Higher NDUFS8 Serum Levels Correlate with Better Insulin Sensitivity in Type 1 Diabetes

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Research Article

Keywords: diabetes mellitus type 1, insulin resistance, e-GDR: estimated glucose disposal rate, NADH dehydrogenase iron-sulfur protein 8

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Higher NDUFS8 serum levels correlate with better insulin sensitivity in Type 1 Diabetes.

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ABSTRACT

Aim: The aim of the study was to evaluate the function of Complex I by measuring NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 serum level and the relationship with insulin resistance in type 1 diabetes. T1DM causes adverse changes in the mitochondria, which can influence the development of chronic complications. The NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 (NDUFS8 protein) is a subunit of NADH dehydrogenase and plays an important role in the mitochondrial function. NDUFS8 serum concentration probably reflects the function of mitochondria.

Methods: All of 36 people suffer from T1DM. All participants were treated with functional intensive insulin therapy. NDUFS8 protein serum concentration was measured using the ELISA test. Insulin resistance was evaluated with indirect marker estimated glucose disposal rate (eGDR). The group was divided on the base of median value of eGDR (higher eGDR – less IR).

Results: The study group consisted of 12 women and 24 men, aged 39.5 (28.0-46.5) years with the duration of the disease 22 (15-26) years. Medians of eGDR and NDUFS8 protein concentration were 7.6 (5.58-8.99) mg/kg/min and 2.25 (0.72-3.81) ng/ml, respectively. The group with higher insulin sensitivity had higher NDUFS8 protein serum concentration, lower WHR, BMI and they were younger. A negative correlation was observed between NDUFS8 protein serum concentration and WHR (rs=-0.35, p=0.03), whereas a positive correlation was observed between NDUFS8 protein serum concentration and eGDR (rs=0.43, p=0.008). Multivariate linear regression confirmed a significant association between insulin sensitivity and better mitochondrial function (beta=0.54, p=0.003), independent of age, duration of diabetes and smoking.

Conclusions: Higher NDUFS8 protein serum concentration is associated with higher insulin sensitivity among people with T1DM and might reflects better mitochondrial function.
INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a disorder characterized by destruction of pancreatic \( \beta \) cells. This process leads to complete insulin deficiency \(^1\,^2\). The etiology is still being analyzed and prevention possibilities investigated \(^3\). The incidence of DM is continually increasing all over the world, in the Polish population as well \(^4\,-\,^6\). Adults with T1DM experience lower health-related quality of life, are more frequently unemployed and have more sick leave per year in contrast to the general population \(^7\). These facts are attributed to the development of chronic complications: neurovascular and macrovascular \(^8\). The development of chronic complications is connected with several risk factors, most prominently insulin resistance (IR) \(^9\) and hyperglycemia \(^10\).

Insulin resistance is mostly connected to obesity and diabetes mellitus type 2. However, more and more young people diagnosed with T1DM are overweight or obese at the moment of diagnosis \(^11\,^12\). Moreover, IR also develops during T1DM as a result of exogenous insulin treatment and aging. Insulin therapy causes abdominal obesity as well as smoking and physical laziness. Widely used indirect marker of IR in DM1 is estimated glucose disposal rate (eGDR), which was created well with IR measured by hyperinsulinemic-euglycemic clamp, the gold diagnostic standard of IR in DM1 \(^13\).

The main pathomechanisms of hyperglycemia based complications other than enhanced glycolysis – the main pathway of glucose metabolism, are increased flux through the polylol pathway, intracellular production of advanced glycation end products (AGE) precursors, protein kinase C (PKC) isoforms activation and increased hexosamine pathway activity. Furthermore, it is also known that IR is strongly associated with micro and macrovascular complications \(^9\,^14\,^15\). All these mechanisms have a common underlying process: increased reactive oxygen species (ROS, free radicals) production \(^16\).

Oxidative stress is defined as a disturbance in the balance between the production of ROS and antioxidant defenses which leads to cellular damage \(^17\). The main organelle responsible for ROS production is the mitochondrion. In the inner membrane of this organelle exist four complexes, creating the mitochondrial respiratory chain. This whole structure is responsible for electron transport. During each one of these four steps, partially reduced oxygen intermediates are being generated. Some of them are very stable and can be stored by the enzyme cytochrome c oxidase until all the electrons are transferred. However, 1-2\% of the oxygen molecules are converted to superoxide anion radical. It
happens mainly in Complex I (mitochondrial NADH:ubiquinone oxidoreductase) and Complex III (ubisemiquinone) \(^{18-20}\).

