SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Cerebral and peripheral microvascular alterations in metabolic diseases

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	Tamás Csonka, MD, PhD

The Examination takes place at the Library of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
8th April 2025, at 11:00

Head of the **Defense Committee**:	Prof. Csongor Kiss, MD, Phd, DSc
Reviewers:	János Tibor Kiss, MD, Phd
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Members of the Defense Committee:	János Tibor Kiss, MD, Phd
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	Tamás Csonka, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
8th April 2025, at 13:00

INTRODUCTION

Atherothrombotic cerebrovascular disorder (CVD) is a major risk factor for the development of neurodegenerative diseases due to its adverse effects on the cerebral blood flow (CBF), which is a major cause of cognitive decline. Dementia-related memory impairment, weakened executive function and difficulties in activities of daily living lead to significant deterioration in quality of life and reduced life expectancy. Already high mortality rates of cerebral circulation disorders are expected to increase in the coming years according to epidemiological forecasts, which will impose a huge burden on societies and healthcare structures worldwide. In light of all this, research in this field is becoming increasingly important.

Several clinical tests prove the close connection between CVD, type 2 diabetes mellitus (T2DM) and obesity. Inadequately treated T2DM and the resulting hyperglycaemia, as well as abnormal cerebrovascular processes related to ischaemia and obesity are shown to be associated with brain perfusion abnormalities. Although the molecular background of the changes in cerebral microcirculation triggered by metabolic diseases is not perfectly known, different pathophysiological factors that accompany T2DM and obesity may explain the close association between central microvascular damage and metabolic diseases. Persistent hyperglycaemia, insulin resistance (IR), abnormal insulin signalling, chronic, mostly subclinical inflammatory conditions, and other metabolic disorders resulting from obesity all contribute to altered rheological states, which are hallmark characteristics of these diseases.

In metabolic diseases, a number of well-known, traditional risk factors may be primarily responsible for the development of CVD, such as hypertension, dyslipidaemia, smoking, advanced age and abnormal weight gain along with the resulting diabetes mellitus. However, in light of significant residual risk that is not related to any of the above factors, so-called "non-traditional" risk factors associated with metabolic diseases have been identified in recent years. The results of certain studies have established a correlation between the frequency/severity of neuropathy (NP) in type 1 diabetes mellitus (T1DM) and impaired secretion of the connecting-peptide (C-peptide). Other studies have identified an increase in the amount of visceral adipose tissue (VAT) as a risk factor for metabolic and atherosclerotic diseases.

Early microvascular changes in the brain associated with metabolic diseases can be a potential indicator of a number of neuro-psychiatric diseases, consequently their timely diagnosis is crucial. In current clinical practice computer tomography (CT) and magnetic resonance imaging

(MRI) are routinely used instruments in the examination of cerebral structural abnormalities. However, the opportunities offered by nuclear medicine should also be utilised accordingly. Single-Photon Emission Computed Tomography (SPECT) is an imaging technique that can be used in the detection of functional and brain perfusion impairments. The most promising radiopharmaceutical of isotope diagnostics is Technetium-99m hexamethylpropyleneamine oxime ([99mTc]Tc-HMPAO), which offers a wide range of possibilities for performing organ perfusion examinations, including the study of brain circulation and the microcirculation of certain muscles through the detection of decreased isotope uptake representing reduced perfusion. In addition to resting brain perfusion tests, the transcranial Doppler (TCD) breath holding test (BHT) is recommended to measure the percentage of provoked regional CBFs and cerebrovascular functional reserve (CVR). Non-invasive Doppler methods can also be used to examine carotid arteries; applying carotid ultrasonography (CUS) to measure the severity of atherosclerotic lesions of the carotid artery, intima-media thickness (IMT/c-IMT), the presence of plaques, and the degree of stenosis or occlusion. Since the IMT of the common carotid artery, measured by B-mode CUS, correlates well with the prevalence as well with the risk of cardiovascular diseases. IMT is considered a potential marker of early stage atherosclerosis.

Apart from the central involvement, peripheral vasculopathy in the form of neuropathy (NP) is another pathological condition associated with metabolic diseases. Peripheral neuropathy (PNP) affects more than half of diabetic patients but has also been shown to be closely linked to obesity. Serious symptoms related to PNP, such as lower limb pain, sensory impairment/loss of sensory function, or gait instability, can lead to reduced quality of life and, in severe cases, permanent disability. Leg ulcers that develop as a result of NP are responsible for a significant proportion of lower limb amputations due to metabolic diseases. To assess peripheral nerve function, the Neurometer®-based current perception threshold (CPT) measurement is recommended to provide a non-invasive assessment of peripheral nerve fibre conduction and functional integrity.

In addition to imaging diagnostics, the regular monitoring of laboratory parameters remains the cornerstone of clinical care for metabolic diseases, primarily diabetes mellitus. Apart from routinely used glycaemic indicators such as plasma glucose, insulin and glycated haemoglobin (HbA1c), the measurement of C-peptide levels has recently received increasing attention. The primary reason for this is that this protein being the product of proinsulin cleavage released from the pancreas, accurately represents beta-cell function and can therefore help therapy decisions. Recent research has also revealed that besides this well-known part, C-peptide plays a beneficial role in the prevention/improvement of diabetic vasculopathy. C-peptide seems to be promising in treating microvascular complications in T1DM and the advanced stages of T2DM, though this correlation has not been fully clinically established. It should be noted however that its role in the early stages of the T2DM is not yet clearly understood.

As detailed above, microvascular changes caused by T2DM and obesity may entail serious adverse consequences on health, quality of life and life expectancy through both central and peripheral involvement. Deeper understanding of microcirculation and sensory nerve function with a particular focus on identifying the relationships with laboratory parameters and anthropometric data characterising glucose homeostasis is becoming a central issue in current medical research and is also the subject of our ongoing investigations.

