analyzed also odds ratios for variables significantly associated with the presence of DM or IGM. We assumed cut-off points for TG and HDL cholesterol level according to diagnostic criterion for MS [8], while for WC we took higher value, ≥ 88 cm, because 92.3% of all study participants had WC ≥ 80 cm. A cut-off point for BMI was ≥ 30 kg/m^2 (obesity), for WHR > 0.85 (abdominal obesity in women), for other variables cut-off points were taken from literature: VAI from Amato et al. [10], TG/HDL from Salazar et al., [11] and WHtR from Ashley & Gibson [12] or from ROC curve (hip circumference) (Figure 2).

In the backward stepwise regression analysis including all anthropometric and metabolic parameters and indices significantly associated with abnormal glucose metabolism, WHtR appeared to be the only significant predictor of having DM, $P < 0.001$, while WHtR and VAI were the only significant predictors of having IGM, $P < 0.001$ and $P = 0.005$ respectively.

Discussion

Our study revealed high prevalence of overt DM and prediabetes in elderly women living in a rural-urban community in central Poland. In this number 55.2% were previously un diagnosed. It is in line with data from other countries. In the United States prevalence of known DM in the elderly population is reaching 20.8%, and 4.4% have unknown DM [13]. In Canada prevalence of known DM among women aged 65–74 years is estimated to be 19.4% of females in this age range [14]. In the United Kingdom, in the age group 60–69 years, prevalence of DM exceeded 26% [15]. Also in China DM is prevalent in over 20% of elderly people, while IFG and/or IGT in roughly 25% [16]. Roughly, every one out of four people aged > 65 years suffers from DM. These data indicate how important epidemiological problem is DM in the elderly.

Both DM as well as IGT and/or IFG are associated with unfavorable clinical outcome including increased risk of cardiovascular disease (CVD) events and elevated CVD and all-cause mortality [17, 18]. In our study the

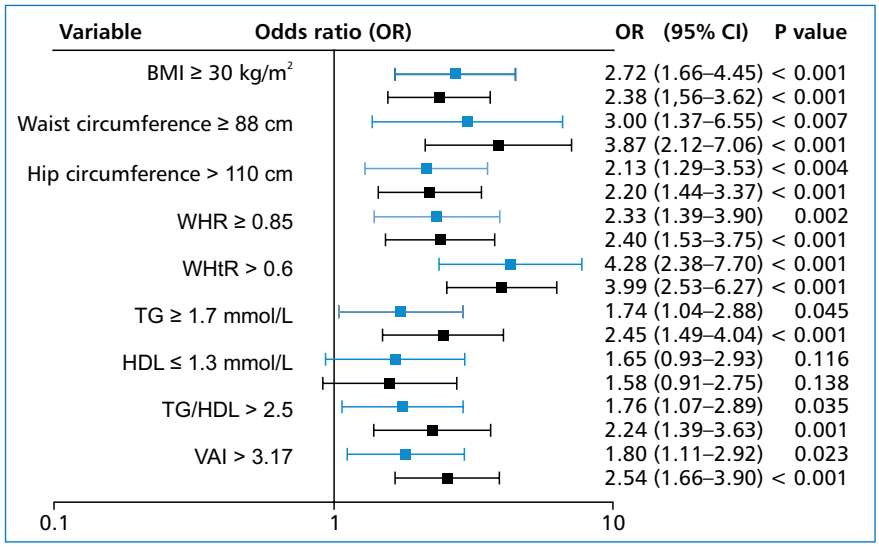

Figure 2. Odds ratios (OR) of anthropometric and metabolic variables for the probability of having diabetes mellitus (blue) or impaired glucose metabolism (black) in the univariate analysis. OR — odds ratio; CI — confidence interval; BMI — body mass index; WHR — waist-to-hip-ratio; WHtR — waist-to-height ratio; TG — triglycerides; HDL — high density lipoproteins; VAI — visceral adiposity index

history of myocardial infarction was significantly associated with overt DM. Interestingly, we revealed also association between lower abdominal aorta diameter in women with newly diagnosed DM. In men, Taimour et al. did not find such a relationship [19]. Thus, this finding requires further investigation. Significantly higher SBP and pulse pressure in women with overt diabetes together with lower abdominal aorta diameter can be considered as a clinical manifestation of the arterial stiffness in females with DM [20] (we did not perform direct measurements of pulse wave velocity and aortic characteristic impedance). In the Atherosclerosis Risk In Communities (ARIC) study low ABI was modestly but independently associated with diabetes incidence [21]. In our study ABI was not associated with any abnormal pattern of glucose metabolism. In the study by Gomez-Marcos et al. CIMT was related to HbA_{1c} and fasting, but not postprandial, plasma glucose [22]. We also revealed relationship between fasting glycemia and CIMT, while we did not measure HbA_{1c} in our study.

MS, non-surprisingly, demonstrated in our study significant relationship with DM and IGM. However, after adjustment to elevated glucose level, this relationship became much weaker. Significant relationship between different components of MS and DM was also found by other authors [16, 23]. In these studies the strongest indicators of DM prevalence were elevated TGs, low HDL cholesterol and elevated WC. In search of the best anthropometric indicator of elevated "early health risk" Ashley and Gibson indicate WHtR as a better indicator of this risk compared to BMI or WC alone [12]. In our study WHtR, although had slightly lower AUC than WC in the ROC curve analysis, it appeared to be significant predictor of prevalent DM and IGM in the backward stepwise analysis.

Amato et al. identified applicable indicator of visceral fat function based on WC, BMI, TGs and HDL cholesterol levels [9]. They called it Visceral Adiposity Index (VAI), and they developed the calculation formula separate for men and women. VAI can be considered as a predictor of cardio-metabolic risk, including diabetes [24, 25]. In our study females with abnormal glucose metabolism had significantly higher VAI score compared to women with NGT. The cut-off point for high metabolic risk (3.17) suggested by Amato et al. for Caucasian women aged ≥ 66 years [10] was in our study significantly associated with both DM and IGM prevalence and, together with WHtR, it was a predictor of prevalent IGM in the backward stepwise analysis.

Elevated TG/HDL cholesterol ratio is considered to be a useful tool in identifying men and women at high cardio-metabolic risk with a cut-off point at 2.5 for females and 3.5 for males [11]. In our study also this metabolic index was significantly higher in women with overt DM and prediabetes compared to females with NGT.

Type 2 DM is considered to be a preventable disease, both through the lifestyle as well as through the pharmacological interventions [26–28]. Early detection of IFG/IGT allows to introduce the efforts to prevent or at least delay diabetes development. Such strategy is not only beneficial for patients, but is also cost-effective

[29]. Early diagnosis of DM as well allows healthcare providers to initiate the treatment earlier in the natural history of diabetes, which gives a chance to avoid the long-term negative consequences of the disease, which was documented in the United Kingdom Prospective Diabetes Study (UKPDS) [30]. In the meta-analysis of 97 prospective studies involving 820,900 individuals, the onset of diabetes at the age of 65 was associated with a shortened life expectancy of almost 5 years [31]. Thus, screening focused on identifying people with abnormal glucose metabolism (in our study over 1/4 of women with DM were unaware of having diabetes and over 80% of women with prediabetes were unaware of this abnormality) can improve long-term prognosis of such persons, increase their life span and may be helpful in maintaining their quality of life. Moreover, it can be cost-effective.

Our study is not free from several limitations. The first one is a relatively small number of participants. The second one is its cross-sectional design, which did not allow us to determine a causal relationship of revealed associations. Also a number of women with the history of CVD events was too small to find more significant associations between analyzed variables and clinical outcomes other than myocardial infarction. Finally, our study was performed solely in Caucasian population. Thus, our results may not be fully applicable to other ethnic groups. On the other hand, our study included representative group of females in the age range 65–74 living in a rural-urban municipality, and a wide spectrum of analyzed variables allowed us to find several factors associated with glucose metabolism abnormalities in this population. We also found potential usefulness of anthropometric and metabolic indices other than BMI and waist circumference.

Conclusions

Diabetes and prediabetes are highly prevalent among women at early elderliness, and many of them were unaware of having these abnormalities.

Assessment of simple anthropometric measurements with the calculation of anthropometric indices seem to be helpful in identifying women at a high probability of having abnormal glucose metabolism. WHtR > 0.6 appeared to be the best predictor of DM, while WHtR > 0.6 and VAI > 3.17 were the best predictors of IGM.

Screening aimed at the detection of diabetes and prediabetes in women with central obesity and impaired lipid metabolism is highly reasonable, and it should be considered as a routine procedure to early diagnose and to early treat women at particularly high CVD risk.

Long-term observation of such a population is required to identify significant predictors of important clinical outcomes (major cardiovascular events, diabetes and cancer incidence, and all-cause death) in the future.

Conflict of interest

All the authors declare no conflict of interest in the field covered by this paper.

