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

# The role of metabolic markers in low-grade, chronic and acute inflammatory disorders

by Imre Zoltán Juhász, M.D.

Supervisor: Professor György Paragh, M.D.


UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF HEALTH SCIENCES

DEBRECEN, 2023

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Doctoral School of Health Sciences, University of Debrecen

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| Head of the **Examination Committee**: | Margit Balázs, MD, PhD, DSc |
| Members of the Examination Committee: | István Ilyés, MD, PhD, DSc |
| | Anikó Somogyi, MD, PhD, DSc |

The Examination takes place at the Library of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen on the 28th of November, 2023 at 11:00 AM

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| Head of the **Defense Committee**: | Margit Balázs, MD, PhD, DSc |
| Reviewers: | Edit Bodolay, MD, PhD, DSc |
| | László Márk, MD, PhD |
| Members of the Defense Committee: | István Ilyés, MD, PhD, DSc |
| | Anikó Somogyi, MD, PhD, DSc |

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen on the 28th of November, 2023 at 14:00 PM

## 1. Introduction

According to the WHO (World Health Organization) definition of obesity, if a patient's BMI (body mass index, $kg/m^2$) is 25-29.9 $kg/m^2$ that person is considered to be overweight. If BMI is 30-34.9 $kg/m^2$ we talk about class I obesity. A BMI between 35-39.9 $kg/m^2$ is known as class II or severe obesity, whereas a BMI 40 $kg/m^2$ < is called class III or morbid obesity. Obesity develops if energy uptake exceeds the energy requirements of the human body over a longer period of time. Energy requirements of the human body depend on 3 main factors: basal metabolic rate (commonly estimated by Owen's equation), physical activity and adaptive thermogenesis which takes place in brown adipose tissue.

Obesity is a multifactorial condition: genetic causes (e.g. Prader-Willi syndrome, leptin or leptin receptor deficiency), demographic and lifestyle factors (e.g. race, educational level, eating habits, sedentary lifestyle), endocrine disorders (e.g. hypothyroidism, Cushing syndrome), medications (e.g. steroids, antipsychotics, antidepressants) as well as physiological changes due to aging could be in the background.

Abdominal obesity (also known as central, android or apple-shaped obesity) typically occurs in men and due to metabolically active, hormone-producing visceral adipocytes significantly increases cardiovascular risk compared to gluteo-femoral obesity (also called peripheral, gynoid or pear-shaped obesity) mainly affecting women. Singificant metabolic changes occur in obesity: the composition and level of adipokines (hormones produced by adipocytes) change, dyslipidemia, mitochondrial and endoplasmic reticulum dysfunction develop, oxidative stress increases and these changes all result in low-grade inflammation and insulin-resistance (decreased response of cells to insulin). Finally, diabetes mellitus develops.

Diabetes is suspected either when blood sugar is elevated during a routine check-up or when classic symptoms (polyuria, polydipsia, nocturia, weight loss) develop. The diagnosis is confirmed by clinical findings, by measuring the level of HbA1c (haemoglobin A1c, gives us information regarding blood sugar levels in the previous 3 months), fasting glucose or random glucose or by performing an OGTT (oral glucose tolerance test). If HbA1c is ≥6.5% or fasting glucose (after at least 8 hours of fasting) is ≥7 mmol/l or random glucose is ≥11.1 mmol/l the diagnosis is certain.

Etiology and risk factors of diabetes and obesity significantly overlap. Poor glycaemic control results in the development of diabetic microvascular and macrovascular complications (microvascular complications include retinopathy, nephropathy, neuropathy, macrovascular complications are transient ischaemic attack, stroke, ischaemic heart disease, peripheral arterial disease), impairs cellular and humoral immunity and thus immune response, increases the risk of sepsis and increases sepsis mortality.

Sepsis is mainly caused by bacteria, however viral and fungal infections could also be in the background. Diagnosis is either based on the SIRS criteria (systemic inflammatory response syndrome) or the qSOFA (quick Sequential Organ Failure Assessment score) and SOFA scores (Sequential Organ Failure Assessment score).

The diagnosis of sepsis is confirmed if at least 2 of the 4 SIRS criteria (pulse > 90/min, temperature > 38 $^{o}$C or < 36 $^{o}$C, respiratory rate > 20/min or partial pressure of arterial carbon-dioxide = $PaCO_2$ < 32 mmHg, white blood cell count > 12 G/L or < 4 G/L or > 10% immature forms) are present and an infectious cause is either suspected or verified. If organ dysfunction/hypoperfusion or hypotension develops we talk about severe sepsis. If hypotension persists despite of adequate fluid resuscitation the diagnosis of septic shock is confirmed.

When using the qSOFA and SOFA scores the diagnosis of sepsis is confirmed if at least 2 of the 3 qSOFA criteria (altered mental status, respiratory rate ≥ 22/min, systolic blood pressure ≤ 100 mmHg) are present and based on the SOFA score (SOFA parameters: partial pressure of arterial oxygen/fraction of insipred oxygen = $PaO_2/FiO_2$, mean arterial pressure = MAP, bilirubin level, creatinine concentration, Glasgow Coma Scale = GCS, platelet count) organ dysfunction is also verified (SOFA score ≥ 2 points) in a patient with a suspected infecion. If vasopressor support is also needed - despite adequate fluid resuscitation - to maintain blood pressure (MAP ≥ 65 mmHg) and lactic acid concentration is ≥ 2 mmol/l we talk about septic shock. If the qSOFA score is <2, but sepsis is still likely organ dysfunction should be assessed (the SOFA score has to be calculated). The diagnosis of sespsis could be excluded if the qSOFA score is <2 and an infectious cause is rather unlikely.

Countless publications have compared the efficiency of the SIRS, qSOFA and SOFA criteria in the diagnosis of sespsis since 2016 (the year when the qSOFA and SOFA criteria were introduced), however results have turned out to be rather inconclusive whether one is superior to the other. As a result, the 2016 recommendations regarding the use of the qSOFA and SOFA score are not universally accepted, and many countries still prefer the use of the previously favoured SIRS criteria in the diagnosis of sepsis.

The main risk factors of sepsis - besides diabetes mellitus - are advanced age (≥65 yrs), previous hospital stay (within the previous 90 days, ICU treatment, nosocomial infection, community-acquired pneumonia), immunosuppression (e.g. neolpasms, severe kidney and liver failure, AIDS, splenectomy) and genetic factors.

## 2. Literature review

### 2.1. Epidemiology

Obesity and diabetes (often as a consequence) are considered to be a huge global burden in the modern era.