AIMS OF THE STUDY

1. The aim of our investigations and the subject of my thesis, was to assess the central microcirculation in patients with T2DM and obesity using several approaches ([99mTc]Tc-HMPAO SPECT and TCD – BHI) and to assess the condition of the main cervical artery (carotid ultrasonoraphy – IMT).

2. We looked for connections between cerebral blood flow and patients' anthropometric indices, CT-based abdominal fat segmentation data and various laboratory parameters characterising glucose homeostasis.

3. We also examined lower limb and central microcirculation in these patients using HMPAO SPECT(/CT) and peripheral sensory nerve function using Neurometer®. Through these studies, we sought to answer the question of how lower limb microcirculation changes in PNP associated with metabolic diseases and whether these abnormalities are accompanied by changes in central microcirculation.

4. Finally, we aimed to identify complex connections between the results of these perfusion and conduction tests and patients' anthropometry and glycaemic indexes.

PATIENTS AND METHODS

Patients

A total of 99 people participated in the part of our study where central microcirculation was examined and 72 in the peripheral microcirculation part. Patient composition in the former part was 52 patients with T2DM (32 men and 20 women) and 47 patients with obesity (21 men and 26 women), while in the latter part it was 40 patients with T2DM (24 men and 16 women) and 32 patients with obesity (14 men and 18 women).

Patients were recruited from the Division of Metabolic Diseases, Department of Internal Medicine, Faculty of Medicine, University of Debrecen (Debrecen, Hungary) and from a private general practice in Miskolc (Miskolc, Borsod-Abaúj-Zemplén county, Hungary). The inclusion criteria for the study were: age between 18 and 70 years and no history of mental disorder or structural/functional disease of the central nervous system (CNS).

Participants were divided into two groups: obese (BMI > 30 kg/m², without confirmed T2DM) and T2DM (regardless of BMI) patients. Patients were under regular medical check-up in a specialist clinic/hospital setting. For the T2DM group, care was given according to current national diagnostic and therapeutic guidelines at the time of the study.

Exclusion criteria for the study were: pregnancy, breastfeeding, current acute or chronic inflammatory condition and liver disease, oral steroid or retinoid treatment, history of hyperthyroidism or inadequately controlled hypothyroidism, history of malignancy (excluding basocellular carcinoma), diagnosed peripheral arterial disease (PAD) (confirmed by physical examination or handheld Doppler ultrasonography), presence of a crural ulcer at the time of the study, long-term anticoagulant therapy or change in regularly taken medications within six months prior to the study.

Prior to inclusion participants were given detailed information concerning the main aims of the study as well as the investigations performed. Each patient signed the informed consent (OGYEI/2829-4/2017).

Anthropometric parameters

The following anthropometric parameters were measured: height (cm), weight (kg), body mass index (BMI; kg/m²) and age (years). Height was measured in a standing position with arms down at the sides and without shoes using a standardised medical height measuring device. Body weight was determined in light clothing, without shoes, using a standard digital scale (MMSZ1, MicraMetriopond Ltd., Hódmezővásárhely), and values were recorded rounded to 100 g. BMI was calculated as the ratio of weight in kilograms squared to height in metres. The age of the participants was also recorded.

Laboratory parameters

Blood samples for laboratory tests were taken from all patients after eight hours of fasting. Four laboratory markers were measured to assess the patients' glucose homeostasis: plasma glucose, HbA1c, insulin and C-peptide. Fasting glucose levels were determined using plasma samples containing sodium fluoride potassium oxalate (NaF-KOx) (reference range: 3.6-6 mmol/L). HbA1c levels were measured by high-performance liquid chromatography (HPLC; BioRad, Hercules, CA, USA) from whole blood samples anticoagulated with K_3-EDTA (reference range: 4.2-6.1%), whereas insulin (reference range: 4.3-20 mU/L) and C-peptide (reference range: 350-1170 pmol/L) concentrations were determined from native plasma samples.

Assessment of abdominal fat distribution

To determine the ratio of VAT to SAT, transaxial low-dose CT images were acquired using the CT component of the AnyScan® PET/CT (Mediso Ltd., Budapest, Hungary) hybrid device with the following parameters: 120 kW and 100 mAs. Abdominal fat segmentation was performed using a semi-automated method. A transaxial CT slice fixed at the level of the 1st lumbar (L1) vertebra was selected for image processing. Subsequently, the region of interest (ROI) representing the body contour and the abdominal cavity was manually marked on the CT scans. Selection of adipose tissue was performed in the range -190 to -30 HU. SAT and VAT were quantified based on the number and volume of pixels segmented within the ROIs. (Seven of the participants did not participate in the CT imaging.)

Perfusion examinations with SPECT

Peripheral perfusion

Lower limb perfusion was determined by SPECT/CT imaging. The scan was performed 15 minutes after intravenous administration of approximately 709±42 MBq [99mTc]Tc-HMPAO into the right cubital vein of the patients (Mediradiopharma, Budapest, Hungary) in supine position with the AnyScan® TRIO SPECT/CT device (Mediso Ltd., Budapest, Hungary). The scan was performed proximal to the foot, including the calf muscles, on a 40 cm long area on both lower limbs. Thirty minutes prior to imaging, all subjects received 1000 mg of perchlorate capsules orally to block unwanted thyroid radiotracer uptake. Low-dose CT was used for radiosensitization correction, and images were post-processed using the Tera-Tomo Q SPECT reconstruction method (Mediso Ltd., Budapest, Hungary). Lower limb tracer uptake was quantified from VOIs of 1 cm diameter placed on the sural muscles. Then, the mean of the standardized radiofrequency uptake values (standard uptake value mean/SUV_{mean}) in each VOI was determined using InterView™ FUSION image analysis software (Mediso Ltd., Budapest, Hungary).