Acknowledgement

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REFERENCES

1. International Diabetes Federation. 2017. IDF Diabetes Atlas. Eighth Edition. http://diabetesatlas.org/resources/2017-atlas.html (dostęp z dnia 2019.07.15).
2. Walicka M, Chlebus M, Brzozowska M, et al. Prevalence of diabetes in Poland in the years 2010–2014. Clin Diabetol. 2016; 4(6): 232–237, doi: 10.5603/dk.2015.0031.
3. Sinclair A, Dunning T, Rodriguez-Mañas L. Diabetes in older people: new insights and remaining challenges. Lancet Diabetes Endocrinol. 2015; 3(4): 275–285, doi: 10.1016/S2213-8587(14)70176-7, indexed in Pubmed: 25466523.
4. American Diabetes Association. 4. Comprehensive medical evaluation and assessment of comorbidities: standards of medical care in diabetes — 2019. Diabetes Care. 2019; 42(Suppl 1): S34–S45, doi: 10.2337/dc19-S004, indexed in Pubmed: 30559230.
5. Central Statistical Office. Life expectancy tables of Poland 2016. Statistical Publishing Establishment. Warszawa 2017.
6. Levey AS, Stevens LA, Schmid CH, et al. CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009; 150(9): 604–612, doi: 10.7326/0003-4819-150-9-200905050-00006, indexed in Pubmed: 19414839.
7. Diabetes Poland. 2019 Guidelines on the management of diabetic patients. A position of Diabetes Poland. Clin Diabetol. 2019; 8(1): 1–95, doi: 10.5603/DK.2019.0001.
8. Alberti KG, Eckel RH, Grundy SM, et al. International Diabetes Federation Task Force on Epidemiology and Prevention, National Heart, Lung, and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society, International Association for the Study of Obesity. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009; 120(16): 1640–1645, doi: 10.1161/CIRCULATIONAHA.109.192644, indexed in Pubmed: 19805654.
9. Amato MC, Giordano C, Galia M, et al. AlkaMeSy Study Group. Visceral Adiposity Index: a reliable indicator of visceral fat function associated with cardiometabolic risk. Diabetes Care. 2010; 33(4): 920–922, doi: 10.2337/dc09-1825, indexed in Pubmed: 20067971.
10. Amato MC, Giordano C, Pitrone M, et al. Cut-off points of the visceral adiposity index (VAI) identifying a visceral adipose dysfunction associated with cardiometabolic risk in a Caucasian Sicilian population. Lipids Health Dis. 2011; 10: 183, doi: 10.1186/1476-511X-10-183, indexed in Pubmed: 22011564.

11. Salazar MR, Carbajal HA, Espeche WG, et al. Relation among the plasma triglyceride/high-density lipoprotein cholesterol concentration ratio, insulin resistance, and associated cardio-metabolic risk factors in men and women. Am J Cardiol. 2012; 109(12): 1749–1753, doi: 10.1016/j.amjcard.2012.02.016, indexed in Pubmed: 22449634.
12. Ashwell M, Gibson S. Waist-to-height ratio as an indicator of 'early health risk': simpler and more predictive than using a 'matrix' based on BMI and waist circumference. BMJ Open. 2016; 6(3): e010159, doi: 10.1136/bmjopen-2015-010159, indexed in Pubmed: 26975935.
13. National Center for Chronic Disease Prevention and Health Promotion. 2017. National Diabetes Statistics Report, 2017. Estimates of diabetes and its burden in the United States. https://www.cdc.gov/diabetes/pdfs/data/statistics/national-diabetes-statistics-report.pdf (dostęp z dnia 2019.07.15).
14. Public Health Agency of Canada. Diabetes in Canada: Facts and figures from a public health perspective. https://www.canada.ca/content/dam/phac-aspc/migration/phac-aspc/cd-mc/publications/diabetes-diabete/facts-figures-faits-chiffres-2011/pdf/facts-figures-faits-chiffres-eng.pdf (dostęp z dnia 2019.07.15).
15. Diabetes UK. 2016. Facts and Stats. https://diabetes-resources-production.s3-eu-west-1.amazonaws.com/diabetes-storage/migration/pdf/DiabetesUK_Facts_Stats_Oct16.pdf (dostęp z dnia 2019.07.15).
16. Chen GY, Li L, Dai F, et al. Prevalence of and Risk Factors for Type 2 Diabetes Mellitus in Hyperlipidemia in China. Med Sci Monit. 2015; 21: 2476–2484, doi: 10.12659/MSM.894246, indexed in Pubmed: 26297334.
17. Liu L, Simon B, Shi J, et al. Impact of diabetes mellitus on risk of cardiovascular disease and all-cause mortality: Evidence on health outcomes and antidiabetic treatment in United States adults. World J Diabetes. 2016; 7(18): 449–461, doi: 10.4239/wjd.v7.i18.449, indexed in Pubmed: 27795819.
18. Huang Yi, Cai X, Chen P, et al. Associations of prediabetes with all-cause and cardiovascular mortality: a meta-analysis. Ann Med. 2014; 46(8): 684–692, doi: 10.3109/07853890.2014.955051, indexed in Pubmed: 25230915.
19. Taimour S, Zarrouk M, Holst J, et al. Aortic diameter at age 65 in men with newly diagnosed type 2 diabetes. Scand Cardiovasc J. 2017; 51(4): 202–206, doi: 10.1080/14017431.2017.1319971, indexed in Pubmed: 28434285.
20. Prenner SB, Chirinos JA. Arterial stiffness in diabetes mellitus. Atherosclerosis. 2015; 238(2): 370–379, doi: 10.1016/j.atherosclerosis.2014.12.023, indexed in Pubmed: 25558032.
21. Hua S, Loehr LR, Tanaka H, et al. Ankle-brachial index and incident diabetes mellitus: the atherosclerosis risk in communities (ARIC) study. Cardiovasc Diabetol. 2016; 15(1): 163, doi: 10.1186/s12933-016-0476-4, indexed in Pubmed: 27923363.
22. Gomez-Marcos MA, Gomez-Sanchez L, Patino-Alonso MC, et al. MARK Group. Association between markers of glycemia and carotid intima-media thickness: the MARK study. BMC Cardiovasc Disord. 2016; 16(1): 203, doi: 10.1186/s12872-016-0380-6, indexed in Pubmed: 27793100.
23. Giannini S, Bardini G, Dicembrini I, et al. Lipid levels in obese and nonobese subjects as predictors of fasting and postload glucose metabolism. J Clin Lipidol. 2012; 6(2): 132–138, doi: 10.1016/j.jacl.2011.09.005, indexed in Pubmed: 22385546.
24. Wang Y, He S, He J, et al. Predictive value of visceral adiposity index for type 2 diabetes mellitus: A 15-year prospective cohort study. Herz. 2015; 40 Suppl 3: 277–281, doi: 10.1007/s00059-014-4175-1, indexed in Pubmed: 25410470.
25. Dereziński T, Zozulińska-Ziółkiewicz D, Uruska A, et al. Visceral adiposity index as a useful tool for the assessment of cardiometabolic disease risk in women aged 65 to 74 living in central Poland. Diabetes Metab Res Rev. 2018; 34(8): e3052, doi: 10.1002/dmrr.3052, indexed in Pubmed: 30064156.
26. Diabetes Prevention Program Research Group. Long-term safety, tolerability, and weight loss associated with metformin in the Diabetes Prevention Program Outcomes Study. Diabetes Care. 2012; 35(4): 731–737, doi: 10.2337/dc11-1299, indexed in Pubmed: 22442396.
27. Ley SH, Hamdy O, Mohan V, et al. Prevention and management of type 2 diabetes: dietary components and nutritional strategies. Lancet. 2014; 383(9933): 1999–2007, doi: 10.1016/S0140-6736(14)60613-9, indexed in Pubmed: 24910231.
28. Balk EM, Earley A, Raman G, et al. Combined Diet and Physical Activity Promotion Programs to Prevent Type 2 Diabetes Among Persons at Increased Risk: A Systematic Review for the Community Preventive Services Task Force. Ann Intern Med. 2015; 163(6): 437–451, doi: 10.7326/M15-0452, indexed in Pubmed: 26167912.
29. Breeze PR, Thomas C, Squires H, et al. The impact of Type 2 diabetes prevention programmes based on risk-identification and lifestyle intervention intensity strategies: a cost-effectiveness analysis. Diabet Med. 2017; 34(5): 632–640, doi: 10.1111/dme.13314, indexed in Pubmed: 28075544.
30. Stratton IM, Adler AI, Neil HA, et al. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ. 2000; 321(7258): 405–412, doi: 10.1136/bmj.321.7258.405, indexed in Pubmed: 10938048.
31. Rao Kondapally Seshasai S, Kaptoge S, Thompson A, et al. Emerging Risk Factors Collaboration. Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med. 2011; 364(9): 829–841, doi: 10.1056/NEJMoa1008862, indexed in Pubmed: 21366474.

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Functional activities of neutrophils in diabetic rats are changed by yacon extracts

ABSTRACT

Introduction. Characterization of the polymorphonuclear neutrophils functional activity during the diabetes is of outmost importance for the understanding of the immunological processes involved in disease pathogenesis. The search for effective drugs that would suppress and/or modulate the immune response, preferably without negative effects, is a promising area of modern research. *Smallanthus sonchifolius* Poepp. et Endl., which has long been used in folk medicine to treat diabetes, was proposed as a potential treatment for immune disorders. The study aimed to search for the effective drug which would be able to regulate neutrophils functional activity in experimentally induced type 1 diabetes mellitus.

Material and methods. The experiments were conducted on male Wistar rats. Diabetes was induced by intraabdominal injection of streptozotocin. For the evaluation of phagocytic activity and the content of cationic proteins and myeloperoxidase in neutrophils cytochemical studies were performed.