In hyperglycemic conditions, in cells there is increased glucose oxidation during tricarboxylic acid (TCA) cycle caused by persistent hyperglycemia and enhanced B-oxidation of free fatty acids (in macrovascular endothelial cells) due to insulin resistance. This leads to a situation where more electron donors, mainly NADH, are released and more protons go through the mitochondrial membrane, thus higher voltage is created \(^{21}\). As a result, more ROS are produced and released. By damaging mitochondrial DNA they activate poly(ADP-ribose) polymerase (PARP), which is a cascade of events inhibits glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Since GADPH is a pivotal enzyme of glycolysis, this inhibition causes not only inhibition of this process but also accumulation of upper level of glycolysis’ substrates and products, which in the end leads to the processes mentioned before like for example PKC activation and the polyol pathway \(^{16}\). As polyol pathway generates NADH, Complex I has to handle more and more NADH, which provides an argument for the assumption that impaired mitochondrial function means increased Complex I’s oxidative activity \(^{22}\). Nonetheless, it is proven that the redox balance in DM between NADH and NAD\(^+\) is highly elevated \(^{23,24}\), leading to enhanced ROS production, which shows the insufficiency in mitochondrial NADH dehydrogenase activity.

It was proven that ROS production is due to high proton potential \(^{21}\). Some trials have been made in patients with T2DM, in which they discovered the possible role of mitochondrial complex I impairment \(^{25}\). Complex I is proposed as a responsible source of oxidative stress also among patients with polycystic ovarian syndrome with IR \(^{26}\). We found that NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 (NDUFS8 protein) can be measured in human serum. It is a subunit of NADH dehydrogenase (ubiquinone) also known as Complex I encoded by the NDUFS8 gene \(^{27}\). The NDUFS8 gene is located on chromosome 11q13. It spans about 6kb and contains seven exons ranging in size from 51 to 186bp. Expression of the gene is ubiquitous but predominant in heart and skeletal muscle \(^{28}\). Mutations in this gene have been associated with Leigh syndrome (neurodegenerative disorder) \(^{29}\). This mutation cause proximal myopathy, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy, encephalopathy, peripheral neuropathy and exercise intolerance \(^{30}\). No studies have been made on patients with T1DM. The aim of the study was to evaluate the function of Complex I by measuring NADH dehydrogenase
[ubiquinone] iron-sulfur protein 8 serum level and the relationship with insulin resistance in type 1 diabetes. As a marker of mitochondrial function, it can help to find patients more prone to the development of chronic complications like retinopathy or neuropathy and hence start the prevention strategies accurately earlier.

**METHODODOLOGY**

**Study Design**

The study design: a cross-sectional, single-center study. The study group consisted of 36 adults with T1DM. Participants were recruited during 1 year (2018-2019). Subjects were informed about the aim of the study and signed a consent form. The study was approved by the local Ethical Committee at the Medical University of Poznan (resolution no. 15/18). We confirm that all methods were performed in accordance with the relevant guidelines and regulations. The inclusion criteria were: age above 18 years old, at least five years history of T1DM confirmed in the past with T1DM antibodies antibodies (to glutamic acid decarboxylase – antiGAD, islet cells – ICA, islet tyrosine phosphatase 2 – IA2), whereas the exclusion criteria were: CRP>5mg/l, unstable hypo/hyperthyroidism (TSH beyond normal range), other endocrinological disorders, contagious diseases, renal or liver diseases, pregnancy, antineoplastic therapy in less than 2 years and any psychological or psychiatric disorder. Everyone was treated with functional intensive insulin therapy at the onset of diabetes. This method aims to mimic physiology, two types of insulin are used: long-acting insulin analogues (so-called basal insulin) and rapid-acting analogues (used before main meals) with insulin pen devices or rapid acting analogues in the insulin therapy with personal insulin pumps. All the participants completed a standardized questionnaire including details of age, sex, chronic diseases, medicines, family history regarding diabetes and pack-years of cigarette smoking, duration of diabetes, blood glucose self-control and medical history. Participants underwent a complete physical examination with anthropometric measurements (weight, height, waist and hip circumference) and blood pressure check.