Cerebral perfusion

Following lower limb imaging with [99mTc]Tc-HMPAO SPECT/CT, a standard cerebral perfusion SPECT examination was performed with AnyScan® S SPECT camera (Mediso Ltd., Budapest, Hungary) as part of a joint study to assess the microcirculation of the CNS. Prior to the cerebral imaging no further radiopharmaceuticals were administered. To achieve baseline relaxation, participants rested for 10 minutes in a stress-free, dimly lit room before brain imaging. (Six of the study participants did not participate in the perfusion SPECT imaging procedure.)

Carotid intima-media thickness (c-IMT)

To determine the c-IMT, we used a Philips HD 11 XE ultrasound machine with a 7.5 MHz linear transducer. After enlarging the image obtained at the end of the diastole, IMT was determined on the wall of the common carotid arteries farthest from the transducer, 10 mm proximal to the carotid bulb on both sides. IMT was measured by millimetre along a 1 cm section of both arteries. A total of 20 measurements were performed on the carotid arteries of the right and left artery; the mean and maximum IMT calculated from the resulting IMT values were provided in case of every patient.

Cerebrovascular reactivity (CVR) – breath-holding test (BHT)

For the BHT, transcranial Doppler studies (DWL, Multi-Dop X, serial number MDX-1156) were performed with a 2 MHz probe. To determine the flow parameters, the probe was placed on the temporal cranial window of the patient at rest, measuring the mean flow velocity (MFV) in the bilateral middle cerebral artery (MCA) at three different test depths: 45, 50 and 55 mm. The optimal acoustic window was identified by recording the pulse waveform of the corresponding shape, after which patients were asked to hold their breath for 30 s. To characterize the velocity profile of the MCA, the mean flow velocities before breath-hold (MFV baseline) and at the end of breath-hold (MFV end) were used to calculate the breath-holding index (BHI). The BHI was calculated using the following formula:

BHI = [(MFV end – MFV baseline) / MFV baseline] × 100/breath-holding time(s).

The resulting percentage surpasses 30% in healthy adults. BHI results between 20 and 30% refer to reduced CVR, while BHI values under 20% indicate the lack of cerebral vasomotor reactivity.

Peripheral nerve conduction tests

The function of lower limb sensory neurons was quantified by determining the CPT values of the big toes and thumbs with Neurometer® (NM-01/CPT; MSB-MET Ltd., Walldorf, Germany). Transcutaneous electrical stimulation of the big toes (5^{th} lumbar fiber (L5)) and thumbs (6^{th} cervical fiber (C6)) was induced manually through electrodes coated with a conductive gel placed on the skin surface; thus, stimulation of the peroneal and median nerves was performed at 3 different frequencies (2000 Hz, 250 Hz and 5 Hz). Then, to automatically generate CPT values, participants were asked to press a button when they felt the stimulus. The mean CPT values for the peroneal nerve were 330±79.5 at 2000 Hz, 123±38.8 at 250 Hz, and 76±31.9 at 5 Hz; for the median nerve, they were 230±55.1 at 2000 Hz, 88.5±27.8 at 250 Hz, and 47.5±18.8 at 5 Hz.

NP severity was judged by deviation of the resulting maximum CPT value from the median, expressed as standard deviation (SD).

Patients were divided into three different groups based on the SD difference of the CPT score from the mean. Patients with CPT values within the mean±2SD range were considered non-neuropathic (non-NP). Patients whose CPT values were between the mean CPT+2 SD and CPT+4 SD were assigned to the mild neuropathy (mild-NP) group. Finally, those whose CPT values exceeded mean values by more than 4 SD or who showed insensitivity (extremely high CPT) were considered as severe-neuropathic (severe-NP) patients.

Statistics

Since many parameters were not normally distributed, we used a non-parametric Wilcoxon rank test for group differences and Spearman correlation tests to examine the monotonic correlation between pairs of parameters. R software (version 4.3.2) was used for statistical analyses, tables and graphs.

RESULTS

Patients: laboratory parameters

The age of the patients included in both the central and peripheral studies ranged from 32 to 73 years. There were no significant differences between the two groups of patients in terms of major anthropometric characteristics, neither age *(central measurement: $p = 0.47$; peripheral measurement: $p = 0.57$)* nor gender (*central measurement: $p = 0.15$; peripheral measurement: $p = 0.26$).* The obese group had significantly higher BMI values than the diabetic group (*central measurement: T2DM group: 33.57 ± 5.86, obese group: 38.07 ± 6.06; peripheral measurement: T2DM group: 32.95 ± 5.10, obese group: 38.81 ± 6.08; $p < 0.001$).* When assessing the patients' glucose homeostasis, fasting plasma glucose concentrations differed significantly between the two study groups *(central measurement: T2DM group: 8.86 ± 3.14, obese group: 5.45 ± 0.55; peripheral measurement: T2DM group: 8.97 ± 3.41, obese group: 5.44 ± 0.52; $p < 0.001$)* and HbA1c values *(central measurement: T2DM group: 7.56 ± 1.25, obese group: 5.51 ± 0.32; peripheral measurement: T2DM group: 7.61 ± 1.18, obese group: 5.53 ± 0.29; $p < 0.001$).* Mean C-peptide levels were also found to be higher and hyperinsulinemia was more pronounced in obese participants *($p < 0.05$)* than in T2DM patients, although the strength of correlation with respect to serum insulin values was slightly above the significance threshold for central measurements *($p = 0.051$).*