Results. Diabetes development was accompanied by reduced phagocytic activity of granulocytes and increased activity of myeloperoxidase. All used extracts intensified phagocytic activity of neutrophil in diabetes, with the yacon root tubers extract demonstrating the strongest effect, which was reflected by rapid degradation of particles engulfed by phagocytes.

Conclusions. It was established that yacon extracts inhibit myeloperoxidase activity in diabetes. Therefore all investigated extracts have a pronounced immunocorrective effect and can become the basis for creating a new generation of antidiabetic drugs. (Clin Diabetol 2019; 8, 5: 248–253)

Key words: yacon, leukocytes, phagocytosis, myeloperoxidase, diabetes mellitus

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Introduction

Polymorphonuclear neutrophil (PMN) is directly involved in pathogenesis of diabetes-associated complications. Neutrophils are characterized by decreased phagocytic and bactericidal activities, which lead to an increased susceptibility to infections in patients with diabetes mellitus type 1 (DM) [1]. Among these antimicrobial agents, myeloperoxidase (MPO) is the most abundant and constitutes 5% dry weight of neutrophils and 25% of azurophilic granular proteins [2]. Accept MPO, several other proteins or enzymes are present in neutrophils which also show antimicrobial properties: defensins (major antimicrobial cationic proteins), serine proteases, NADPH oxidase, *etc.* [3]. Yacon (*Smallanthus sonchifolius* Poepp. et Endl.) has long been used in folk medicine to treat diabetes. Active components from this plant are able to stimulate immune defense by promoting antioxidant, antimicrobial, and anticancer effects [4]. This study aimed to search for the most effective yacon extract that would be able to regulate neutrophils functional activity under the experimental DM.

Material and methods

Preparation of water extracts of yacon

Preparation of water extracts of yacon was performed according to Nagalievska et al. [5]. For the research water solutions of evaporated extracts were used.

Induction of diabetes

Following an overnight starvation, diabetes was induced by intraabdominal injection of streptozotocin (STZ, Sigma, USA) (freshly dissolved in 10 mM citrate buffer [pH 5.5]) at a dose of 0.055 g/kg body weight.

Experimental animals

Experiments were conducted using male Wistar rats weighing 150 to 220 g. The rats were kept in the animal house and fed with a standard laboratory diet and water *ad libitum*. Study was carried out according to the "General ethical principles of experiments on animals" (Kyiv, 2001) and the European Convention (Strasbourg, 1986). Animals were randomly divided into following groups (n = 5–8/group): control animals (C); control animals that were treated with extract of yacon leaves (C + Y_L); control animals that were treated with extract of yacon root tubers (C + Y_R); animals with DM (D); animals with DM that were treated with extract of yacon leaves (D + Y_L); animals with DM that were treated with extract of yacon root tubers (D + Y_R). In the D + Y_L and D + Y_R, groups the studied extracts started to be administered on the 14th day after induction of diabetes. Groups C + Y_L, D + Y_L, C + Y_R and D + Y_R were treated by extracts at dose 70 mg/kg per day orally through a tube with preservation of precise dosage for 2 weeks.

Collection of blood and leukocytes separation

At the end of experimental period, the rats were starved for 15 h and then anesthetized using deep ether anesthesia method. Whole blood was collected and immediately transferred to heparinized tubes. Leukocytes were separated on gradient of Histopaque® 1083 (density of 1.083 g/mL).

Cytochemical studies

Phagocytic Activity Assay was conducted according to Levinsky et. al [6]. Based on obtained results next phagocytosis indexes were calculated: PI — phagocytic index — percentage of cells started the phagocytosis, of the total number of cells (PI_{30} and PI_{120} after 30 min and 120 min of incubation, respectively); PN — phagocytic number — average number of yeasts cells that were inside phagocytes (PN_{30} and PN_{120} respectively after 30 min and 120 min of incubation); IPC — index of phagocytosis completeness — division quotient of PN_{30} on PN_{120}. Determination of cationic proteins content was made according to Shubych [7]. Determination of MPO level was performed by Graham-Knol method [8].

Evaluation of cytochemical studies

Evaluation of cytochemical studies were performed by semiquantitative method using Astraldi principle that is based on the differentiation of a specific color varying intensity (0, +, ++, +++). The results were expressed as the average cytochemical coefficient (ACC) — that was calculated using the formula:

$$ACC = \frac{A \times 0 + B \times 1 + C \times 2 + D \times 3}{n}$$

where a number of cells with A — negative reaction, B — poorly positive reaction, C — moderately positive reaction, D — sharply positive reaction, n — a number of counted cells.

Statistical analysis

Quantitative data obtained from the study was performed by arithmetic mean — M, the standard deviation of the arithmetic mean — m. Reliability of the difference between statistical characteristics of two alternative data sets was performed by one-way analysis of variance. The difference was considered significant if $P < 0.05$.

Results

In the case of DM we have established the decrease in PI_{30} by 16.5% and PN_{30} by 19.3% compared to control. A slightly different picture was observed in the case of a longer incubation of neutrophils with yeast cells, in particular it was established the increase in PI_{120} at 28.8%, compared with the control. In animals with DM the increase in average number of yeasts cells inside phagocytes was observed (Table 1). The decrease in IPC in DM by 19.7% was shown compared to control value (Figure 1).

Y_L extract administration to animals with DM resulted in 2.1-fold increase in PI_{30} and PI_{120} growth both in control animals and in animals with DM, respectively at 97.8% and at 75.1% compared to control. Administration of Y_L extract to animals with DM leads to growth of PN_{30} (39.4%) and PN_{120} (8.2%) compared with diabetes. A similar growth of PN_{120} (20.9%) has been demonstrated when the extract was administrated to healthy animals (Table 1). We have established the increase in IPC after Y_L extract treatment compared to diabetic animals (Figure 1).

Using Y_R extract in the control animals cause 20.3% reduction of PI_{30}, with subsequent growth of neutrophils that entered the phagocytosis at 120 min (Table 1).

In DM animals, the administration of Y_R extract caused the PI_{30} increase at 50.0%, compared with untreated DM animals. In the later stages of the phago-

Table 1. Neutrophils phagocytic activity indexes under administration of yacon extracts

	(%)		Number of yeast cells	
	PI_{30}	PI_{120}	PN_{30}	PN_{120}
C	13.17 ± 0.73	14.75 ± 0.88	1.76 ± 0.03	1.53 ± 0.09
C + Y_L	12.83 ± 0.44	29.17 ± 4.78*	1.62 ±0.03	1.85 ± 0.08*
C + Y_R	10.50 ± 0.29*	17.83 ± 1.42	1.71 ± 0.06	1.77 ± 0.07
D	11.00 ± 3.5	19.00 ± 0.50*	1.42 ± 0.08*	1.84 ± 0.08*
D + Y_L	23.50 ± 1.04*#	25.83 ± 1.17*#	1.98 ± 0.04*#	1.99 ± 0.04*
D + Y_R	16.50 ± 1.19*#	20.50 ± 2.46*	1.52 ± 0.04*	1.55 ± 0.16

*P < 0.05 compared with controls; #P < 0.05 compared with diabetic rats; PI_{30} — percentage of cells (phagocytic index) started the phagocytosis, of the total number of cells after 30 min of incubation; PI_{120} — percentage of cells started the phagocytosis, of the total number of cells after 120 min of incubation; PN_{30} — average phagocytic number of yeasts cells that were inside phagocytes after 30 min of incubation; PN_{120} — average phagocytic number of yeasts cells that were inside phagocytes after 120 min of incubation; C — control animals; C + Y_L — control animals that were treated with extract of yacon leaves; C + Y_R — control animals that were treated with extract of yacon root tubers; D — animals with diabetes mellitus (DM); D + Y_L — animals with DM that were treated with extract of yacon leaves; D + Y_R — animals with DM that were treated with extract of yacon root tubers

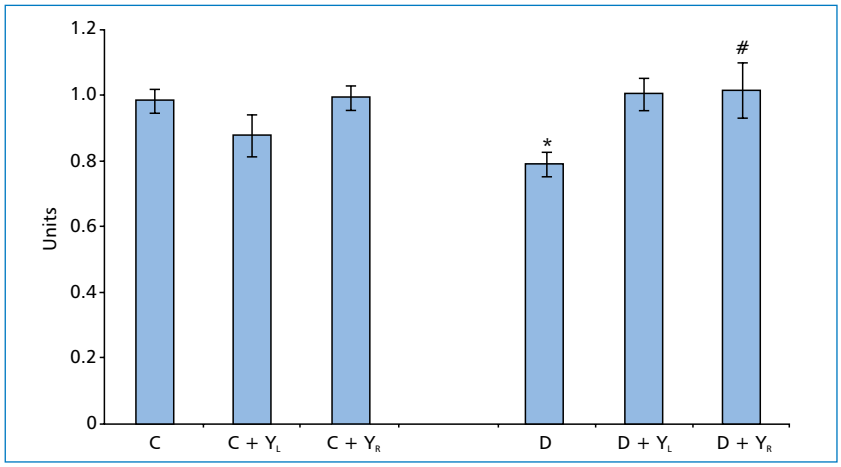

Figure 1. Changes in index of phagocytosis completeness under yacon extracts administration. *P < 0.05 compared with controls; #P < 0.05 compared with diabetic rats; C — control animals; C + Y_L — control animals that were treated with extract of yacon leaves; C + Y_R — control animals that were treated with extract of yacon root tubers; D — animals with diabetes mellitus (DM); D + Y_L — animals with DM that were treated with extract of yacon leaves; D + Y_R — animals with DM that were treated with extract of yacon root tubers

cytosis process (120 minute), the amount of consumed yeast cells were reduced almost to control level (Table 1). Thus, the usage of Y_R extract causes the growth of IPC to 28.5%, comparing to DM (Figure 1).