The number of overweight and obese people is increasing globally. Some studies estimate that over one third of the global population is either overweight or obese. The incidence of obesity seems to have reached its peak in high-income countries whereas in states with middle-income or low-income incidence is still on the rise. The European Union is no exception to this tendency, and obesity seems to be more common among men, the elderly and city dwellers. According to a 2015 study the prevalence of overweight in men was 40% and 32% in women, whereas the prevalence of obesity was 32% regardless of gender in Hungary. This study also pointed out that the prevalence of obesity significantly increased with age in both males and females. Overweight mainly occured among men with higher educational levels, while obesity mostly affected undereducated women. Incidence is also on the rise among Hungarian children.

Obesity significantly increases mortality, which is often related to cardiovascular complications or obesity-related neoplasms. One of the commonest metabolic disorders associated with obesity is insulin resistance and this could easily result in the development of type 2 diabetes mellitus.

The number of diabetics - similarly to obesity - is rising worldwide. According to a publication the number of diabetics was approximately 56 million in Europe in 2013 and some even estimate that this number can be more than 66 million by 2035. Similar tendencies have been reported in Hungary. Cardiovascular causes are responsible for the majority of mortality in type 2 diabetics.

Based on a meta-analysis published in 2016 the number of septic patients was 31.5 million and the number of patients diagnosed with severe sepsis was 19.4 million worldwide, and sepsis was responsible for the death of more than 5 million people annualy. According to another article sepsis was responsible for 19.7% of all-cause mortality in 2017. Although sepsis-related incidence and mortality data may differ significantly, most authors agree that sepsis is still a huge burden and a major cause of global mortality.

## 2.2. Metabolic changes in obesity, the development of low-grade inflammation and insulin resistance

### 2.2.1. Adipokines és hepatokines

*Adipokines*

Human adipose tissue - via the production of adipokines - has a major role in the maintenance and regulation of the homeostasis of the human body, as these adipokines exert countless endocrine, paracine and autocrine effects. The composition and level of adipokines significantly depend on the size of adipose tissue. Some of them have antiatherogenic qualities, while others promote atherosclerosis (proatherogenic).

The most well-known adipokine is adiponectin and its level correlates well with insulin resistance: the higher its concentration the better the cellular response to insulin. Adiponectin - via the activation of AMPK (AMP-activated protein kinase) and PPAR-α (peroxisome proliferator-activated receptor) - improves insulin sensitivity, thus enhances free fatty acid oxidation, promotes glucose uptake by muscle cells and decreases hepatic glucose production. Additionally, adiponcetin has a direct effect on pancreatic β-cell function and has anti-apoptotic qualities. It also decreases the expression of CD36 in the liver (which is responsible for the transport of fatty acids), decreases the number of adhesion molecules in vessels, increases endothelial nitrogen oxide production and angiogenesis, and also prevents foam cell formation. The level of adiponectin is lower in obese people compared to normal weight individuals.

Leptin inhibits hypothalamic NPY/AgRP (neuropeptide Y) neurons, promotes the function of POMC (proopiomelanocortin) neurons, thus increasing alfa-MSH (melanocyte-stimulating hormone) and CART (cocaine and amphetamine regulated transcript) levels, resulting in decreased appetite. As a proatherogenic factor it enhances endothelin 1 production contributing to vasoconstriction, induces the migration and proliferation of vascular smooth muscle cells, promotes vascular calcification, stimulates inflammatory cells, increases TNFα (tumor necrosis factor-α), IL-6 (interleukin 6), IL-12 (interleukin 12) production by macrophages in adipose tissue, raises ROS (reactive oxygen species) levels, therefore contributes to the development of low-grade inflammation. Leptin levels are significantly higher in obesity.

Resistin is mainly produced by macrophages and monocytes. Inflammatory cytokines enhance its production and resistin itself also increases proinflammatory cytokine levels. It inhibits insulin signalling, promotes gluconeogenesis and glycogenolysis and decreases glucose uptake.

*Hepatokines*

Molecules exerting autocrine, paracrine and endocrine effects are not only produced by adipose tissue but also other organs such as the liver. These liver-produced bioactive agents are known as hepatokines and they also play a significant role in the development of insulin resistance and obesity-related metabolic changes (e.g. afamin, fetuin-A, FGF21).

*Afamin*

Afamin is a member of the albumin family. It is mainly produced by the liver however this glycoprotein can also be found in human ovarian follicies and cerebrospinal fluid. It has special α- and γ-tocopherol binding sites, therefore plays a crucial role in vitamin E metabolism. Mice carrying the human afamin gene tend to be obese, have higher lipid and glucose levels compared to non-carrier mice. Afamin concentations are higher in obesity and metabolic syndrome, and levels correlate well with the components of the metabolic syndrome in obese patients and diabetics (hip circumference, BMI, triglyceride, glucose, LDL = low-density lipoprotein). Concentrations are elevated in polycystic ovary syndrome as well. In a multi-center study there was a significant positive correlation between the incidence and prevalence of type 2 diabetes and afamin levels. According to another publication afamin may turn out to be a biomarker of increased hepatic lipid content in type 2 diabetics. Afamin - due to its anti-apoptotic and antioxidant properties - plays an important role in oxidative stress: levels are significantly increased in conditions associated with increased oxidative stress (e.g. metabolic syndrome, type 2 diabetes, obesity).

Afamin concentrations are significantly lower in patients with ovarian cancer and there seems to be a significant correlation between afamin levels and prognosis in these patients. On the other hand, in benign gynaecological conditions (e.g. endometriosis) afamin levels are not decreased.

It also has neuroprotective qualities and in certain neurological disorders (e.g. Alzheimer's disease, multiple sclerosis) it may have a role as a potential biomarker.

In uncomplicated pregnancies levels double and after delivery concentrations almost immediately return to baseline. In preeclampsia afamin levels are significantly higher compared to uncomplicated pregnancies. Based on these observations afamin may have a prognostic value in certain gynaecological conditions.

*Fetuin A*

Fetuin A is encoded by the Ahsg gene and mainly produced by the liver however it can be also found in the human placenta and adipose tissue in smaller amounts. Levels are significantly higher in type 2 diabetics, in patients with non-alcoholic fatty liver

disease and atherosclerosis. High concentrations of free fatty acids and glucose - via the NF-kB (nuclear factor kappa B) and ERK-1/ERK-2 (extracellular signal-regulated kinase) pathways - increase fetuin A levels. Fetuin A inhibits insulin receptor tyrosine kinases and the production of adiponectin, and - as a ligand of TLR (toll-like receptor 4) - inceases proinflammatory response, promotes the production of cytokines in monocytes. Fetuin A therefore plays an important role in the development of insulin resistance in obesity.