**Insulin resistance markers**

Insulin resistance was evaluated with indirect markers (the estimated glucose disposal rate – eGDR, waist to hip ratio – WHR, body mass index – BMI). The clinical characteristics of the whole study group and according to the presence of insulin resistance are shown in Table 1. The estimated glucose disposal rate (eGDR) was calculated by the
following mathematical formula: 24,31-12,22 (WHR) – 3,29 (hypertension 0/1) – 0,57 (HbA1c) [mg/kg/min] 31, waist to hip ratio (WHR) was checked using the non-elastic measuring tape with the resolution of 0.5 cm and calculated from the following equation: WHR=waist circumference [cm] / hip circumference [cm]. It was assumed that the higher the eGDR, the higher tissue sensitivity for insulin.

**Blood tests**

Blood samples were collected in a fasting state using the S-Monovette blood collection system. We evaluated: glycated hemoglobin level (A1c), serum total cholesterol, high-density lipoproteins (HDL) cholesterol, low-density lipoproteins (LDL) cholesterol, triglycerides (TG).

**NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 measurement**

NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 (NDUFS8 protein), mitochondrial (also known as NADH-ubiquinone oxidoreductase 23 kDa subunit, Complex I-23kD (CI-23kD), or TYKY subunit) serum concentration was measured using the ELISA test (this immunoassay kit allows for the in vitro quantitative determination of human NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial, its concentrations in serum, plasma, urine, tissue homogenates and cell culture supernates and other biological fluids). The NDUFS8 protein is a subunit of NADH dehydrogenase (ubiquinone) also known as Complex I, which is located in the mitochondrial inner membrane and is the largest of the five complexes of the electron transport chain. The reference range for Elisa kit is 0.312-20 ng/ml, sensitivity 0.1 ng/mL.

**Statistical analysis**

The statistical analysis was performed using the STATISTICA 13.3 program. The normality of distributions was tested using Kolmogorov-Smirnov’s test with Lilliefors correction. Due to lack of normality, non-parametrical tests were performed. All data are expressed as medians and IQR-interquartile range or percentage of subjects. Usually, the value of the cut-off point for eGDR is 7.5 mg/kg/min according to the clamp technique of DeFronzo 31. In this case, the patients were divided into two groups, below and above eGDR median, because a higher eGDR value shows insulin sensitivity better (less IR). The Mann-Whitney U and Chi² tests were used to assess differences between groups. The multivariate linear regression, R Spearman correlation were performed. Differences with a probability value <0.05 were considered statistically significant.
RESULTS

The study group consisted of 36 adults with T1DM, 12 women (33%) and 24 men (67%), aged 39.5 (28.0-46.5) years with the duration of the disease 22 (15-26) years and HbA1c 8.35 (6.92-9.78) % (table 1). Medians of chosen insulin resistance indicators among investigated group were: eGDR 7.6 (5.58-8.99) mg/kg/min and WHR 0.88 (0.83-0.92). Median of NDUFS8 protein concentration was 2.25 (0.72-3.81) ng/ml (Table 1). People with eGDR above the median (less IR) were proved to have higher NDUFS8 protein serum concentration, lower WHR, BMI and they were younger (Table 1).

*Table 1. Comparison of whole subjects, group above and below eGDR (Glucose Disposal Rate). Median of eGDR was 7.6 mg/kg/min. Data presented as median (IQR)/n(%).*