Studies of central microcirculation

Circulation tests

Group comparison did not demonstrate a significant difference in CBF between diabetic and obese subjects **(***$p > 0.05$***);** however, a more pronounced increase in blood flow was recorded in the obese subgroup after BHT-based provocation *($p < 0.05$)*. T2DM patients had significantly higher maximum c-IMT values *($p < 0.05$).* However, the group difference regarding the average IMT values only approached the statistical significance threshold *($p = 0.07$).*

In the correlation analyses performed, a significant positive correlation was observed between BMI and resting cerebral perfusion *($p < 0.005$, $rho = 0.36$)* and between BMI and maximum *($rho = 0.31$)/average ($rho = 0.29$)* BHI values (*$p < 0.005$*). Conversely, there was a negative correlation between BMI and maximum *($p < 0.01$)/average ($p < 0.05$)* IMT values *($rho = -0.27$*

and -0.23 for maximum and average IMT, respectively). Besides, BHI values showed a significant decrease with age *($p < 0.005$, rho = -0.29 and rho = -0.31 for maximum and average BHI, respectively).*

Significant positive correlation was found between maximum *(rho = 0.28)/average (rho = 0.27)* IMT and intra-abdominal visceral fat *($p < 0.01$),* while maximum (*rho = -0.31)/average (rho = -0.29)* IMT values and the quantity of subcutaneous adipose tissue showed a negative correlation. *($p < 0.005$ and $p < 0.01$ for maximum and average IMT, respectively*). Statistically, there was a particularly strong positive correlation between age and maximum *(rho = 0.46)/*average *(rho = 0.46)* IMT values *($p < 0.001$).*

Our findings showed a BMI related increase in the abdominal fat in both subgroups, i.e. the higher the BMI, the more significant the increase in abdominal fat. Nevertheless, as BMI increased, the pace of increment was more pronounced for subcutaneous fat compared to visceral fat *(rho = 0.25 and rho = 0.73 for visceral and subcutaneous fat, respectively)*.

None of the cerebral perfusion parameters tested showed a correlation with the gender of the patients. However, there was a significant difference between the IMT of male/female patients, with males having higher IMT (*$p < 0.005$).*

Although we observed a positive correlation between resting [99mTc]Tc-HMPAO and reactive brain perfusion *($p < 0.05$, rho = 0.26),* there was no significant correlation between brain perfusion and IMT.

There was also a positive correlation between C-peptide levels and both resting *($p < 0.005$, rho = 0.31)* and post-provocation (*$p < 0.005$, rho = 0.32 and rho = 0.30 for maximum and mean BHI, respectively)* cerebral microcirculation values. Additionally, a positive correlation was observed between C-peptide levels and BMI *($p < 0.001$,rho = 0.38).* As opposed to this, no link was found between IMT data and C-peptide levels (*$p > 0.05$*). Finally, no significant correspondence was demonstrated between plasma glucose, HbA1c, the insulin levels and central microcirculation *($p > 0.05$).*

Nerve conduction tests

Based on SPECT imaging and nerve conduction tests no statistically significant discrepancies were detected in the cerebral perfusion in the three NP groups (*$p > 0.05$*).

Studies of peripheral microcirculation

Perfusion studies

A group comparison of lower limb SPECT scans demonstrated considerable differences in peripheral perfusion between the two study populations *(SUV_{mean} left leg: 0.53±0.11 and 0.64±0.13 for diabetic and obese subjects, respectively; SUV_{mean} right leg: 0.52±0.09 and 0.6±0.13 for diabetic and obese subjects, respectively)*, with higher values on both sides in obese subjects than in diabetic patients *($p < 0.001$ and $p < 0.005$ for left and right leg, respectively).*

In correlation analyses, lower limb perfusion was positively associated with BMI *($p < 0.001$, $rho = 0.44$),* C-peptide *($p < 0.05$, $rho = 0.29$)* and insulin levels *($p < 0.01$, $rho = 0.30$).* In contrast, HbA1c levels showed negative correlation with lower limb radiofrequency uptake *($p < 0.05$, $rho= -0.24$)*. Although plasma glucose concentrations were also inversely correlated with leg SUV values, this did not prove to be statistically significant *($p= 0.081$, $rho= -0.21$).*

Nerve conduction tests

Regardless of the type of metabolic disease present, group comparisons of Neurometer® studies showed notable differences in the peripheral microcirculation of NP subtypes. The lower limb SPECT results of the mild-NP subgroup were remarkably different from the non-NP results, i.e. lower SUV values were measured in patients with mild-NP compared to individuals without sensory nerve damage. Interestingly, there was also a huge gap between the SUV values of the mild and severe-NP subgroups *($p < 0.05$),* which proved to be higher in the severe subgroup compared to the mild-NP group. The SUV values of the severe-NP subgroup were lower than those of the non-NP subgroup ($p = 0.18$), nevertheless, this was not statistically significant.

Changes in C-peptide levels showed a similar tendency with the lower limb microcirculation. The mild-NP group had significantly lower C-peptide levels than patients with severe-NP *($p < 0.01$).* C-peptide levels in patients without NP were higher compared to the mild-NP subgroup and lower compared to the severe-NP subgroup, although the latter group differences remained below the limit of statistical significance ($p = 0.078$, $p = 0.25$).

We also categorised the patients in different NP subgroups according to the type of metabolic disease, where we observed notable deviations in lower limb microcirculation between diabetic and obese patients in both mild and severe NP subgroups *($p < 0.05$)*, with obese patients having better values than diabetic patients. A further finding was that obese patients with severe-NP had considerably higher C-peptide levels than the same subgroup of diabetic patients *($p < 0.05$)*.

Fasting blood glucose and HbA1c levels also showed significant divergence when comparing NP subgroups ($p < 0.001$).