*FGF21 (fibroblast growth factor 21)*

FGF 21 consists of 209 amin oacids. It has beneficial effects on oxidative stress, endoplasmic reticulum dysfunction, mitochondrial dysfunction and low-grade inflammation in obesity. It also slows down the progression of non-alcoholic fatty liver disease and improves insulin resistance. TNFα decreases the production of FGF 21.

### 2.2.2. Dyslipidemia

#### 2.2.2.1. Lipid metabolism in healthy, normal weight individuals

Cholesterol and triglyceride are not only structural components of cell membranes, but also play an important role as signalling molecules and as an energy resources. As they are lipid-soluble they are transported by special molecules (e.g. lipoproteins) in the human body. Triglyceride is either secreted into the circulation by the gut (in the form of chylomicrons) or by the liver (in the form of VLDL, very-low-density lipoprotein). Orally consumed fat is first digested by pancreatic lipases, then absorbed in the gut and later secreted into the circulation as chylomicrons. These molecules reach their final targets (adipose tissue, muscles), where they are hydrolyzed by endothelial LPL (lipoprotein lipase, mainly produced by adipose tissue and muscles) resulting in the formation of non-esterified fatty acids which are then uptaken by cells. LPL synthesis and function is regulated by insulin: in a well-fed state insulin increases LPL activity in adipose tissue and at the same time decreases LPL activity in muscles. The human liver - in response to insulin - is capable of synthetizing triglyceride from fatty acids and glycerol, and this triglyceride is secreted into the circulation in the form of VLDL. Insulin - via SREBF1 (sterol regulatory element-binding transcription factor 1), found in hepatocyte cell membranes - activates genes responsible for de novo lipid synthesis. The fatty acids required in this process either originate from the circulation taken up by the liver or newly synthesized. Fatty acid uptake by the liver is an unregulated process and its rate depends on the free fatty acid concentration of plasma (if fatty acid uptake exceeds the needs fat deposition occurs in the liver). Fasting on the other hand, results in glucagon mediated HSL

(hormone-sensitive lipase) activation, thus triglyceride breakdown and the production of non-esterified fatty acids that can be used as energy resources.

#### 2.2.2.2. Lipid metabolism in obesity

In obesity - as well as in insulin resistance and type 2 diabetes - proatherogenic dyslipidemia develops, which is characterized by elevated levels of triglyceride, FFA (free fatty acid) and small, dense LDL (prone to oxidation) and decreased levels of HDL (high-density lipoprotein). Increased plasma FFA concentrations result in enhanced lipid uptake by tissues responsible for the regulation of carbohydrate metabolism (e.g. muscle, liver, pancreas). Coenzyme A is then attached intacellularly to FFAs resuting in their activation and the formation of LCACoAs (long-chain acyl-CoA). Activated fatty acids then are either used in de novo lipid synthesis or undergo β-oxidation in the mitochondria. When the fatty acid oxidation capacity of cells is exceeded, lipids start to accumulate in the mitochondria leading to lipotoxicity and insulin resistance: LCACoAs in excess activate certain isoforms of protein kinase C which results in the serin phosphorylation of IRS-1 (insulin receptor substrate 1), thus inhibit the attachment and activation of PI3K (phosphoinositide 3-kinase). Previously triacylglycerols were assumed to play a key role in the development of insulin resistance. However nowadays we think that insulin resistance in obesity is rather due to bioactive lipids which can directly affect enzymes involved in insulin signalling pathways (LCACoA, Cer -ceramide, DAG - diacylglycerol).

Enhanced VLDL production and the decreased breakdown of triglyceride rich lipoproteins lead to hypertriglyceridemia. As lipid particles and apolipoproteins from triglyceride breakdown are precursors of HDL the maturation of HDL becomes altered, HDL formation decreases, and HDL levels drop. The main antiatherogenic effect of HDL is due to reverse cholesterol transport, a process in which cholesterol is transported from peripheral tissues to the liver, from where it is later excreted. First ApoA-I (apolipoprotein A-I) is secreted from the liver and the gut and later - due to the interaction with ABCA-1(ATP-binding cassette transporter) - phospholipids and cholesterol are taken up. Cholesterol is then esterified by LCAT (lecithin-cholesterol acyltransferase), and the newly formed cholesterol ester moves into the inner parts of HDL. Thanks to this process HDL structure changes and it takes up its well-known spherical form. Via CETP (cholesteryl ester transfer protein) HDL interacts with triglyceride-rich particles: triglyceride is taken up by HDL while cholesterol ester is transported to LDL and VLDL particles. In the end - due to SR-BI (scavenger receptor class B type I) - cholesterol gets eliminated in the liver. Additionally to its antiatherogenic effect, HDL also possesses anti-infalmmatory, antithrombotic and antioxidant properties. In obesity CETP is present in larger amounts, therefore the triglyceride concentration of HDL is higher, whereas its cholesterol ester content is lower and thanks to this HDL becomes smaller, denser and is metabolized easier which

leads to decreased HDL levels. In obesity - thanks to CETP - not only the production of HDL decreases, but also the clearence of triglycerol-rich HDL increases by hepatic lipase. Additionally, the composition of LDL changes: due to the increased lipolysis of triglyceride-rich LDL by hepatic lipase the production of small, dense LDL increases.

### 2.2.3. Mitochondrial and endoplasmic reticulum dysfunction, oxidative stress

Mitochondrial dysfunction means the decreased number, density, or function of mitochondria. The mitochondrium is the key intracellular organ of glucose and lipid metabolism, and its dysfunction leads to FFA and lipid accumulation. Certain products of fatty acid metabolism (e.g. DAG) inhibit insulin signalling pathways, while excess amount of FFA and glucose in the mitochondrium - in conjunction with hypoxia - results in increased ROS production. ROS - via the activation of PKC (protein kinase C), JNK (c-Jun N-terminal kinase) and NF-κB - impairs insulin signalling and activates the TNF-α pathway. It is still unclear whether mitochondrial dysfunction leads to insulin resistance or vica versa.

The dysfunction of the endoplasmic reticulum results in the production of UPRs (unfolded proteins), the transcription of UPR target genes and decreased insulin sensitivity.

### 2.2.4. Low-grade, chronic inflammation

Low-grade, chronic inflammation in obesity is characterized by leukocytosis and elevated levels of proinflammatory cytokines. Inflammation leads to the development of insulin resistance both directly (via the competitive inhibition of tyrosine phosphorylation of IRS and the IRS-1/PI3K/Akt pathway) and indirectly (via decreased PPAR-gamma expression and increased lipolysis resulting in elevated free fatty acid levels). Changes is adipocytokine levels, dyslipidemia, mitochondrial and endoplasmic reticulum dysfunction, oxidative stress and the increased production of proinflammatory cytokines are all responsible for the activation of intracellular inflammatory pathways.