| All Subjects n=36 | eGDR above median n=16 | eGDR below median n=20 | p   |
|-------------------|------------------------|------------------------|-----|
| Sex males n (%)   | 24 (67)                | 10 (63)                | 6 (30) | 0.091 |
| Smokers n (%)     | 13 (36)                | 6 (38)                 | 7 (35) | 1.000 |
| Pack-years [years]| 0.0 (0.0-2.4)          | 0.0 (0.0-1.25)         | 0.0 (0.0-4.0) | 0.690 |
| Age [years]       | 39.5 (28.0-46.5)       | 29.0 (24.5-35.0)       | 43.0 (39.5-48.5) | 0.003 |
| Diabetes duration [years]| 22.0 (15.0-26.0) | 18.5 (12.5-23.5)       | 25.0 (20.0-27.5) | 0.039 |
| Weight [kg]       | 74.6 (64.8-84.8)       | 66.5 (61.5-74.6)       | 78.5 (69.1-88.8) | 0.063 |
| BMI [kg/m²]       | 24.3 (22.4-27.1)       | 23.0 (22.2-24.3)       | 25.9 (23.4-27.3) | 0.028 |
| Waist circumference [cm] | 0.88 (0.83-0.92) | 82.0 (76.0-91.5)       | 91.0 (85.5-105.5) | 0.003 |
| Systolic blood pressure [mmHg] | 122 (113-130) | 122 (113-127)       | 125 (114-134) | 0.278 |
| Diastolic blood pressure [mmHg] | 80 (73-85) | 80 (70-85)       | 80 (74-86) | 0.405 |
| A1C [%]           | 8.3 (7.3-9.1)          | 8.4 (7.3-9.3)          | 8.2 (7.3-9.1) | 0.762 |
| Total cholesterol [mmol/l] | 172.0 (157.0-203.5) | 170.5 (161.5-199.0) | 177.5 (151.0-219.0) | 0.664 |
| LDL [mmol/l]      | 93.5 (73.5-116.5)      | 93.5 (77.0-116.5)      | 92.5 (68.0-124.5) | 0.985 |
| HDL [mmol/l]      | 64.0 (54.0-70.0)       | 64.0 (53.5-69.5)       | 66.0 (54.5-71.0) | 0.584 |
| Triglycerides [mmol/l] | 101.5 (87.0-114.5) | 102.5 (84.0-112.5)       | 94.0 (88.0-128.0) | 0.895 |
| WHR waist [cm] / hip [cm] | 0.88 (0.83-0.92) | 0.83 (0.79-0.87)       | 0.89 (0.86-0.94) | 0.002 |
The negative correlation was observed between NDUFS8 protein serum concentration and WHR (rs= -0.35, p=0.03) (Figure 1), whereas positive correlation was observed between NDUFS8 protein serum concentration and eGDR (rs=0.43, p=0.008) (Figure 2).

Figure 1. Negative relationship between NDUFS8 protein serum concentration and WHR. (rs=-0.35, p=0.03).
Figure 2. Positive relationship between NDUFS8 protein serum concentration and eGDR. (rs=0.43, p=0.008).

Multivariate linear regression confirmed a significant association between insulin sensitivity and better mitochondrial function (beta = 0.54, p = 0.003), independent of age, duration of diabetes and smoking (Table 2).

Table 2. Multivariate logistic regression, dependent variable: mitochondria function according NDUFS8 protein serum concentration. R=0.527; R^2= 0.278; F(4,31)=2.9828; p<0.03412; Standard error of estimation: 1.8427.

| N=36 | b* | Standard error b* | b | Standard error b | t(31) | p     |
|------|----|-------------------|---|-----------------|-------|-------|
| Free word | -3.06635 | 1.952344 | -1.57060 | 0.126428 |
| Age | 0.281414 | 0.205495 | 0.04566 | 0.033343 | 1.36945 | 0.180699 |
| Duration of diabetes | -0.030173 | 0.189812 | -0.00747 | 0.046986 | -0.15896 | 0.874729 |
| Pack-years | -0.085595 | 0.158055 | -0.00010 | 0.000176 | -0.54155 | 0.591998 |
| e-GDR | 0.554108 | 0.173041 | 0.54410 | 0.169915 | 3.20218 | 0.003147 |
DISCUSSION

The study was designed to assess the mitochondrial function in adults with T1DM and its relationship with insulin resistance (IR). We found that people with T1DM who had better insulin sensitivity (eGDR above the median) had higher NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 (NDUFS8) serum concentration. The relationship was independent of other important factors. There is still lack of information on the exact mechanisms of how Complex I function is indeed impaired: increased or decreased in activity among patients with IR neither what is the best way to check it. The relationship is probably two-sided. There are also no reference range of NDUFS8 protein serum concentration in healthy people, nor in people with T1DM. NDUFS8 protein concentration was measured in serum as a simple, non-invasive way that reflects the function of mitochondria. As a marker of mitochondrial function, it can help us to find patients more prone to the development of IR and hence start the prevention strategies accurately earlier.