In animals with experimentally induced diabetes MPO ACC increased by 16.4%, compared to control animals. The use of Y_L and Y_R extracts caused no significant change in myeloperoxidase ACC in control animals. In DM reduction of ACC was established when using Y_L extract (15.3%) and when Y_R extract (11.0%) (Figure 2).

Discussion

The assessment of changes in phagocytic and bactericidal activity of PMN in diabetes and impact of agents with pronounced hypoglycemic effect on neutrophils functional activity is believed to be a promising research area. To test the phagocytic activity, rats' neutrophils were allowed to phagocytize unopsonized yeast cells. Changes in phagocytosis activity indexes (Table 1, Figure 1) indicate that neutrophils require more time for an adequate response to foreign cells and particles [5].

Considering that neutrophils generate low amounts of ATP in their mitochondria and thus rely primarily on glycolytic metabolism, altered glucose levels in diabetes impact on neutrophil function [9].

Abnormal leukocyte function in DM might be caused by the formation of an advanced glycation end products (AGEs). AGEs may cause aberrant signal processing with some serious consequences in terms of stimulus-response coupling, in particular leads to transient actin polymerization, which is required for chemotactic, phagocytic, and secretory responses.

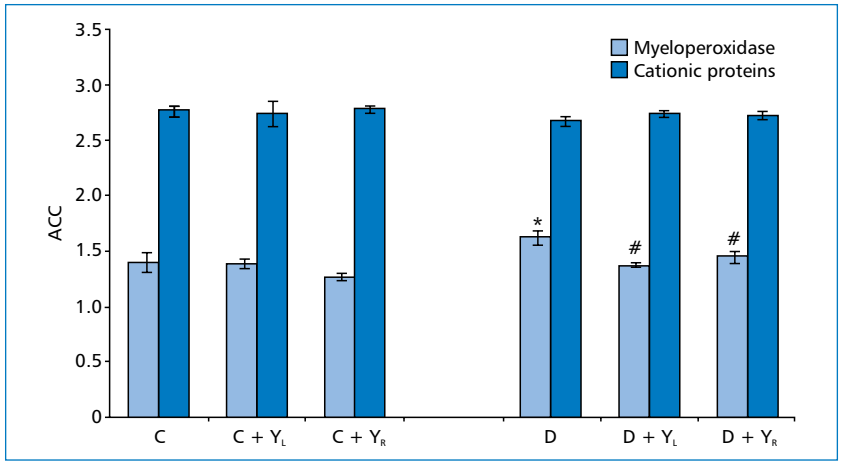

Figure 2. Changes in average cytochemical coefficient (ACC) of myeloperoxidase and cationic proteins under yacon extracts administration. *P < 0.05 compared with controls; #P < 0.05 compared with diabetic rats; C — control animals; C + Y_L — control animals that were treated with extract of yacon leaves; C + Y_R — control animals that were treated with extract of yacon root tubers; D — animals with diabetes mellitus (DM); D + Y_L — animals with DM that were treated with extract of yacon leaves; D + Y_R — animals with DM that were treated with extract of yacon root tubers

The increased number of ingested yeast cells by neutrophils can also be associated with increased concentrations of AGEs. Products of nonenzymatic glycosylation cause the increase in the number of ingested bacteria per cell [10].

Intensification of phagocytosis process under Y_L extract administration may be due to its pronounced hypoglycemic effect. This sugar lowering effect was shown in our previous research. Yacon leaves extracts in the condition of diabetes cause a decrease in glucose concentration by 13.4%, while the root tubers extract caused a decrease in the content of the studied indicator by 49.9% [11]. Established effect may be due to the presence in yacon leaves sesquiterpene lactones, quercetin and other phenolic compounds, which have been shown to exhibit anti-inflammatory activity [12–14].

Demonstrated effect of Y_R extract (Table 1, Figure 1) can be caused by high content in their composition of inulin type β (2 → 1) fructooligosaccharides (FOS) [13]. Short-term supplementation of FOS cause the increase in white blood cells phagocytic activity through binding to carbohydrate receptors [4, 15].

The phagocytic event stimulates the release of agents into the phagolysosome that will kill and digest the ingested microorganism. The non-oxygen-dependent mechanisms include the action of the enzymes contained in granules, such as antibacterial cationic proteins, lysozyme, various proteases, and direct effects of lactoferrin. The oxygen-dependent mechanisms are again of two types: MPO-dependent and MPO-independent. As a part of oxygen-dependent mechanisms of microorganism killing, we investigate the amount of MPO in PMN. Activated neutrophils release MPO from azurophilic granules at the sites of inflammation. MPO is able to interact with diverse ionic, atomic, and molecular entities via the interface with H_2O_2, including $HOCl^-$, hydroxyl radicals, singlet oxygen, ozone, chloramines, and aldehydes. These species are potent oxidants, which under normal and controlled circumstances are toxic to several microorganisms and play an important role in the immune system [2].

The overproduction of MPO by neutrophils from animals with DM (Figure 2) can causes oxidative damage of proteins and DNA in host cells. Thus, the enhanced level of MPO is one of the best inflammatory and oxidative stress markers of diabetes [2]. This enzyme can bind other cell surfaces like epithelial cells, fibroblasts, endothelial cells, macrophages, platelets, neutrophils. The binding of enzyme to these cell surfaces alters some functional properties: interaction with neutrophil integrins causes enhanced tyrosine phosphorylation of some proteins. This activates protein tyrosine kinase, which results in degranulation and leads to respiratory burst. The binding of MPO to platelets causes the reorganization of platelet cytoskeleton, and thus alters the aggregation properties [2, 16].

Reduction of MPO ACC when using Y_L leave extracts (Figure 2) can be caused by the presence in its composition phenolic antioxidants (ferulic acid, chlorogenic acid, gallic acid, caffeic acid, rosmarinic acid and quercetin) and flavonoids (apigenin and luteolin), which can inhibit MPO [13, 17, 18]. Somewhat less pronounced effect on MPO activity possessed Y_R extract that may be due to lower content or lack of components that are capable to inhibit the activity of

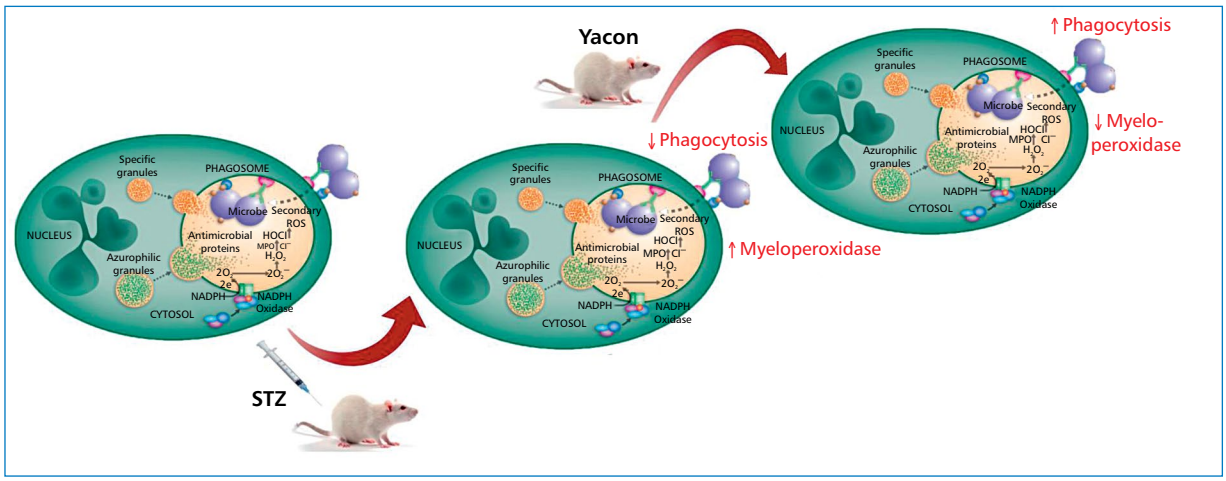

Figure 3. Generalizing scheme of yacon extracts influence on neutrophils functional activity under conditions of experimental diabetes mellitus (modified from Quinn et al. [20])

enzyme. FOS, which are the major biologically active substances in yacon root tubers also tended to reduce MPO activity [4].

In order to obtain additional data on the violation of bactericidal properties of neutrophils in diabetes, we investigate the amount of antibacterial cationic proteins as part of non-oxygen-dependent microbe killing mechanisms [19]. In our studies, we did not find significant change in cationic proteins ACC (Figure 2).

Conclusions

DM development was accompanied by reduced phagocytic activity of granulocytes with activation of oxygen-dependent mechanisms of microorganism killing. All used extracts intensified phagocytic activity of neutrophil in diabetes, with the yacon root tubers extract demonstrating the strongest effect, which was reflected by rapid degradation of particles engulfed by phagocytes. Yacon extracts possess inhibiting effect on myeloperoxidase activity in diabetes (Figure 3).