TNF-α is produced not only by macrophages and monocytes, but also adipose tissue and muscle. TNF-α - via the p55 receptor - inhibits the function of IRS-1 and PPARγ (the phosphorylation of IRS increases by serin kinases), prevents the translocation of GLUT 4 (glucose transporter type 4) into the cell membrane, decreases fatty acid and glucose metabolism, and reduces adiponectin concentration. On the other hand, TNF-α enhances the production of proinflammatory cytokines (e.g. IL1, IL6) by macrophages, activates the NF-κB pathway and increases oxidative stress in adipose tissue. The previously mentioned activation of the NF-κB pathway is partly responsible for the inflammation of pancreatic β cells and hence decreased insulin production. As TNF-α

levels are higher in obesity, TNF-α plays an important role in the development of insulin resistance.

IL6 - another proinflammtory cytokine - decreases insulin receptor expression and apiponectin levels and impairs insulin signalling via the phosphorylation of IRS-1.

IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta)/NF-κB is an important inflammatory pathway and it can block insulin signalling in adipose tissue (IKKβ also phosphorylates IRS-1).

Fatty acid induced TLR-4 receptor activation causes IRS phosphorylation and thus alters insulin signalling, while the activation of the TLR-4/NF-kB increases the production of proinflammatory cytokines.

In obesity macrophage concentration significantly increases in adipose tissue. These macrophages produce TNF-α and other proinflammatory cytokines more actively compared to adipose tissue. There are 2 main types of adipose tissue macrophages: the proinflammatory type M1 (it produces large amounts of IL 12 and nitrogen oxide synthase, expresses mainly MHC II and produces only small amounts of IL-10) and M2, which has rather anti-inflammatory qualities. Obesity is characterized by M1 dominance.

To date it is still unclear whether inflammation leads to insulin resistance or vica versa.

### 2.2.5. Insulin resistance

Obesity significantly inceases the risk of developing insulin resistance and type 2 diabetes. Insulin resistance is caused by all the above-mentioned factors (changes is adipocytokine levels, dyslipidemia, mitochondrial and endoplasmic reticulum dysfunction, oxidative stress and consecutive low-grade, chronic inflammation).

#### 2.2.5.1. Insulin effects

Insulin is produced by pancreatic β cells. It echances cellular glucose uptake and glucose utilization (oxidation), increases glycogenesis, lipogenesis, and protein synthesis, inhibits gluconeogenesis, glycogenolysis, proteolysis and lipolysis. Insulin binds to the alpha subunit of its receptor, resulting in the autophosphorylation of the receptor and the phosphorylation of IRS. The latter activates PKB (protein kinase B)/Akt and as a result GLUT4 is relocated into the cell membrane where it increases glucose uptake. Insulin also phosphorylates SCH proteins (Sch adaptor protein) resulting in cell growth and proliferation via the MAPK (mitogen-activated protein kinase)/RAS pathway.

Insulin increases lipogenesis and inhibits lipolysis. It enhances the activity of lipoprotein lipase therefore promotes the hydrolysis of TAG (triacylglycerol), chylomicron and VLDL, thus making it possible for cells to take up FFAs. Intracellularly

free fatty acids are activated by acetyl-coenzyme A synthetase, and acetyl-coenzyme A serves as a substrate for de novo lipid synthesis. In the process of de novo TAG synthesis GPAT (glycerl-3-phosphate acyltransferase) catalyses the creation of phosphatidic acid, which - after dephosphorylation by phosphatidic acid phosphatase - turns into DAG. DAG is then transformed into TAG by the enzyme DGAT (diacylglycerol acyltransferase) If adipose tissue has reached its maximum storing capacity lipid deposition continues in other organs (mainly in muscles and the liver) leading to insulin resistance. Insulin - via the inhibition of hormone-sensitive lipase - decreases lipolysis in adipose tissue, promotes the breakdown of ApoB100 (apolipoprotein B100) and decreases hepatic VLDL secretion. Insulin also enhances the LPL mediated hydrolysis of triglyreide from VLDL and the activity of hepatic lipase. It also contributes to the dephosphorylation of HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme-A reductase) in the liver which results in increased cholesterol synthesis.

If energy demand of the body increases lipolysis is going to take place and TAG is hydrolyzed into FFA and glycerol. Lipolysis is activated by catecholamines and other hormones (e.g. growth hormone, glucagon, natriuretic peptide, sTSH = thyroid-stimulating hormone). Catecholamines act via adrenergic receptors found in adipocyte cell membranes (mainly on β1, β2 and partly on β3 receptors). Activation of these receptors result in elevated cAMP (cyclic adenosine monophosphate) levels, the activation of protein kinase A, the consecutive phosphorylation and activation of hormon-sensitive lipase, the breakdown of TAG and the production of diglycerol and monoglycerol. Natriuretic peptides act via the activation of the cGMP (cyclic guanosine monophosphate) and HSL pathways (the levels of natriuretic peptide are lower in obesity and type 2 diabetics).

#### 2.2.5.2. Insulin resistance and its consequences

Insulin resistance means the decreased response of cells to insulin. In insulin resistance insulin cannot successfully prevent HSL mediated lipolysis in adipose tissue. Additionally, the activation of LPL is altered, and this leads to the accumulation of triglyceride in circulation. CETP enhances triglyceride and cholesterol ester transfer between apolipoproteins, thus making LDL and HDL rich in triglyceride, and smaller and denser after hydrolysis. Significant postprandial lipaemia develops and the level of non-esterified fatty acids dramatically increase. These non-esterified fatty acids are then mainly taken up by the liver. To compensate for this hepatic VLDL production (mainly large VLDL synthesis) increases. As the rate of excretion depends both on the production and excretion of VLDL the excess amount of triglyceride accumulates in the liver resulting in the development of non-alcoholic fatty liver disease. Hyperinsulinemia - even in mild insulin resistance - stimulates genes involved in de novo lipid synthesis via SREBP 1-c (sterol regulatory-element binding protein, found in

hepatocyte membranes). Hyperglycaemia can also contribute to increased lipogenesis via the activation of ChREBP (carbohydrate response element binding protein).

Insulin resistance increases the chance of developing type 2 diabetes, ischemic heart disease, metabolic syndrome (characterized by central obesity, hyperglycaemia, dyslipidemia, hypertension), polycystic ovary syndrome, non-alcoholic fatty liver disease and certain neoplasms (e.g. colon, breast and ovarian cancer).