Apart from the factors described in the introduction, the main point of improving insulin sensitivity is endurance training and the related activation of AMP-activated protein kinase (AMPK). AMPK increases ATP generation and decreases ATP consumption. When cellular energy is low, AMPK is activated and influences physiological processes, resulting in increased energy production. Catabolic pathways, including glucose and fatty acid uptake, glycolysis, fatty acid oxidation are activated by AMPK. Another important processes activated by AMPK is mitochondrial biogenesis and special form of autophagy – mitophagy. Mitochondria are the primary production site of reactive oxygen species in cells. By recycling damaged mitochondria – mitophagy is important to the ability to produce ATP, as is the production of new mitochondria. We do not have data on the relationship between the activity of complex 1 subunits and the function of mitochondria but the interruption of the activity of complex 1 either by toxins or due to genetic disorders such as Leigh’s Syndrome or Leber hereditary optic neuropathy, has debilitating consequences. Petrosillo G. et al. in studies on rats confirmed a strong positive correlation between a decreased complex 1 functionality and an increase in ROS production. We therefore assume that a higher concentration of single subunits determines good performance and better mitochondrial function.

Oxidative stress is the process that is known as the one that causes IR and in general may be involved in T1DM pathogenesis. Oxidative stress induces insulin resistance and
arises from chronic low-grade inflammation due to increased amounts of free fatty acids (FFA) and pro-inflammatory cytokines released from adipose tissue \(^{39,40}\). Also there is an increased flux of FFA from adipocytes, which then undergo B-oxidation. In this process NADH is produced as well as Aceto-CoA. NADH goes to electron transport chain together with NADH produced by tricarboxylic acid (TCA) cycle started by the delivery of Acetyl-CoA from B-oxidation. It is exactly the same mechanism of ROS production as hyperglycemia causes. Free radicals damage DNA, proteins and lipids \(^{41}\). Protein oxidation leads to degradation, fragmentation and cross-linking of their functional groups which together leads to loss of their function \(^{42-44}\). Furthermore, ROS cause mitochondrial DNA mutations which result in an accumulation of errors in the replication and ineffective damage repair. Disturbances in oxidative phosphorylation decreased adenosine triphosphate (ATP) production and increased ROS production lead together to cell function disturbance. Finally, mitoptosis (also known as auto-destruction of mitochondria) occurs \(^{45}\). We can assume that the mitochondria by producing an excessive amount of ROS, destroy themselves. Moreover, it is also proved that the antioxidant levels in DM patients are decreased \(^{46}\). There also studies which indicate that glucose induced ROS overproduction can lead to IR in skeletal muscle in the process of mitochondrial fusion \(^{47}\). All of this constitutes the late micro- and macrovascular complications \(^{16}\). The conclusion is: by restricting the mitochondrial ROS production, we can prevent the development of IR but also act later in pathomechanism of IR and hyperglycemia based complications development.

Victor M. V. et al. reported that there can be an association between impaired mitochondrial oxidative metabolism and IR, which plays huge role in pathogenesis of polycystic ovary syndrome (PCOS), as well as in T2DM and T1DM. The study demonstrated reduction in NADH oxidation in leukocytes, which indicates that Complex I is the place that is mostly affected by IR \(^{26}\). Similar study was conducted by Hernandez – Mijares A. et al., who also checked the rates of oxidative stress and mitochondrial impairment among people with T2DM. The results, proved the same conclusion – impaired mitochondrial oxidative metabolism that takes place at complex I was present among DM2 in comparison with healthy counterparts \(^{25}\).