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Hodgson K, Morris J, Bridson T, et al. Immunological mechanisms contributing to the double burden of diabetes and intracellular bacterial infections. Immunology. 2015; 144(2): 171–185, doi: 10.1111/imm.12394, indexed in Pubmed: 25262977.
2. Khan AA, Alsahli MA, Rahmani AH. Myeloperoxidase as an active disease biomarker: recent biochemical and pathological perspectives. Med Sci (Basel). 2018; 6(2), doi: 10.3390/medsci6020033, indexed in Pubmed: 29669993.
3. Yamaguchi R, Kawata J, Yamamoto T, et al. Mechanism of interferon-gamma production by monocytes stimulated with myeloperoxidase and neutrophil extracellular traps. Blood Cells Mol Dis. 2015; 55(2): 127–133, doi: 10.1016/j.bcmd.2015.05.012, indexed in Pubmed: 26142328.
4. Paredes LLR, Smiderle FR, Santana-Filho AP, et al. Yacon fructans (Smallanthus sonchifolius) extraction, characterization and activation of macrophages to phagocyte yeast cells. Int J Biol Macromol. 2018; 108: 1074–1081, doi: 10.1016/j.ijbiomac.2017.11.034, indexed in Pubmed: 29126938.
5. Nagalievska M, Sabadashka M, Hachkova H, et al. Galega officinalis extract regulate the diabetes mellitus related violations of proliferation, functions and apoptosis of leukocytes. BMC Complement Altern Med. 2018; 18(4): 1–13, doi: 10.1186/s12906-017-2079-3, indexed in Pubmed: 29310643.
6. Levinsky RJ, Harvey BA, Paleja S. A rapid objective method for measuring the yeast opsonisation activity of serum. J Immunol Methods. 1978; 24(3–4): 251–256, doi: 10.1016/0022-1759(78)90129-1, indexed in Pubmed: 363954.
7. Shubych MH. [Identification of cationic protein in the cytoplasm of leukocytes using bromophenol blue]. Tsytolohyia. 1974: 1321–1322.
8. Graham RC, Karnovsky MJ. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructure cytochemistry by a new technique. J Histochem Cytochem. 1966; 14(4): 291–302.
9. Ratter JM, Tack C, Netea M, et al. Environmental signals influencing myeloid cell metabolism and function in diabetes. Trends Endocrinol Metab. 2018; 29(7): 468–480, doi: 10.1016/j.tem.2018.04.008.
10. Collison KS, Parhar RS, Saleh SS, et al. RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs). J Leukoc Biol. 2002; 71(3): 433–444, indexed in Pubmed: 11867681.
11. Horbulinska O, Khokhla M, Hachkova G, et al. [Influence of yacon (Smallanthus sonchifolius Poepp. & Endl.) on rats' blood cells the condition of experimental diabetes mellitus. Visnyk of Lviv University]. Biological Series. 2016; 71: 31–42.
12. Schorr K, Mefort I, Da Costa FB. A novel dimeric melampolide and further terpenoids from Smallanthus sonchifolius (Asteraceae) and the inhibition of the transcription factor NF-κB. Nat Prod Commun. 2007; 2: 367–374.
13. Lachman J, Fernandez EC, Orsak M. Yacon [Smallanthus sonchifolia (Poepp. et Endl.) H. Robinson] chemical composition and use — a review. Plant soil environ. 2003; 49(6): 283–90, doi: 10.17221/4126-PSE.

14. Bub A, Watzl B, Blockhaus M, et al. Fruit juice consumption modulates antioxidative status, immune status and DNA damage. J Nutr Biochem. 2003; 14(2): 90–98, indexed in Pubmed: 12667600.
15. Vogt L, Ramasamy U, Meyer D, et al. Immune modulation by different types of β2→1-fructans is toll-like receptor dependent. PLoS One. 2013; 8(7): e68367, doi: 10.1371/journal.pone.0068367, indexed in Pubmed: 23861894.
16. Gorudko IV, Kostevich VA, Sokolov AV, et al. Functional activity of neutrophils in diabetes mellitus and coronary heart disease: role of myeloperoxidase in the development of oxidative stress. Bull Exp Biol Med. 2012; 154(1): 23–26, doi: 10.1007/s10517-012-1865-7, indexed in Pubmed: 23330081.
17. Luan H, Kan Z, Xu Y, et al. Rosmarinic acid protects against experimental diabetes with cerebral ischemia: relation to inflammation response. J Neuroinflammation. 2013; 10: 28, doi: 10.1186/1742-2094-10-28, indexed in Pubmed: 23414442.
18. Zhang Xu, Huang H, Yang T, et al. Chlorogenic acid protects mice against lipopolysaccharide-induced acute lung injury. Injury. 2010; 41(7): 746–752, doi: 10.1016/j.injury.2010.02.029, indexed in Pubmed: 20227691.
19. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003; 3(9): 710–720, doi: 10.1038/nri1180, indexed in Pubmed: 12949495.
20. Quinn MT, Ammons MC, Deleo FR. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clin Sci (Lond). 2006; 111(1): 1–20, doi: 10.1042/CS20060059, indexed in Pubmed: 16764554.

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A case series of five hypertensive type 2 diabetes patients showing reduction in blood pressure and mean arterial pressure reduction in ambulatory blood pressure monitoring with remogliflozin etabonate 200 mg coprescribed with recent onset anti-hypertensive drugs

ABSTRACT

Introduction. Hypertension is commonly occurring in type 2 diabetes and metabolic syndrome and inflammation are a well-known part of this disease entity. The data of using remogliflozin in Indian patient is not known as this is a very recently approved molecule for the treatment of type 2 diabetes. Here we look into a case series of five patients who had their ambulatory blood pressure monitoring (ABPM) done at baseline and again after 14 days of therapy of adding remogliflozin etabonate to recent onset antihypertensive druges.

Methods. We analysed the ABPM results of five patients after taking their informed consent at baseline and two weeks post-treatment initiation with remogliflozin alongside with recent onset antihypertensive drugs. We used paired t test for statistical analysis of the two readings of each patient to come to a conclusion.

Results. We found a statistically significant decrease in mean arterial pressure (MAP) reflected by a p value of 0.0277 and the reduction in mean awake time systolic blood pressure (SBP) was also very close to statistical significance as seen by the p value of 0.0541.

Conclusions. Remogliflozin etabonate when co-prescribed with antihypertensive drugs shows a significant reduction in MAP as well as reduction in SBP although most of the contribution seems to be coming from the antihypertensive molecule itself. (Clin Diabetol 2019; 8, 5: 254–257)

Key words: type 2 diabetes, remogliflozin etabonate, mean arterial pressure, systolic blood pressure, ambulatory blood pressure monitoring

Introduction

Hypertension is a common entity among diabetic patients, with the prevalence determined by type as well as the duration of diabetes with the other contributing factors being race/ethnicity, sex, age, BMI, presence of kidney disease and glycemic control [1]. A large subset of participants (60%) in the Avoiding Cardiovascular Events Through Combination Therapy in Patients Living With Systolic Hypertension (ACCOMPLISH) trial were at high risk of cardiovascular events and benazepril plus the dihydropyridine calcium channel blocker amlodipine showed a decrease in morbidity and mortality when compared with the benazepril plus

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Table 1. Baseline characters and drugs used in the case series analysis

Serial Number	Age	Sex	Duration of hypertension	Anti-diabetic drugs	Duration of diabetes	Anti-hypertensive drugs	BMI [kg/m^2]	HbA_{1c} (%)
1	62 years	M	3 years	4.5 months	Metformin 1000 mg; glimepiride 1 mg	Olmesartan 20; chlorthalidone 12.5 mg	25.7	7.8
2	63 years	M	4.5 years	4 months	Metformin 1500 mg; glimepiride 2 mg	Telmisartan 40; chlorthalidone 12.5 mg	26.9	7.7
3	60 years	M	4 years	2 months	Metformin 2000 mg; glimepiride 2 mg	Amlodipine 5; chlorthalidone 12.5 mg	27	8.1
4	65 years	M	5 years	2 months	Metformin 2000 mg; teneligliptin 20 mg; glimepiride 1 mg	Chlorthalidone 12.5 mg	28.8	7.3
5	60 years	F	2 years	3 months	Metformin 1500 mg; glimepiride 2 mg	Amlodipine 5; chlorthalidone 12.5 mg	25.3	7.4

the thiazide-like diuretic hydrochlorothiazide [2, 3]. There is a decrease in blood pressure of ~1 mm Hg with the loss in body weight by 1 kg [4]. In recent times SGLT2Is are widely used to control glycemia along-with many other parameters as they have shown multiple benefits including reduction in nephropathy and hypertension. Amongst the SGLT2Is marketed presently in India, remogliflozin is the newest one to get approval for diabetes control in type 2 diabetes. The data of remogliflozin in blood pressure reduction is sparse as the molecule is in its nascent state. Dobbins et al. in their study have shown that administration of remogliflozin etabonate for 12 days leads to clinically significant improvements in plasma glucose as well as changes in body weight and blood pressure in type 2 diabetic patients [5]. This case series is the first of its kind from India demonstrating the marked reduction in SBP, DBP and MAP when remgoliflozin etabonate 200 mg is co-prescribed with anti-hypertensive druges.