### 2.2.6. The relationship between carbohydrate metabolism, diabetes mellitus and sepsis

Abnormal carbohydrate metabolism is relatively common in critically ill patients. It is initiated by stress, which causes hyperglycaemia and high glycemic variability. Inflammatory mediators in sepsis enhance hepatic gluconeogenesis and insulin resistance. At the same time catecholamines and cortisol - released from the adrenal glands - further increase glucose levels. High blood sugar concentrations at admission as well as hypoglycemia during intensive insulin therapy both are bad prognostic signs in sepsis. Strict glycemic control on the other hand suggests a favourable outcome.

Diabetes - especially if we consider its prevalence and the altered immune response in diabetics - is a major risk factor of sepsis. Chronic hypeglycaemia in diabetics, microorganisms causing sepsis and their toxins all contribute to the worsening of inflammatory response. Proinflammatory cytokine release (due to both diabetes and sepsis) and the activation of the immune system result in endothelial dysfunction and organ failure. Infections caused by multi-resistant organisms and post-septic complications are more common in sepic, diabetics compared to non-diabetic, septic patients.

In one British cohort study they found a significant increase in infection rate in diabetics if HbA1c levels were <6% and 7%< compared to those whose HbA1c levels were optimal (6-6.9%).

A Danish publication (involving approximately 70000 patients) pointed out that in type 2 diabetics whose HbA1c was <5.5% or 6.5%< the number of hospital admissions was significantly higher compared to those whose HbA1c was between 5.5 and 6.5%.

In a prospective Swedish study (involving more than 500000 diabetics, published in 2021) they found a U-shaped correlation between HbA1c and the risk of sepsis (the risk of sepsis was 1.15x if HbA1c was <6.1%, 1x if HbA1c was 6.1-6.5%, 0.93x if HbA1c was 7-7.8%, 1.05x if HbA1c was 7.9-8.7%, 1.14x if HbA1c was 8.8-9.7%, 1.52x if HbA1c was 9.7%< compared to patients with HbA1c between 6.5 and 6.9%). They also pointed out that most diabetic, septic patients had poor glycemic control and mortality was 4.16x compared to non-septic, diabetics. They found no significant correlation between HbA1c and mortality in this study.

Some publications even pointed out the potential benefits (better prognosis and outcome) of slightly elevated glucose levels in diabetic, septic patients.

It is also well-known that certain anti-diabetic medications have beneficial properties in sepsis. Insulin for example prevents adverse, hyperglycaemia induced immunological changes and has both direct and indirect anti-inflammatory effects. Metformin has anti-inflammatory, vasoactive and antimicrobial qualities. Thiazolidinediones promote neutrophil migration thus enhance immune response.

## 2.3. Objectives

### 2.3.1. Examining afamin levels and their correlation with oxidative and lipid parameters in non-diabetic, obese patients

In our prospective study we were interested in the relationship between lipid parameters, oxidized LDL, α- and γ-tocopherol levels, lipoprotein subfractions, carbohydrate parameters and afamin in obese, non-diabetic patients (n = 50) compared with normal-weight, healthy individuals (n = 32). We also wanted to find the independent predictors of afamin.

### 2.3.2. Examining the potential diagnostic and predictive role of HbA1c in diabetic, septic patients

In our retrospecive, single-center study we examined diabetic, septic patients who had HbA1c levels measured either in the previous 30 days (SIRS 30d subgroup, n=39) or within 24 hours after their Emergency Department admission (SIRS 24h subgroup, n=73). Sepsis was diagnosed based on the SIRS criteria (SIRS group, n=112). We later selected those patients from the SIRS group, who were also considered septic based on the SOFA criteria (SOFA group, n=55). SOFA patients were also divided into two subgroups based on the time of measurement of HbA1c (30d subgroup, n=21 vs. SOFA 24h subgroup, n=34). We analyzed the relationship between laboratory parameters (urea and electrolytes, glucose levels, liver function tests, pancreatic enzymes, C-reactive protein - CRP, procalcitonin - PCT, albumin, full blood count), length of hospital stay and HbA1c in every group and subgroup. Additionally we examined the possible independent predictors of HbA1c.

## 3. Patients and methods

### 3.1. Non-diabetic, obese patients

A total of 50 non-diabetic, obese (BMI > 30 kg/m$^2$) patients and 32 normal-weight, healthy, age- and sex-adjusted control patients were enrolled in our study. Patients with liver, kidney, endocrine disorders (including both type I and II diabetes mellitus) or malignancies were excluded. Exclusion criteria also included pregnancy, breast feeding, smoking and regular alcohol consumption. All subjects gave their informed consent before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University of Debrecen and the Medical Research Council (registration number: DE RKEB/IKEB 5513B-2020 and ETT TUKEB IV/7989-1/2020/EKU, respectively).

### 3.2. Diabetic, septic patients

We collected all cases from the Department of Emergency Medicine and later Emergency Clinic at the University of Debrecen, between 1$^{st}$ January 2017 and 31$^{st}$ December 2018 (27737 patients, 42766 cases). First, we selected patients who had their HbA1c measured in the study period (3743 patients) and later from these patients we collected those diabetic, septic patients who had HbA1c levels measured either in the previous 30 days or within 24 hours after their Emergency Department admission. Sepsis was diagnosed based on the SIRS criteria. Patients with autoimmune disease, end-stage renal failure, liver cirrhosis and active cancer were excluded from our study. HbA1c levels strongly depend on the turnover of red blood cells: slow turnover (e.g. in iron, vitamin B12, or folate deficiency anemias) often results in higher, whereas fast turnover (e.g., hemolytic anemia and erythropoietin therapy) leading to lower HbA1c levels. Therefore, all patients with the above-mentioned disorders have been excluded from the study. HbA1c levels also vary among different racial and ethnic groups (e.g. higher levels in Afro-Americans and Asians), therefore we enrolled only Caucasian patients. This way 112 diabetic, septic patients were included in our study (SIRS group) from whom 39 had HbA1c measured in the previous 30 days (SIRS 30d subgroup) and 73 within 24 hours after their Emergency Department admission (SIRS 24h subgroup). The past medical history (type of diabetes, hypertension, dyslipidaemia, ischaemic heart disease, previous myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting surgery, transient ischaemic attack, stroke, peripheral arterial disease, chronic renal failure), anti-diabetic therapy (metformin, sulfonylureas, dipeptidyl peptidase-4 inhibitors, other oral anti-diabetic agents, insulin), laboratory results (arterial blood gas results, urea and electrolytes, glucose levels, liver function tests, pancreatic enzymes, C-reactive protein - CRP, procalcitonin - PCT, albumin, full blood count), HbA1c levels and time of measurement, SIRS and SOFA scores, microbiological results, type of infection, length of hospital stay and

mortality data of all patients were summarized in a table. Most laboratory parameters - with sometimes the exception of HbA1c - were measured upon arrival. We later selected those patients from the SIRS group, whose SOFA score was ≥ 2 (55 patients, SOFA group). SOFA patients were also divided into two subgroups based on the time of measurement of HbA1c (patients with HbA1c measured in the previous 30 days - SOFA 30d subgroup vs. patients with HbA1c measured within 24 hours after their Emergency Department admission - SOFA 24h subgroup). The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University of Debrecen and the Medical Research Council (registration number: DE RKEB/IKEB H.0172–2020 and ETT TUKEB: 7324-9/2017/EÜIG, respectively).