Wu J. et al. demonstrates that complex I up-regulated activity in either T1DM or T2DM pancreas can possibly be a major source of ROS production, oxidative stress and \(\beta\) cell failure in diabetes \(^{48}\). The activity of complex I was performed by an in-gel based blue native
polyacrylamide gel electrophoresis (BN-PAGE) analysis which allows to analyze high-molecular-weight protein complexes such as the ones building the mitochondrial inner membrane \(^{49}\). As a proof of complex I hyperactivity, it is also reported that metformin, a widely used drug to treat IR among people with diabetes, inhibits Complex I as one of its main mechanisms to help reducing IR \(^{50}\). These studies showed that IR development and mitochondrial damage are an ongoing process.

There are studies that tried to find out which exactly part of the electron transport chain is responsible for the augmented ROS production that drives the described spiral of unwanted events in T1DM patients’ cells. T1DM mice model has shown that mitochondria with single nucleotide polymorphism in the mitochondrial gene encoding NADH dehydrogenase subunit 2 (mt-ND2) are characterized by lower reactive oxygen species production and are more resistant to nitric oxide \(^{51}\). Another study showed that subjects with three mtDNA RFLPs (restriction fragment length polymorphism ) (morphs), two in the subunit 5 of the NADH dehydrogenase gene and one in the tRNA for threonine, were characterized a higher maximal oxygen uptake (VO2max) in the untrained state than noncarriers. VO2 max is a marker associated with insulin sensitivity. Studies carried out in healthy people suggest that certain mtDNA variants may contribute to increasing in VO2max and its response to training \(^{52}\).

We assume that higher NDUFS8 protein concentration confirms good mitochondrial function. In this case the results proved an association between good mitochondrial oxidative function and better insulin sensitivity. Therefore by diminishing IR there is a possibility to influence and improve the mitochondrial function, meaning also delay the onset of late complications. A simple way to do this is physical activity. It is due to modulating the effectiveness of antioxidative defense, by disturbing the prooxidative-antioxidant balance. Long-term, moderate physical activity causes prolonged high activity of antioxidant enzymes, which prevents the development of late complications and improves prognosis \(^{53}\).

Study has several limitations. First of all, the number of patients is small. Furthermore, we measured only the NDUFS8 protein serum level using the Elisa test, excluding other methods of evaluating NADH dehydrogenase activity and also other mitochondrial enzymes. However, there are no studies in the literature that check NDUFS8 protein serum level among people with T1DM which makes this study unique. Also, we have
not assessed insulin resistance using the DeFronzo clamp technique \cite{31}, which is the gold standard. Our results provide the basis for future research, assessing function of mitochondria, its relationship with IR and the factors that can influence both mitochondrial function and IR. All of this in order to slow down or even stop the development of chronic complications and improve prognosis of T1DM.

CONCLUSIONS
The higher NDUFS8 protein serum concentration is associated with higher insulin sensitivity among people with T1DM and might reflect better mitochondrial function.

The results showed in this study confirmed the importance of insulin sensitivity as a factor which improves mitochondrial function. People with T1DM are much more likely to develop insulin resistance, which is why exercise should be a very important therapeutic point in T1DM.

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**CONTRIBUTIONS**

J.F. conducted and completed the data analysis and manuscript writing. D.K. wrote manuscript D.K., M.K., A.C. enroled the patients J.F., A.U. provided ideas and aims of study. D.Z.Z. provided support on the verification of the targets and supervision. M.M., M.P., performed a biochemical analysis All authors contributed to the article and approved the submitted version.

**Figure legend**

Figure 1. Negative relationship between NDUFS8 protein serum concentration and WHR. (rs=-0.35, p=0.03).

Figure 2. Positive relationship between NDUFS8 protein serum concentration and eGDR. (rs=0.43, p=0.008).
Figure 1

Negative relationship between NDUFS8 protein serum concentration and WHR. (rs=-0.35, p=0.03).
Figure 2

Positive relationship between NDUFS8 protein serum concentration and eGDR. (rs=0.43, p=0.008)
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Figures

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Negative relationship between NDUFS8 protein serum concentration and WHR. (rs=-0.35, p=0.03).
Figure 2

Positive relationship between NDUFS8 protein serum concentration and eGDR. (rs=0.43, p=0.008)
Negative relationship between NDUFS8 protein serum concentration and WHR. ($r_s=-0.35$, $p=0.03$).
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Negative relationship between NDUFS8 protein serum concentration and WHR. \((r_s = -0.35, p = 0.03)\).
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