Case series presentation

There were five type 2 diabetic patients (after taking proper written informed consent) who had a baseline ambulatory blood pressure monitoring (ABPM) report as well as ABPM report done after two weeks available. All of the patients were put on antihypertensive drugs three days prior to recording the baseline ABPM to see the dipping status of each patient and again the ABPM machine was re-installed after fourteen days of initiating remogliflozin 200 mg to see the final outcome of antihypertensive therapy and diurnal variation after drug initiation. Baseline characters of the patients and the drugs used are depicted in Table 1 who were all put on remogliflzoin 200 mg per day (BID).

Methods

Informed consent was taken from all the participants. All protocols were followed as per declaration laid down in the Declaration of Helsinki.

Statistical analysis

We analysed the clinic data record of the author of five selected type 2 diabetic patients with hypertension who were recently put on antihypertensive drugs and three days after starting it, each one was put on remogliflozin etabonate 200 mg. We used paired t test for comparison between the baseline and post-treatment values of ABPM for each individual. We took a p value of < 0.05 as statistically significant.

Results

This case series showed a reduction in SBP, DBP and a statistically significant drop in MAP after computing the results in paired t-test (Table 2). The p-value of mean changes in SBP during day time was 0.0541, p-value of mean changes in SBP during night time was 0.7828, p-value of mean changes in DBP during day time was 0.0607, p-value of mean changes in DBP during sleep time was 0.1533 and the change in p-value in MAP was 0.0277 which was statistically significant. The mean awake SBP showed a marked reduction with the p-value close to statistical significance ($p = 0.0541$). An interesting finding of this ABPM was a change in the dipping status (Table 3) which was present in both the systolic and diastolic arms at baseline but there was not much change after treatment in the diastolic arm while it actually increased in the systolic arm making it a reverse dipping once the average is seen. The reason

Table 2. Paired t test analysis of baseline and follow-up values of different parameters of ABPM

	Baseline	Follow-Up	Absolute change from baseline	95% CI	P value
Mean awake time SBP [mm Hg]	129 ± 8.09	122 ± 6.98	–6.8	–13.79 to 0.19	0.0541
Mean awake time DBP [mm Hg]	79 ± 6.78	66.4 ± 6.84	–12.6	–26.11 to 0.91	0.0607
Mean sleep time SBP [mm Hg]	123.8 ± 14.77	125.2 ± 23.66	1.4	–11.79 to 14.59	0.7828
Mean sleep time DBP [mm Hg]	72.6 ± 10.83	65.4 ± 10.92	–7.2	–18.56 to 4.16	0.1533
MAP [mm Hg]	94 ± 8.37	85.4 ± 6.11	–8.6	–15.66 to –1.54	0.0277*

*Signifies statistically significant; DAB — diastolic blood pressure; SBP — systolic blood pressure; MAP — mean arterial pressure; CI — confidence interval

Table 3. Dipping property at baseline and after follow-up

Mean awake SBP (B/L) Avg	Mean sleep SBP (B/L) Avg	Mean awake SBP (F/UP) Avg	Mean sleep SBP (F/UP) Avg	Mean awake DBP (B/L) Avg	Mean sleep DBP (B/L) Avg	Mean awake DBP (F/UP) Avg	Mean sleep DBP (F/UP) Avg	% change of SBP	% change of DBP	Dipping status B/L	Dipping status F/UP
129	123.8	122.2	125.2	79	72.6	66.4	65.4	B/L: 4.03 F/UP: –2.45	B/L: 8.1 F/UP: 1.5	Present	Reduced but present for DBP but lost in SBP

Avg — average; B/L — baseline; DBP — diastolic blood pressure; SBP — systolic blood pressure; F/UP — follow-up

to it seems to come from the reading of a single patient who had a very substantial increase in his SBP and DBP readings that surpassed the reduction property seen with the others but the reason of this increase is not known (could have been any severe stress or compliance issue).

Discussion

In lower-income and developing countries we are getting an epidemic in the prevalence of obesity and type 2 diabetes (T2D) attributed to changed lifestyles with high caloric intake and low energy expenditure [6]. India has the highest prevalence of diabetes in the world mostly because of the above mentioned reasons and cheap medicines of the class of SGLT2Is are in huge demand to tackle this epidemic in India as they have multi modalities of action due to their class effect. The Sodium-Glucose Cotransporter 2 (SGLT2) Inhibitor and Angiotensin Receptor Blocker (ARB) Combination Therapy in Patients with Diabetes and Uncontrolled Nocturnal Hypertension study (SACRA) was done to investigate changes in blood pressure with existing antihypertensive therapy and empagliflozin and it showed older diabetes patients who are nonseverely obese having uncontrolled hypertension at night-time had a significant blood pressure reduction without significant reductions in glycemic parameter [7]. In an animal study by Nakano et al. it was seen that out of canagliflozin, dapagliflozin and remogliflozin, only remogliflozin showed a reduction in oxygen radical absorbance capacity (ORAC) as well as there was marked lowering of both serum alanine aminotransferase (76%) and aspartate aminotransferase (48%), and there was also reduction in hepatic triglyceride content by 40% and liver weight by 42% [8]. The use of approved SGLT2 inhibitors was associated with mean reduction of systolic and diastolic blood pressure of 4.0 mm Hg and 1.6 mm Hg, respectively, compared with baseline [9]. This case series is the first data from Indian patients who were co-prescribed remogliflozin and antihypertensive drugs with chlorthalidone being present in all the groups showing improvements in mean SBP and mean DBP and a statistically significant reduction in MAP. There were few limitations of this case series analysis: small sample size makes it impossible to conclude that remogliflozin has potent BP reducing capacity as there was concomitant administration of antihypertensives; there was no chance to intervene in the lifestyle of the patients to find out the modifications they are applying themselves to correct their hypertension other than those prescribed by the author; they also did not tell the author about any illness which might have happened during the time of ABPM recording.

Conclusions

Our case series analysis shows for the first time the possibility of significant SBP and DBP reduction with remogliflozin and antihypertensives but we need large randomised trials or larger real-world data to have a final conclusion. Till that time we can presume that remogliflozin also shares the same pleotropic benefit of BP reductions to some extent like shown by the existing three SGLT2Is. The significant BP reduction shown here may be a chance finding as in real-world setting the BP reductions seen with SGLT2I is much lesser than seen in this case series and hence it is mostly contributed by the antihypertensive therapy that was given.

Conflict of interest

The author declares no conflict of interest.

REFERENCES

1. de Ferranti SD, de Boer IH, Fonseca V, et al. Type 1 diabetes mellitus and cardiovascular disease: a scientific statement from the American Heart Association and American Diabetes Association. Circulation. 2014; 130(13): 1110–1130, doi: 10.1161/CIR.0000000000000034, indexed in Pubmed: 25114208.
2. Weber MA, Bakris GL, Jamerson K, et al. ACCOMPLISH Investigators. Cardiovascular events during differing hypertension therapies in patients with diabetes. J Am Coll Cardiol. 2010; 56(1): 77–85, doi: 10.1016/j.jacc.2010.02.046, indexed in Pubmed: 20620720.
3. Bakris G, Briasoulis A, Dahlof B, et al. ACCOMPLISH Investigators, ACCOMPLISH Trial investigators, ACCOMPLISH Trial Investigators. Benazepril plus amlodipine or hydrochlorothiazide for hypertension in high-risk patients. N Engl J Med. 2008; 359(23): 2417–2428, doi: 10.1056/NEJMoa0806182, indexed in Pubmed: 19052124.
4. Semlitsch T, Jeitler K, Berghold A, et al. Long-term effects of weight-reducing diets in people with hypertension. Cochrane Database Syst Rev. 2016; 3: CD008274, doi: 10.1002/14651858.CD008274.pub3, indexed in Pubmed: 26934541.
5. Dobbins RL, O'Connor-Semmes R, Kapur A, et al. Remogliflozin etabonate, a selective inhibitor of the sodium-dependent transporter 2 reduces serum glucose in type 2 diabetes mellitus patients. Diabetes Obes Metab. 2012; 14(1): 15–22, doi: 10.1111/j.1463-1326.2011.01462.x, indexed in Pubmed: 21733056.
6. Petrie JR, Guzik TJ, Touyz RM. Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. Can J Cardiol. 2018; 34(5): 575–584, doi: 10.1016/j.cjca.2017.12.005, indexed in Pubmed: 29459239.
7. Kario K, Okada K, Kato M, et al. Twenty-Four-Hour Blood Pressure-Lowering Effect of a Sodium-Glucose Cotransporter 2 Inhibitor in Patients With Diabetes and Uncontrolled Nocturnal Hypertension. Circulation. 2019; 139(18): 2089–2097, doi: 10.1161/circulationaha.118.037076.
8. Nakano S, Katsuno K, Isaji M, et al. Remogliflozin Etabonate Improves Fatty Liver Disease in Diet-Induced Obese Male Mice. J Clin Exp Hepatol. 2015; 5(3): 190–198, doi: 10.1016/j.jceh.2015.02.005, indexed in Pubmed: 26628836.
9. Baker WL, Smyth LR, Riche DM, et al. Effects of sodium-glucose co-transporter 2 inhibitors on blood pressure: a systematic review and meta-analysis. J Am Soc Hypertens. 2014; 8(4): 262–75.e9, doi: 10.1016/j.jash.2014.01.007, indexed in Pubmed: 24602971.