### 3.3. Sample collection and laboratory measurements

Venous blood samples were obtained from both obese and control patients after 12 h of fasting. A Cobas c501 (Roche Ltd., Mannheim, Germany) analyzer was used to measure carbohydrate and lipid parameters. Oral glucose tolerance test (OGTT) was performed in order to exclude diabetes mellitus in obese patients (75 g of glucose). HbA1c and insulin levels were also measured at the same time. The widely used formula was used to calculate homeostasis model assessment-estimated insulin resistance (HOMA-IR) values (fasting insulin concentration (μU/mL) × fasting glucose concentration (mmol/L)/22.5). Serum samples were separated by centrifugation at 4 °C at 3500× *g* for 10 min. Routine laboratory parameters were determined from fresh sera with a Cobas c501 analyzer (Roche Ltd. Mannheim, Germany). Total cholesterol levels were measured by using enzymatic, colorimetric tests (cholesterol oxidase-p-aminophenazone—GPOD-PAP; Modular P-800 analyzer; Roche/Hitachi). HDL cholesterol and LDL cholesterol levels were determined by a homogenous enzymatic, colorimetric assay (Roche HDL-C plus third generation and Roche LDL-C plus second generation, respectively). Immunoturbidimetric assays (Tina-quant apolipoprotein A-I ver. 2 and Tina-quant apolipoprotein B ver. 2, respectively) were used to measure Apo A-I and ApoB levels. All the tests were performed according to the recommendations of the manufacturer. Sera were kept frozen at −70 °C for subsequent lipoprotein subfraction analysis and ELISA measurements.

### 3.4. Determination of serum afamin levels

Serum afamin concentrations were measured by a commercially available ELISA kit (Afamin Human ELISA, cat. number: RD194428100R, BioVendor, Asheville, NC, USA), according to the recommendations of the manufacturer. The intra- and inter-assay variation coefficients were <3.61% and <3.4%, respectively.

### 3.5. Measurement of serum oxidized LDL concentration

Serum concentrations of oxidized LDL (oxLDL) were detected by a commercially available solid phase two-site enzyme immunoassay (ELISA) kit (Mercodia AB, Uppsala, Sweden). Measurements of oxLDL levels in the sera were performed according to the recommendations of the manufacturer. The intra- and inter-assay coefficients of variations were 5.5–7.3% and 4.0–6.2%, respectively, and the sensitivity was <1 mU/L.

### 3.6. Measurement of serum α- and γ-tocopherol levels by gas chromatography-mass spectrometry

Plasma α- and γ-tocopherol determination was based on the modified method described by Zerbianti et al. Gas chromatography-mass spectrometry measurements were performed with Finnigan Trace GC Ultra connected to Polaris Q mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were injected manually into Agilent J&W column (DB-5MS UI; 60 m × 0.25 m × 0.25 μm) using helium as carrier gas (1 mL/min, constant flow). In total, 2 μL of the previously prepared samples were injected into each column. Initial oven temperature (150 °C) was held for 2 min, then raised to 300 °C with a rate of 25 °C/min, and maintained for further 15 min, giving a total run time of 23 min. The mass spectrometer was operated in selective ion monitoring mode and the chosen ions were: α-tocopherol-TMS 237.3 m/z, γ-tocopherol-TMS 488.4 m/z, 2,2,5,7,8-Pentamethyl-6-chromanol-TMS 292.3 m/z.

### 3.7. HDL subfraction analysis

HDL subfractions were measured by using an electrophoretic method on polyacrylamide gel with the Lipoprint System (Quantimetrix Corp., Redondo Beach, CA, USA), according to the manufacturer's instructions. Ten HDL subfractions were differentiated between VLDL + LDL and albumin peaks, and were grouped into three major classes: large, intermediate and small HDL subfractions. The percentage of large (HDL1-3), intermediate (HDL4-7) and small (HDL8-10) HDL subfractions were analyzed with Lipoware software (Quantimetrix Corp., Redondo Beach, CA, USA). Cholesterol concentrations of HDL particle subsets were calculated by multiplying HDL-C concentrations of samples by the relative area under the curve (AUC) of subfraction bands.

### 3.8. LDL subfraction analysis

LDL subfractions were also detected using the Lipoprint System (Quantimetrix Corp., Redondo Beach, CA, USA) according to the instructions of the manufacturer. AUC% for the VLDL, Midband A, B, C (comprising primarily IDL), up to seven LDL subfractions and HDL peaks were calculated by Lipoware computer software (Quantimetrix Corp., Redondo

Beach, CA, USA). Percentage of large LDL (large LDL%) was defined as the sum of the percentage of LDL1 and LDL2, whereas percentage of small LDL (small-dense LDL%) was defined as the sum of LDL3-LDL7. Cholesterol concentrations of LDL subfractions were determined by multiplying the relative AUC of subfractions by total cholesterol concentration of the sample. Mean LDL size was calculated by Lipoware software (Quantimetrix Corp., Redondo Beach, CA, USA).

### 3.9. Statistical analysis

The STATISTICA (ver: 13.7; StatSoft Inc., Tulsa, OK, USA) program was used to analyze data. The Kolmogorov–Smirnov test was used to analyze the distribution of our results. In case of normal distribution, the two sample Student T-test, whereas in case of non-normal distribution the Mann–Whitney u test was performed. Parameters with normal distribution are given as mean ± standard deviation (SD). Other parameters with non-normal distribution are given as median with lower and upper quartiles.

Pearson's correlation was used to analyze the relationship between continuous variables. In case of non-normal distribution parameters, we calculated base 10 logarithm prior to analysis.

Multivariate analysis (backward-stepwise method) was used to find those parameters that correlated with afamin levels the most.

We performed backward stepwise multiple regression analysis to determine independent predictors of HbA1C. The model included age, gender, $\log_{10}$ length of hospital stay in survivors, insulin use, platelet count, $\log_{10}$ bilirubin, white blood count, and $\log_{10}$ glucose. Those parameters that showed no correlation with HbA1c were excluded prior to analysis.