No meaningful difference was confirmed between glucose and HbA1c values of NP subgroups *($p > 0.05$)*. Furthermore, changes in these glycaemic indicators did not follow the same tendency as the observations on lower limb microcirculation.

Finally, during the correlation analyses with baseline parameters, no age-related differences were found within subgroups classified according to degree of severity of NP *($p > 0.05$)*. However, for BMI, regardless of NP severity, the discrepancy between obese and diabetic participants was remarkable *($p < 0.001$)*.

DISCUSSION

Due to intensive research in recent years, significant progress has been made in investigating the links between metabolic diseases and microcirculatory disorders, but many aspects of this topic are still not well understood. Considering the extremely high and rapidly increasing prevalence of T2DM and obesity as well as the related, severe, life-limiting and life-shortening health consequences, a detailed analysis of the pathophysiology of these diseases and their impact on microcirculation is of utmost importance and has become a major priority for research in this field today.

During our investigations, we employed a comprehensive approach to gain in-depth insights into changes in the microcirculation of individuals with metabolic diseases using various diagnostic methods. The aim of our study was to map and compare the central and peripheral microvascular patterns of patients with T2DM and obese individuals, as well as to explore the relationships of these patterns with peripheral nerve functions, the glycemic status of the patients, and their various anthropometric parameters.

Studies of the central microcirculation

Based on our findings, we first performed a group comparison, where we found no significant difference in global and hemispheric cerebral perfusion between the obese and T2DM groups using brain SPECT imaging. Although full exploration of the background remains to be done, we speculate that the lack of difference in our study may have been caused by closer diabetic care of the participating T2DM patients, minimizing the number of hyperglycemic periods and, through this, prevention of abnormal cerebral perfusion changes. In contrast to our results, *Képes et al*. detected a perfusion difference between T2DM and obese individuals using [99mTc]Tc-HMPAO SPECT imaging, although this difference only affected one brain region, the insula. In fact, they assessed regional perfusion, whereas we analysed global patterns. Although there is a relatively small literature comparing brain perfusion in diabetic and obese individuals, much more research findings are available concerning the CBF examination of metabolic diseases. *Birdsill et al*. reported a 15% lower cortical blood flow in patients with metabolic syndrome compared to controls. Furthermore, *Cui et al*. measured reduced CBF in the posterior cingulate cortex, precuneus and bilateral occipital lobes of patients with T2DM. One potential explanation for the discrepancies between the available literature and our current findings lies in the methodological differences: the studies by *Birdsill* and *Cui* compared cerebral perfusion in T2DM patients to a healthy reference group, whereas our investigation did not include a metabolically healthy control group. Additionally, we lack precise data regarding the extent to which obesity, independent of T2DM, contributes to cerebral microcirculatory impairment in obese individuals included in our study.

Looking at healthy subjects, *Willeumier et al.* found reduced regional CBF at higher BMI values in several Broadmann's areas as well as in the prefrontal cortex. Similar research findings include those of *Képes et al*., who demonstrated hypoperfusion in the brainstem associated with higher BMI and linked this phenomenon to obesity-related pathophysiological processes, such as leptin resistance and impaired insulin signaling. Additionally, a previous publication by *Káplár et al.* confirmed that cerebral perfusion in the frontal cortex is more significantly reduced in T2DM compared to T1DM.

Based on the better CVR and lower maximum IMT values observed in the obese patient group compared to those with T2DM, we concluded that vascular damage is more pronounced in diabetic patients. Although resting cerebral perfusion showed no significant differences between the two groups, the vascular dysfunction in diabetic patients limits their ability to accommodate increased circulatory demands. Moreover, this group exhibits more advanced subclinical atherosclerotic processes, indicating a greater extent of underlying vascular pathology. Consistent with our results, the studies of *Tchistiakova et al.* also confirmed the poorer cerebrovascular reactivity observed in diabetic patients compared to those with hypertension. Further similarity was established with our findings in a study by *Novak et al.*, which compared patients with T2DM with healthy controls. Their measurements revealed reduced mean cerebral blood flow rate/volume and reduced carbon dioxide inhalation reactivity in the latter group of patients. Unlike our present study, they performed the assessment of the cerebral vascular autoregulation in subjects with impaired glucose homeostasis after acetazolamide provocation, still *Selvarajah et al.* found significantly lower vasoreactivity in the common carotid artery of T2DM and impaired glucose tolerance (IGT) patients compared to controls.

Finally, when patients were grouped according to the severity of neuropathy, there was no difference in brain perfusion between subgroups (without NP, mild-NP and severe-NP). This suggests that central perfusion is independent of NP severity.

Based on our detailed correlation analyses, similarly to the measurements of *Képes et al*., BMI was positively correlated with resting cerebral perfusion and BHI, while a significant negative correlation was found between BMI and IMT. Surprisingly, these results may even suggest that in a group of patients with metabolic disorders, an increase in BMI may have a "paradoxically positive” effect on brain reactivity and carotid IMT. The observed negative correlation between BMI and IMT can be attributed to the stronger impact of risk factors associated with diabetes (including the disease itself) compared to those linked to obesity. Consequently, vascular characteristics such as IMT are more adversely affected in the diabetic group. However, the significantly higher BMI values in the obesity group understandably lead to an inverse correlation. The positive correlation observed between BMI and cerebral perfusion, as well as between BMI and BHI, is primarily driven by the role of C-peptide, which is elevated in obesity compared to diabetes. (A detailed explanation of this phenomenon is provided in the section discussing peripheral circulation.) Overall, our results appear to contradict the currently available research data on the relationship between BMI and IMT. The study by *Liu et al.* found that BMI was positively correlated with IMT in patients with CVD, whereas the study by *Rodríguez-Flores et al*. established a negative connection between BMI and CVR. When comparing vasomotor reactivity in normal weight and obese participants, a significantly lower BHI was found in the latter group. Although it is difficult to directly compare our current research results with those of *Rodríguez-Flores et al.,* mainly due to the lack of a healthy control group, in addition to the factors mentioned above, we attribute the conflicting data to the different number of subjects, different patient composition as well as to differences in the methods used to determine the BHI. Contrary to our results, in a study by *Sugiura et al.* who investigated the relationship between obesity-related anthropometric parameters and early stage atherosclerosis in CVD-free individuals, a positive correlation ($rho = 0.170$) was established between BMI and mean c-IMT.