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From islets of Langerhans to insulin analogs. It's been almost 100 years since the discovery of insulin

ABSTRACT

Until the discovery of insulin, diabetes was a fatal disease. The diet was the only form of treatment for this disease. Since the discovery of the Langerhans Islands, huge progress has been made in the treatment of diabetes. In 1921 due to Banting's research and collaborators, there has been a revolution in the treatment of diabetes. A disease that was once incurable could be controlled by insulin injections. Today insulin is not only a life-saving medicine. After the breakthrough discovery of scientists, research was conducted to improve insulin. Ultimately, these actions led to the creation of insulin analogs to make diabetes therapy even more perfect, by reducing the incidence of hypoglycemia. (Clin Diabetol 2019; 8, 5: 258–261)

Key words: insulin, diabetes, Paul Langerhans, Frederick Banting, insulin analogs

Introduction

Insulin is an anabolic peptide hormone produced and secreted by the beta cells of the pancreas. This hormone is involved in the metabolism of carbohydrates, fats, and proteins. Moreover, insulin influences genes expression and has an antiapoptotic effect. In people with diabetes, depending on the type of the disease, there is a problem with its production in appropriate amounts or with proper functioning. In 1951, researchers and physicians Lawrence and Bornstein (United Kingdom) measured blood levels of insulin in 10 diabetic patients and recorded major differences in the results of these measurements. Researchers have observed a lack of insulin in young people, while in older and obese people, they found significant amounts of this hormone in the blood [1]. Modern medicine owes the discovery of insulin to many scholars who over the course of several decades have conducted a number of studies aimed at improving insulin therapy. Insulin-dependent diabetes, which for many hundreds of years was a fatal disease, has become a disease that could be treated, controlled more effectively, and reduced the adverse effects of its action on patients. Frederick Grant Banting is a scientist recognized as the discoverer of insulin. However, before him, there were many researchers who explored the topic of diabetes and who have gone down in the history of medicine. Term 'insulin' had been introduced by Jean de Meyer (1878–1934) in 1909 [2]. In this, he was followed by English physiologist Edward Albert Sharpey-Schafer (1850–1935), who in 1913, in lectures at Stanford University, he suggested the name 'insuline' for a substance in the islets of Langerhans then unknown [3]. Using radioimmunoassay technology, Solomon Berson, and Rosalyn Yalow in 1960 developed a method for measuring insulin in the blood. They observed that some people with diabetes still make their own insulin, and on this basis, they determined the occurrence of 'insulin-dependent' and 'non-insulin-dependent' diabetes [4].

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The islets of Langerhans, or how did it begin?

The history of insulin therapy begins in 1869. The German 22-year-old medical student Paul Langerhans (1847–1888), while working on a microscope, identifies for the first time a group of cells within the pancreas, about which he writes: "(…) small cells of almost perfect homogeneous content and of a polygonal form, with round nuclei without nucleoli, mostly lying together in pairs or small groups" [5]. Langerhans admitted that he did not know the function of these special cells. The discovery of a student from Berlin did not find interest among scholars for many years, until 1893 when Gustav Laguesse bent over them. A group of cells was named by him 'Langerhans islands'. The scientist proposed that the pancreatic islets may play the endocrine role and produce a substance that reduces blood glucose levels [6]. The concept of 'pancreatic diabetes' was introduced in 1877 by Frenchman Etienne Lancereaux (1829–1910) [7]. In 1901, pathologist Eugene Lindsay Opie (1873–1971) described the occurrence of hyaline degeneration within the Langerhans islands in diabetes. At the same time, he hypothesized that these changes may condition the occurrence of the disease [8]. Despite the above-mentioned discoveries, the substance produced by the pancreas has not yet been used as a drug in people with diabetes. However, the researchers attempted to isolate island secretion, which proved to be ineffective. The German physician George Ludwig Zuelzer (1870–1949) obtained and applied pancreatic extract for the first time in 1906. The results of the experiment were promising at first because the sugar was reduced or the total disappearance of sugar from the urine was observed. However, the preparation caused side effects in the form of allergic-toxic reactions and hypoglycemia [9]. On 28 May 1912, Zuelzer patented his method in the United States. In contrast, Dr. Ernest Scott (1877–1966) (University of Chicago) in 1911 and Israel Kleiner (1885–1966) (Rockefeller University in New York) in 1919, giving an aqueous extract of the pancreas to dogs, obtained a temporary reduction of glucose level in urine and blood [10, 11]. Romanian professor of physiology Nicolas Paulesco (1869–1931) (Medical University of Bucharest) in 1916 refined the acquisition of aqueous extract from the pancreas, which in diabetic dogs had a normalizing effect on sugar level in the blood and urine. In addition, he observed a decrease in urea in the blood and urine. In 1921 Paulescu published his data in two prominent French journals, eight months before the first publication of Banting and Best from February 1922 [12]. Unfortunately, the work of scientists interrupted the outbreak of World War I or the disapproval of university authorities. Despite attempts to isolate the substance that reduces blood glucose, it has not been able to be used in clinical practice. However, these studies have had a huge impact on further work leading to the final isolation of insulin and its administration to the patient. By the time of significant progress in the treatment of diabetes, i.e. the nineteenth and twentieth centuries, it was not possible to determine what causes diabetes, and it was not known how to treat it. People with diabetes died from exhaustion and due to complications. The only known treatment at that time was diet.

The breakthrough time has come...

The discovery of insulin by Canadian scientists changed the treatment of diabetes completely and improved the prognosis of patients. It was a breakthrough moment, a milestone in the history of diabetes and one of the greatest achievements in medicine. In addition to research activities, Frederick Grant Banting (1891–1941), a young orthopedic surgeon, lectured for medical students. In October 1920, when preparing for a lecture on the subject of the pancreas, he found an article about *pancreatolithiasis*. Due to the mechanical blockade of pancreatic ducts, the disease caused atrophy of cells secreting digestive enzymes, but the function of the islets of Langerhans remained unchanged. Mering and Minkowski, in their experiments, ligated pancreatic ducts in dogs, which caused similar results. This prompted Banting to repeat the experiment. The dogs examined had the pancreas removed or ligated pancreatic ducts. The pancreas with ligated ducts underwent the extraction process and the solution he obtained was injected into diabetic dogs. Banting observed a short-term reduction in the glucose level in the blood and urine, which resulted from contamination of the extract by digestive enzymes. In addition, adverse reactions at the injection site in the form of abscesses were observed due to the administration of a poorly purified pancreatic extract. Banting's colleague suggested re-conducting experiments in a specialized laboratory at the University of Toronto under the supervision of medical professor John James Richard Macleod (1876–1935), a well-known Scottish physiologist and diabetes specialist from Aberdeen. Macleod was one of the greatest specialists in the field of carbohydrate metabolism. In the spring of 1921, Macleod agreed to make the laboratory and experimental animals available to Banting and offered him the assistance of two medical students, Charles Herber Best (1899–1978) and Edward Clark Noble. Best became Banting's closest associate, and their work in the Toronto laboratory resulted in exceptional results. The extract obtained by the researchers, which they called the isletine, was

subjected to purification at the request of Professor Macleod. This task belonged to the Canadian biochemist James Bertram Collip (1892–1965). Obtaining a maximum purified and minimally toxic pancreatic extract allowed for its clinical use and subcutaneous administration to a diabetic patient. This treatment saved the life of 14 year-old Leonard Thompson, a native of Toronto. The boy had diabetes for three years and his body weight was only 30 kilograms. Leonard was in a diabetic coma, in which he collapsed and woke up alternately. His father agreed to give his son a newly discovered drug, previously tested on animals. The first injection of insulin occurred on 11 January 1922. However, its first clinical application did not bring about a spectacular effect, because only a 25% reduction in glucose was obtained. In addition, glucose and ketone bodies remained in the urine of the patient. An abscess appeared at the injection site, and the patient developed a severe allergic reaction to the newly discovered drug. Insulin therapy was considered ineffective, but the researchers decided to improve the effectiveness of the discovered drug. Cooperation with Connaught Laboratories allowed for achievement the more purified insulin. On 23 January 1922, the obtained preparation was given to the boy in several injections a day. Then the expected effect was observed — the glucose concentration dropped significantly in the blood and urine. Also, the symptoms of diabetic acidosis have disappeared, which was accompanied by the complete disappearance of ketone bodies in the urine. The boy died at the age of 27, but the cause of his death was not diabetes and its complications, but pneumonia. Researchers continued testing the extract and in February 1922 qualified six people for treatment, but in these cases, the treatment was successful [13–19]. For the first time, the researchers announced the results of their work in May 1922 during the meeting of the Association of American Physicians. Discoverers were represented by Macleod, who talked about the discovery and use of insulin. Soon the production of insulin on a large scale began. The first company that chose to do this was the Connaught Laboratories in Indianapolis, but the insulin they produced was ineffective clinically. Another company that took up the challenge was Eli Lilly from Toronto. They were stronger than their predecessors because production was very large, but also their activity proved to be ineffective. In November 1922, the cause of the lack of efficacy of insulin produced by Lilly was discovered. Their chief chemist George Walden found that insulin was reduced in purity due to precipitation at the improper pH [20]. However, failures in the production process were not the only obstacles. These were eliminated, while issues such as production standardization or patent law remained to be resolved. The interest in insulin has reached large proportions and its production has been taken up by numerous scientific and commercial centers. One of them was a non-profit body, the Nordisk Insulin Laboratory, which is currently one of the leading insulin companies. This undertaking was initiated in 1923 by an eminent physiologist Nobel laureate for research on metabolism and physical effort, August Krogh from the University of Copenhagen, whose wife was diabetic. During a meeting with Banting and Macleod, he got the knowledge of insulin and received authorization from the University of Toronto to bring insulin to Scandinavia [21]. During the work on the discovery of insulin, there were conflicts between the members of the research team. The source of the conflict was the first public report on the results of the experimental treatment of diabetes in animals with a partially purified solution of insulin. Macleod wanted to be recognized as a leading researcher, while Banting's position in the medical world was not strong enough. However, both scientists were noticed and honored. In 1923, Banting and MacLeod received the Nobel Prize in medicine and physiology. However, they did not forget about their closest collaborators, without which we would not use insulin today. Banting donated half of the award to Charles Herbert Best, while MacLeod shared the prize with James Bertram Collip. Never before has a scientist received the Nobel Prize in such a short time after the discovery.