Results were considered significant if $p$ value was <0.05.

## 4. Results

### 4.1. Non-diabetic, obese and normal-weight control patients

Lipid and carbohydrate values significantly differed between obese and age- (44.2 ± 13.5 and vs 41.87 ± 6.71 yrs) and sex-adjusted (n = 50, 43 males and 7 femles vs n= 32, 27 males and 5 females) control patients; however, these parameters were still in normal range (BMI 41.96 ± 8.63 kg/m$^2$ vs 24.24 ± 2.54 kg/m$^2$, p <0.001). Triglyceride [1.4 mmol/l (1.1-2.0) vs 1.0 mmol/l (0.75-1.39), p <0.01] and LDL (3.17 ± 0.74 mmol/l vs 2.86 ± 0.55 mmol/l, p <0.05) levels were significantly higher, HDL (1.36 ± 0.33 mmol/l vs 1.59 ± 0.47 mmol/l, p <0.001) and ApoA-I (1.48 ± 0.24 g/l vs 1.71 ± 0.31 g/l, p <0.001) concentrations were significantly lower in obese patients. There was no significant difference between cholesterol (5.04 ± 0.83 mmol/l vs 5.02 ± 0.75 mmol/l) and ApoB levels (0.86 ± 0.2 g/l vs 0.88 ± 0.23 g/l). HbA1c (5.07 ± 0.33 % vs 5.76 ± 0.54 % p <0.001) and fasting glucose levels

(5.41 ± 0.71 mmol/l vs 4.79 ± 0.47 mmol/l, p <0.001) were significantly higher in obese, non-diabetic patients; however, even these parameters were in normal range. Two-hour OGTT and HOMA-IR values were used to exclude diabetes. Elevated hsCRP in obese patients (high-sensitive C-reactive protein, 8.24 mg/l vs 1.4 mg/l, p <0.001) was due to the previously mentioned low-grade systemic inflammation. We also found significantly elevated uric acid (315.24 ± 91.61 µmol/l vs 254.53 ± 63.73 µmol/l, p <0.001), GOT (23.52 ± 8.98 U/l vs 18.71 ± 3.87 U/l, p <0.01), GPT (29.4 ± 15.25 U/l vs 18.13 ± 7.87 U/l, p <0.001), GGT (33.58 ± 21.44 U/l vs 24.34 ± 15.35 U/l, p <0.05) and LDH (355.58 ± 83.95 U/l vs 222.24 ± 73.23 U/l, p <0.001) concentrations in obese patients. There was no significant difference in sTSH levels between the two groups (1.98 ± 0.98 mU/L vs 2.06 ± 1.22 mU/L).

Total HDL concentrations were significantly lower in obese, non-diabetic patients (1.36 ± 0.33 mmol/l vs 1.59 ± 0.47 mmol/l, p <0.001). There was also a shift in HDL particles to small, dense HDL subfractions. The percentage and absolute amount of large HDL particles were significantly lower (0.319 ± 0.157 mmol/l vs 0.528 ± 0.308 mmol/l, p<0.05, 22.5 ± 5.7 % vs 30.9 ± 9.4 %, p<0.05), while the percentage and absolute amount of small HDL were significantly higher in obese patients (0.333 ± 0.070 mmol/l vs 0.282 ± 0.060 mmol/l, p<0.01, 25.2 ± 5.9 % vs 18.9 ± 5.7 %, p<0.05). There was a mild, but significant, decrease in the amount of intermediate HDL in obese patients (0.708 ± 0.169 mmol/l vs 0.781 ± 0.170 mmol/l, p<0.001, 52.3 ± 3.4 % vs 50.2 ± 4.7 %, p<0.001). Total LDL concentrations (3.17 ± 0.74 mmol/l vs 2.86 ± 0.55 mmol/l, p <0.05), as well as the percentage and amount of both large (1.317 ± 0.361 mmol/l vs 1.077 ± 0.352 mmol/l, p<0.01, 25.8 ± 4.1 % vs 21.4 ± 5.9 %, p<0.05) and small LDL (0.113 ± 0.118 mmol/l vs 0.051 ± 0.109 mmol/l, p<0.05, 2.0 ± 1.6 % vs 1.0 ± 2.1 %, p<0.001) subfractions were higher in obese patients. The mean size of LDL particles was significantly smaller in obese patients (26.980 ± 0.314 nm vs 27.253 ± 0.346 nm, p<0.01).

Serum afamin (70.43 ± 12.87 µg/ml vs 47.56 ± 8.46 µg/ml, p<0.0001), oxidized LDL (46.8 ± 9.95 U/L vs 40.20 ± 10.10 U/L, p<0.005), α- [9.4 µg/ml (7.9-13.17) vs 8.22 µg/ml 7.21-9.69), p<0.05] and γ tocopherol [0.2 µg/ml (0.16-0.31) vs 0.12 µg/ml (0.1-0.17), p<0.001] levels were significantly higher in obese patients. This difference was still present after α- and γ tocopherol levels were normalized to cholesterol values [α-tokoferol/cholesterol 1.953 (1.620-2.507) vs 1.640 (1.491-1.9464), p<0.05, γ-tokoferol/cholesterol 0.042 (0.031-0.062) vs 0.0255 (0.021-0.0327), p<0.0001].

### 4.2. Correlations between anthropometric values, laboratory parameters and afamin

As previously published in different articles, significant positive correlations were found between BMI (r = 0.582, $p < 0.001$), waist circumference (r = 0.434, $p < 0.005$), glucose (r = 0.468, $p < 0.001$), HbA1c (r = 0.661, $p < 0.001$), uric acid (r = 0.573, $p < 0.001$), hsCRP (r = 0.537, $p < 0.001$), and afamin levels. A significant positive correlation between afamin and oxLDL levels and a significant negative correlation between afamin and mean

LDL size were seen. However, the α- and γ-tocopherol levels did not correlate with afamin concentrations in obese patients (r = 0.20; $p = 0.2$ and r = 0.22; $p = 0.1$, respectively).

There were significant negative correlations between the percentage and absolute amount of large HDL and afamin levels and significant positive correlations between the percentage and amount of small HDL and afamin concentrations.

A multiple regression analysis was performed to determine which parameter (age, BMI, waist circumference, glucose, HbA1c, α- and γ-tocopherol, α-tocopherol/cholesterol, γ-tocopherol/cholesterol, oxLDL, the amount and percentage of large and small HDL) affects afamin levels the most. Afamin levels were mainly influenced by waist circumference (β = 0.685, $p < 0.001$), HbA1c (β = 0.291, $p < 0.01$) and the amount of small HDL subfraction (β = 0.282, $p < 0.05$).