In order to gain a deeper understanding of the connections with BMI, we measured visceral and subcutaneous abdominal fat distribution patterns in our subjects using low-dose CT. Utilizing this approach, we determined that in the patient groups studied, the accumulation of subcutaneous and visceral fat mass does not occur proportionally with the increase in BMI. Subcutaneous adipose tissue, considered "metabolically neutral," exhibits a relatively greater increase compared to visceral adipose tissue, which is linked to a heightened risk of metabolic and atherosclerotic diseases. This imbalance may also provide an explanation for the less favorable cerebral circulation parameters observed in individuals with lower BMI during the study. The results of our research on adipose tissue distribution were only partially consistent with a previous study by *Liu et al*. in which they found a positive association between IMT and both visceral and subcutaneous adipose tissue in CVD-free individuals.

As a result of our measurements, we have found that advanced age has an extensive and, as expected, adverse effect on cerebral circulatory parameters and it may be a risk factor not only for a decrease in CVR but also for an increase in c-IMT. Consistent with our results, *Ko et al.* found a strong association between advancing age and abnormal trends in IMT in a critically obese group of adults. In an evaluation of healthy volunteers' DUS scores, *Zavoreo and Demarin* measured a decreasing BHI with increasing age, a result also consistent with our current findings.

Our IMT measurement results also suggest that male gender increases only the likelihood of developing early stage atherosclerosis (represented by elevated IMT); whereas with regard to the other cerebral circulatory parameters examined (resting CBF and CVR) no gender differences have been revealed. This observation was in agreement with the results of *van Qu et al*, who also reported significantly higher IMT values in men compared to women. Consistent with our results, in the [99mTc]Tc-HMPAO study by *Catafau et al.* gender did not affect regional CBF in either young or older subjects; however, hypoperfusion was observed in certain brain areas, including the left frontal lobe and posterior region of the left temporal lobe, in the older group.

Of the glycaemic parameters studied, the positive correlation of C-peptide levels, which was detected by both resting and post-provocation brain perfusion, emphasises the unique contribution of this protein to the preservation/enhancement of central microvascular function. Whereas in our study C-peptide correlated with cerebral blood perfusion, in the study by *Kim et al.* basal C-peptide levels in T2DM patients showed a positive correlation with IMT. This phenomenon is likely attributable to elevated insulin levels observed in insulin-resistant states, which, in conjunction with increased C-peptide production, may promote cell proliferation and contribute to atherosclerosis in large arteries. Moreover, it is important to emphasize that the patients included in the referenced study exhibited higher C-peptide levels, longer disease duration, and poorer carbohydrate metabolic profiles compared to the cohort analyzed in our current investigation.

However, contrary to expectations, no significant negative correlation was found between the most commonly used clinical indicators of glycaemic control, such as fasting plasma glucose, HbA1c and CBF. This is primarily attributed to the effective antidiabetic management and the favorable glycemic profiles of the patients enrolled in our study.

Studies of peripheral microcirculation

The result of a group comparison of lower limb microcirculation studies using [99mTc]Tc-HMPAO SPECT/CT, according to which diabetic patients have a significantly reduced lower limb perfusion compared to obese patients, may indicate the appearance of microvascular damage associated with T2DM in this patient group. The underlying cause of this phenomenon is most certainly the increased vascular risk associated with diabetes. (It is noteworthy that, as detailed above, the enhanced risk/damage to cerebral circulation in diabetes leads to a reduction of the CVR in these patients, whereas disturbances in microcirculation related to foot perfusion can be detected even at rest.)

There is limited literature available on peripheral microcirculation in diabetic patients. Although some cases were reported to have used nuclear medicine techniques to map the lower limb circulation in PAD, while *Képes and colleagues* applied the [99mTc]Tc-HMPAO SPECT/CT method for such purposes in their "proof-of-concept" study. In addition, previous findings have suggested that MRI, ankle-arm index or transcutaneous tissue partial oxygen pressure measurements appear to be appropriate non-isotopic diagnostic methods for the assessment of disease-related vascular damage. Using MRI, *Zheng et al.* demonstrated reduced peripheral blood flow in diabetes, but in contrast to our current study, they involved a healthy control group. Non-invasive angiology/microreology tests have shown worse perfusion parameters in diabetic patients with more advanced stage and more pronounced peripheral circulatory insufficiency symptoms than in those with milder symptoms. Although the results of these studies overlap with ours, differences in patient populations and the applied methods make direct comparisons difficult. In addition, the contrasting digital photoplethysmography results, which exhibit more pronounced peripheral microcirculation/ blood flow in T2DM compared with a healthy control group, highlight the critical importance of optimizing the appropriate methodology for drawing long-term conclusions.

In our study, glycaemic parameters and lower limb SPECT data were also compared. After reviewing the literature, we found that our current study is the first to directly address the relationship between C-peptide levels and peripheral perfusion parameters. The correlation analyses showed a positive link between lower limb perfusion and C-peptide suggesting that this protein may play a role in attenuating peripheral vasculopathy induced by metabolic dysfunction. Although no pathophysiological studies have been performed in our current research, literature confirms the beneficial effects of C-peptide on microvasculature, including stimulation of eNOS, enhancement of red blood cell deformability, or anti-inflammatory and anti-apoptotic roles.