From NPH insulin to analogs

Insulin derived from animals has been used to treat diabetes for many years. Despite the effectiveness and increase in survival, insulin was not devoid of imperfections due to the occurrence of allergic reactions in many patients. Research has been carried out on improving insulin. One of the shortcomings of insulin therapy at the time was the necessity of administering the drug in several injections a day. The researchers sought to create long-acting insulin. The year 1936 is the time when the long-acting insulin, containing an admixture of protein — protamine, was first developed. This was accomplished by the assistant of August Krogh, Hans Christian Hagedorn. The preparation was named as insulin with a prolonged action type NPH (neutral protamine Hagedorn) [22]. At the same time, in Toronto Scott and Fisher further prolonged the effect of insulin by adding zinc [23]. These findings led to the launch of longer-acting animal insulins. The world of science got to know insulin more and more. In 1955, a scientist from the University of Cambridge, Frederick Sanger determined the amino acid sequence of insulin, for which he received the Nobel Prize [24]. The year 1958

is also a breakthrough in genetic engineering because insulin as the first protein was successfully synthesized chemically as the first drug produced by genetic engineering methods. In 1959, Paul Lacy confirmed with immunohistochemistry that the cell responsible for the production of insulin is the β cell [25]. In 1969, Dorothy Hodgkin of the University of Cambridge determined the spatial structure of the insulin molecule, for which she was awarded the Nobel Prize.

The era of human insulins and their analogs have come. In 1978, David Goeddel, Ph.D., and colleagues at Genentech prepared the first recombinant human insulin by utilizing and combining the insulin A- and B-chains expressed in Escherichia coli. Afterward, Genentech and Lilly signed an agreement to commercialize human insulin produced by recombinant DNA. The first recombinant insulins launched in 1982 were Humulin R (short-acting) and N (NPH, intermediate-acting) [26]. To make diabetes therapy even more perfect, by reducing the incidence of hypoglycemia, insulin preparations were sought that mimicked the physiological secretion of insulin. The change in pharmacokinetics due to the modification of the amino acid site in insulin led to the formation of insulin analogs. These insulins were characterized by faster absorption and shorter duration of action. The first rapid-acting analog of insulin introduced to the market in 1996 was lispro. In 2000 and 2005, aspart and glulisine were introduced respectively. In contrast, long-acting insulin analogs were approved in 2000 (glargine) and 2005 (detemir). The next generation of insulin analogs are ultralong-acting insulin analogs glargine U300 and degludec (2015).

Conclusions

Over the decades, there has been undeniable progress in the treatment of diabetes. The disease, once considered deadly, become a chronic disease. However, complications of diabetes continue to be an important problem. Insulin has become a drug of opportunity for people with diabetes, and its continuous refinements have made metabolic control of the disease often achieved perfectly.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Bornstein J, Lawrence RD. Plasma insulin in human diabetes mellitus. Br Med J. 1951; 2(4747): 1541–1544, doi: 10.1136/bmj.2.4747.1541, indexed in Pubmed: 14879146.
2. De Meyer J. Action de la sécrétion interne du pancréas sur différents organes et en particulier sur la sécrétion rénale. Arch Fisiol. 1909; 7: 96–99.
3. Schäfer EA. An introduction to the study of the endocrine glands and internal secretions. Stanford University, Palo Alto 1914: 18, 84, 86.
4. Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. J Clin Invest. 1960; 39: 1157–1175, doi: 10.1172/JCI104130, indexed in Pubmed: 13846364.
5. Sakula A. Paul Langerhans (1847–1888): a centenary tribute. J R Soc Med. 1988; 81(7): 414–415, indexed in Pubmed: 3045317.
6. Goet JP. Gustave Edouard Laguesse; his demonstration of the significance of the Islands of Langerhans. Diabetes. 1953; 2(4): 322–324, doi: 10.2337/diab.2.4.322, indexed in Pubmed: 13083474.
7. Lancereaux E. Note et réflexions sur deux cas de diabète sucré avec alteration du pancréas. Bull Acad Méd. 1877; 6(2): 1215.
8. Opie EL. The relation oe diabetes mellitus to lesions of the pancreas. Hyaline degeneration of the islands oe langerhans. J Exp Med. 1901; 5(5): 527–540, doi: 10.1084/jem.5.5.527, indexed in Pubmed: 19866956.
9. Zuelzer G. Ueber Versuche einer specifischen Fermenttherapie des Diabetes. Zeitschrift fü Experimentelle Pathologie und Therapie. 1908; 5(2): 307–318, doi: 10.1007/bf02661302.
10. Scott E. On the influence of intravenous injections of an extract of the pancreas on experimental pancreatic diabetes. Am J Physiol. 1912; 29(3): 306–310, doi: 10.1152/ajplegacy.1912.29.3.306.
11. Kleiner IS. The action of intravenous injections of pancreas emulsions in experimental diabetes. J Biol Chem. 1919; 40: 153–170.
12. Ionescu-Tirgoviste C, Buda O. Nicolae Constantin Paulescu: the first explicit description of the internal secretion of the pancreas. Acta Med Hist Adriat. 2017; 15(2): 303–322, doi: 10.31952/amha.15.2.8, indexed in Pubmed: 29402120.
13. Tan SY, Merchant J. Frederick Banting (1891–1941): Discoverer of insulin. Singapore Med J. 2017; 58(1): 2–3, doi: 10.11622/smedj.2017002, indexed in Pubmed: 28111693.
14. Stylianou C, Kelnar C. The introduction of successful treatment of diabetes mellitus with insulin. J R Soc Med. 2009; 102(7): 298–303, doi: 10.1258/jrsm.2009.09k035, indexed in Pubmed: 19605862.
15. Vecchio I, Tornali C, Bragazzi NL, et al. The discovery of insulin: an important milestone in the history of medicine. Front Endocrinol (Lausanne). 2018; 9: 613, doi: 10.3389/fendo.2018.00613, indexed in Pubmed: 30405529.
16. Karamanou M, Protogerou A, Tsoucalas G, et al. Milestones in the history of diabetes mellitus: the main contributors. World J Diabetes. 2016; 7(1): 1–7, doi: 10.4239/wjd.v7.i1.1, indexed in Pubmed: 26788261.
17. Rosenfeld L. Insulin: discovery and controversy. Clin Chem. 2002; 48(12): 2270–2288, indexed in Pubmed: 12446492.
18. Unger J. Diabetes managementt in primary care. Lippincott Williams & Wilkins, Philadelphia 2007.
19. Holt RIG, Cockram C, Flyvbjerg A, et al. Textbook of diabetes. Fifth edition. Wiley-Blackwell, Hoboken 2016.
20. Hilgenfeld R, Seipke G, Berchtold H, et al. The evolution of insulin glargine and its continuing contribution to diabetes care. Drugs. 2014; 74(8): 911–927, doi: 10.1007/s40265-014-0226-4, indexed in Pubmed: 24866023.
21. Poulsen JE. The impact of August Krogh on the insulin treatment of diabetes and our present status. Acta Med Scand Suppl. 1975; 578: 7–14, indexed in Pubmed: 1098401.
22. Hagedorn HC, Jensen BN, Krarup NB. Protamine insulinate. J Am Med Assoc. 1936; 106(3): 177, doi: 10.1001/jama.1936.02770030007002.
23. Scott D, Fisher A. The prolongation of insulin action by protamine and zinc. Proc Am Soc Biol Chem. 1936; 88: 8.
24. Berg P. Fred Sanger: a memorial tribute. Proc Natl Acad Sci U S A. 2014; 111(3): 883–884, doi: 10.1073/pnas.1323264111, indexed in Pubmed: 24449828.
25. Lacy PE. Electron microscopy of the islets of Langerhans. Diabetes. 1962; 11: 509–513.
26. Goeddel DV, Kleid DG, Bolivar F, et al. Expression in Escherichia coli of chemically synthesized genes for human insulin. Proc Natl Acad Sci USA. 1979; 76(1): 106–110, doi: 10.1073/pnas.76.1.106, indexed in Pubmed: 85300.