Although we found a positive correlation between plasma insulin levels and lower limb perfusion, no meaningful clinical conclusions can be drawn from this, as patients on regular insulin therapy were not excluded from our studies. Consistent with previous findings, HbA1c and fasting glucose levels showed a negative correlation with lower limb perfusion, which, in contrast to the excellent adaptability of the cerebral circulation, may indicate a susceptibility of the peripheral myocirculation to hyperglycaemia and systemic "metabolic inflammation".

During our research we also investigated peripheral perfusion patterns in relation to the severity of metabolic disease-related neuropathy, an approach that is unique in the literature and had not been previously published in this area. We measured significantly reduced peripheral perfusion in the mild-NP group compared to the neuropathy-free group. However, a surprising finding was that we recorded improved perfusion parameters with further exacerbation of neuropathy (mild/severe-NP). The changes in C-peptide and SUV data also showed a similar U-shaped trend in the different neuropathy subgroups, suggesting that the effect of C-peptide concentrations on peripheral microcirculation, with a positive correlation between them, may explain the peripheral perfusion patterns observed. Thus, the "paradoxical phenomenon" can be explained by the fact that, due to their higher C-peptide levels, patients with severe NP show more favorable lower limb perfusion indices than those with mild NP. Similarly, the nearly identical perfusion observed in neuropathy-free patients compared to the severe NP group can be attributed to the lower C-peptide levels in the former group. In our studies, within each NP subgroup, the better lower limb SPECT perfusion indices in obese patients, compared to those with T2DM, can also be associated with higher C-peptide levels.

Overall, our results provide evidence of the positive biological effect of C-peptide on microvascular function, which – in line with previous research data – suggests that C-peptide exerts a significant protective effect on peripheral microvascular function in metabolic diseases.

Unlike C-peptide, the other glycaemic indicators tested (glucose, HbA1c) did not follow the U-shaped pattern mentioned above, and there was no significant difference between the three neuropathy subgroups. Consistent with the literature that suggests that the development of NP may not be necessarily/solely determined by glycaemic control, our results point in the direction that, independently of blood glucose and other associated glycaemic markers, C-peptide has a beneficial effect on microvascular regulation. However, it is important to emphasise that in our research, the study population was under regular medical control to maintain glucose homeostasis within an acceptable range, which may have influenced the results of the correlation tests. Our study results support the notion that the pathophysiology of PNP differs between obesity and T2DM. In diabetes mellitus, vascular factors seem to be the main determinants of NP, whereas in obesity, metabolic aspects seem to be dominant.

Although there are relatively few publications on the relationship between NP and microcirculation, it is worth mentioning the similar findings of *Tomesová et al.*, which indicate impaired microvascular reactivity in diabetic neuropathy. Conversely, in their study, *Képes et al.* found no connection between CPT-based neuropathy indicators and lower limb perfusion in T2DM and obese patients. Furthermore, their study showed a significant positive correlation of HbA1c levels with neuroconductive function in diabetes, which also contradicts our current results, albeit, in their case analyses were independent of neuropathy severity. In addition, they calculated the values based on the individual frequencies, whereas we used a scoring system-based comparison, which was formed based on deviations from the mean.

Conclusion - outlook

My thesis provides a comprehensive analysis of the effects of T2DM and obesity on microcirculation and peripheral nerve function. The connections between central and peripheral microcirculation, C-peptide levels and PNP severity, published by us for the first time in the literature, provide a strong and scientifically sound evidence for a protective role of this protein against microcirculatory complications associated with metabolic diseases. The correlations obtained with BMI and abdominal adipose tissue distribution highlight the clinical importance of regular monitoring of anthropometric parameters during patient follow-up. However, a deeper understanding of the relation between body mass gain and cerebral perfusion parameters suggests the need for further research.

Our studies have significantly expanded our knowledge on the links between T2DM/obesity and microcirculatory impairments in the brain and lower limb, which can be exploited both to reduce disease-associated vascular risk and to prevent the development of dementia. The findings originating from the present study may provide a basis for the development of novel therapeutic targets, targeted drug treatment options, which may ultimately aid personalised patient care.

NEW FINDINGS

1. In our multivariate/multi-parameter comparison of central circulatory parameters (brain perfusion at rest and following breath-holding provocation, as well as c-IMT measurement), we found that, while no differences in global brain perfusion patterns were observed between the two patient groups (type 2 diabetes mellitus and obesity) at rest, provocation resulted in a significantly lower BHI value in the diabetic group compared to the obese one, suggesting functional impairment of cerebral vasculature in diabetic patients. Moreover, the diabetic patients exhibited higher c-IMT values.

2. A novel finding in our study is that, in patients with this metabolic disorder, brain reactivity "paradoxically improved" and c-IMT decreased as BMI increased. The likely explanations for these seemingly contradictory associations include the distribution of abdominal fat (where, with increasing BMI, the amount of metabolically neutral subcutaneous fat increases disproportionately), the significantly higher BMI in the obesity group, and the relationship between cerebral reactivity and microcirculation with C-peptide levels.

3. C-peptide was identified as a protective factor against peripheral and central microcirculatory abnormalities in T2DM and obesity; this was confirmed by its plasma contraction as well as the significant positive correlation with both central and peripheral perfusion. Indeed, in our current study, the improved central nervous perfusion values with higher BMI (along with the distribution of abdominal fat) were due to the beneficial effect of rising C-peptide levels on cerebral circulation with increasing body weight.

4. From a scientific perspective, a significant research finding in our study was the complex relationship that we found between lower limb microcirculation, peripheral neuropathy severity and C-peptide levels in the examined metabolic diseases. We confirmed that patients with mild neuropathy had significantly reduced microcirculation compared to non-neuropathic patients, whereas in more severe cases of neuropathy we found a minor, non-significant deterioration of perfusion, which was explained by higher C-peptide levels. This result underlines the role of C-peptide in preserving microcirculation in the presence of severe neuropathy.

5. In examining severe neuropathy cases, the microcirculation of obese patients - due to their higher C-peptide levels - was found to be better than that of T2DM patients. This finding supports the view regarding the development of neuropathy that, in obesity, metabolic factors, rather than vascular factors, play the primary role.

6. In response to one of the central questions of our research, we determined that there is no correlation between the severity of central circulation and peripheral neuropathy; in this regard, the peripheral changes precede the central abnormalities.

7. We confirmed that while there is a strong negative correlation between peripheral microcirculation and conventional glycaemic parameters (fasting plasma glucose and HbA1c), the central circulation is not affected by this correlation (presumably due to central nervous compensatory/protective mechanisms).

8. Finally, our research has introduced a new element in the composition of the study population concerning the investigation of the relationship between certain anthropometric parameters and central circulation. We determined that, similar to healthy individuals or those with other underlying conditions (targeted studies have thus far only been conducted in critically obese patients), the presence of metabolic diseases (T2DM and obesity) makes advancing age a significant risk factor. With increasing age, we observed a decline in BHI and worsening of IMT. Moreover, we revealed that masculine gender is only unfavourable from the point of view of early atherosclerosis, it does not affect other brain perfusion parameters studied.

SUMMARY

Investigating the microcirculatory abnormalities associated with T2DM and obesity is a major research focus today, given the widespread prevalence of these diseases and the serious health complications they cause.

In our study we included and compared individuals with metabolic disease (T2DM/obese, non-diabetic) and further grouped them according to the severity of PNP (NP free/mild NP/severe NP). In our complex study, we used isotopic diagnostic ([99mTc]Tc-HMPAO SPECT) and Doppler ultrasonographic methods (BHI, c-IMT determination) to map the microvascular patterns of these patients and analyzed the effects of peripheral nerve function, glycemic status and anthropometric parameters. A central question of our study was whether the lower limb microcirculatory disturbances seen in PNP associated with metabolic disease are accompanied by central microcirculatory changes.

Our results have provided a new perspective on a number of issues. At rest, we found no significant difference in brain perfusion between the T2DM and obese groups. However, during BHT, we observed a smaller increase in blood flow in diabetic patients, which may indicate the presence of functional central circulatory abnormalities that are more sensitive to detection by these provocation tests. In parallel, the peripheral microcirculation of patients with T2DM was also found to be significantly poorer, and this condition showed a strong correlation with the deterioration of glycaemic parameters.

When anthropometric parameters were examined, age was identified as a significant risk factor for central nervous system vascular abnormalities, especially in the presence of metabolic diseases. Furthermore, we established that when assessing central circulatory parameters, in addition to BMI, it is of particular importance to consider the distribution of abdominal fat.

A significant scientific observation was the complex relationship identified between the degree of neuropathy, peripheral microcirculation, and C-peptide levels. We observed that higher C-peptide levels have beneficial effects on both central and peripheral circulation, and this positive impact could explain the relatively better peripheral circulation in patients with severe-NP and obesity. Our results suggest that, in addition to traditional glycaemic markers, the measurement of C-peptide levels may be critical in predicting microvascular complications, which may be

explained by the vascular protective effects of C-peptide in diabetes and its potential metabolic benefits in obesity.

Another important observation was that, in contrast to the clear association between peripheral microcirculation and neuropathy, no direct connection could be revealed between central microcirculation and the severity of peripheral neuropathy. This phenomenon suggests that the microcirculatory abnormalities associated with peripheral neuropathy are not accompanied by central microcirculatory disturbances.

In conclusion, our research has made a significant contribution to a deeper understanding of the links between metabolic diseases, microcirculatory impairment and peripheral neuropathy. Our results have highlighted the particular importance of considering abdominal fat distribution, in addition to traditional BMI-based body weight assessment, in estimating vascular risk. Apart from this, the impact of C-peptide on microcirculation and metabolic homeostasis may represent a new therapeutic target in the treatment of T2DM and obesity, either to prevent cognitive decline, dementia or lower limb microcirculatory disorders, offering personalized treatment options for patients with metabolic diseases.

PUBLICATIONS

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List of publications related to the dissertation

1. **Esze, R.**, Barna, S., Fülöp, P., Kempler, P., Mikó, M., Páll, D., Paragh, G., Somodi, S., Emri, M., Képes, Z., Garai, I., Káplár, M.: C-peptide: an essential ally in microvascular complications of type 2 diabetes mellitus and obesity.
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2. **Esze, R.**, Balkay, L., Barna, S., Egeresi, L., Emri, M., Páll, D., Paragh, G., Rajnai, L., Somodi, S., Képes, Z., Garai, I., Káplár, M.: Impact of Fat Distribution and Metabolic Diseases on Cerebral Microcirculation: a Multimodal Study on Type 2 Diabetic and Obese Patients.
   J Clin Med. 13 (10), 1-14, 2024.
   DOI: http://dx.doi.org/10.3390/jcm13102900
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List of other publications

3. Képes, Z., Péli-Szabó, J., Kálmán-Szabó, I., Sass, T., **Esze, R.**, Opposits, G., Jószai, I., Szikra, D. P., Fenyvesi, F., Hajdu, I., Trencsényi, G.: 52Mn-labelled Beta-cyclodextrin for Melanoma Imaging: a Proof-of-concept Preclinical Study.
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