## 4.3. Diabetic, septic patients with HbA1c levels measured within 24 hours after emergency department admission

### 4.3.1. SIRS 24h patients

SIRS 24h patients were 72.8±12.7 years old (73 patients, 47 females, 26 males). All our patients were type II diabetics. Hypertension was the commonest comorbidity among patients (n = 67, 91.8%), but other - mainly cardiovascular - disorders were also frequent (dyslipidaemia n = 31, 42.5%, ischaemic heart disease/previous myocardial infarction/percutaneous coronary intervention/coronary artery bypass grafting surgery n = 32, 43.8%, transient ischaemic attack/stroke n = 15, 20.6%, peripheral arterial disease n = 42, 57.5%, chronic renal failure n = 27, 37%). Most patients were taking metformin (n = 30, 41.1%), while the use of other oral anti-diabetic medications was less common (sulfonylurea n = 24, 32.9%, dipeptidyl peptidase-4 inhibitors n = 4, 5.5%). Only 18 patients were on insulin therapy prior to admission (24.7%). Average laboratory values in SIRS 24h patients were the following: blood sugar 11.5 mmol/l (7.7-16.3), HbA1c 7.47 ± 1.8%, urea 8.4 mmol/l (6-12.3), creatinine 99 mmol/l (77-137), GFR 52 ml/min$^{x}$1,73 m$^{2}$ (38-75), CRP 77 mg/l (21-151.5), GOT 21 U/L (17-33.5), GGT 35 U/L (21-69), GPT 21 U/L (14-32), bilirubin 10 µmol/l (6.5-17.4), white blood cell count 15.8 ± 6.1 G/L, Hgb 129.8 ± 22.3 g/L, platelet count 252.6 ± 76.9 G/l. Survivors spent 8 days (6-11.5) in hospital on average.

We analyzed the relationship between laboratory parameters, length of hospital stay and HbA1c. Additionally we examined the relationship between leukocyte count and glucose, platelet count and glucose, and length of hospital stay and glucose levels. In these patients there was a significant positive correlation between glucose and HbA1c levels ($p<0.001$). We found significant negative correlations between white blood cell count and glucose ($p = 0.01$), white blood cell count and HbA1c levels ($p = 0.001$). Correlations were observed in most cases even if patients were divided based on

gender, anti-diabetic therapy (oral anti-diabetic agents vs. insulin therapy), age (<65 yrs vs.≥65 yrs) and hospitalization in the previous 90 days. Due to lack of data, we could not conclude anything regarding the relationship between HbA1c and mortality.

### 4.3.2. SOFA 24h patients

34 type II diabetic, septic patients were in the SOFA 24h group (21 females, 13 males, age: 73.9±12.3 years). Hypertension was also the commonest comorbidity in this group (n = 31, 91.2%), but other disorders were also frequent (dyslipidaemia n = 12, 35.3%, ischaemic heart disease/previous myocardial infarction/percutaneous coronary intervention/coronary artery bypass grafting surgery n = 18, 52.9%, transient ischaemic attack/stroke n = 6, 17.7%, peripheral arterial disease n = 18, 52.9%, chronic renal failure n = 13, 38.2%). The same number of patients used metformin and insulin in the SOFA 24h group (n = 11, 32.4%). Sulfonylureas were taken by 10 patients (29.4%), while no patient was on dipeptidyl peptidase-4 inhibitor treatment prior to admission. Average laboratory values in SOFA 24h patients were the following: blood sugar 12.05 mmol/l (8.5-19.2), HbA1c 7.26 ± 1.9%, urea 9.85 mmol/l (6.2-19.4), creatinine 118 mmol/l (95-172), GFR 42 ml/min$^{x}$1.73 m$^{2}$ (27-61), CRP 108 mg/l (21.3-246.3), GOT 25 U/L (16-42), GGT 40 U/L (16-124), GPT 21 U/L (14-37), bilirubin 11,2 µmol/l (6.3-33.6), white blood cell count 17.3 ± 7.3 G/L, Hgb 133.8 ± 19.4 g/L, platelet count 251.0 ± 92.8 G/l. Survivors also spent 8 days (7-11.5) in hospital on average.

We analyzed the relationship between laboratory parameters, length of hospital stay and HbA1c. Additionally we examined the relationship between leukocyte count and glucose, platelet count and glucose, and length of hospital stay and glucose levels. There was a significant positive correlation between glucose and HbA1c levels in the SOFA 24h group, similarly to the one we found in SIRS 24h patients ($p<0.001$). We also found significant negative correlations between white blood cell count and glucose ($p = 0.02$) and white blood cell count and HbA1c levels in SOFA 24h patients ($p = 0.02$). Additionally, there was a significant positive correlation between HbA1c levels and length of hospital stay in survivors ($p = 0.01$). The previous correlations in the SOFA 24h group were observed in most cases even if patients were divided based on gender, anti-diabetic therapy (oral anti-diabetic agents vs. insulin therapy), age (<65 yrs vs.≥65 yrs) and hospitalization in the previous 90 days. Due to lack of data, we could not conclude anything regarding the relationship between HbA1c and mortality.

### 4.4. Diabetic, septic patients with HbA1c levels measured in the previous 30 days before their emergency department admission

#### 4.4.1. SIRS 30d and SOFA 30d patients

There were 39 diabetic, septic patients in the SIRS 30d group. We studied the same correlations that were previously examined in the SIRS 24h group: we did not find any significant correlation in this population even if we later selected and examined patients whose SOFA score was positive (≥ 2) (SOFA 30d group, 21 patients). Due to lack of data, we also could not conclude anything regarding the relationship between HbA1c and mortality in these groups.

### 4.5. Backward stepwise multiple regression analysis

We performed backward stepwise multiple regression analysis to determine independent predictors of HbA1C. The model included age, gender, $\log_{10}$ length of hospital stay in survivors, insulin use, platelet count, $\log_{10}$ bilirubin, white blood count, and $\log_{10}$ glucose. Glucose levels ($\beta = 0.324$; $p = 0.02$) and insulin use ($\beta = 0.612$; $p = 0.003$) were significant independent predictors of HbA1c.

## 5. Discussion

### 5.1. Non-diabetic, obese patients

Internationally we were among the first to examine the relationship between carbohydrate parameters, lipid, α- and γ-tocopherol levels, lipoprotein subfractions and afamin concentrations in non-diabetic, obese patients. Afamin levels were 48.1% higher in obese, non-diabetics compared to control patients. This elevation is similar to that observed in diabetics compared to healthy individuals; however, due to methodical differences, the values are not totally comparable.

Lipid and glucose levels were significantly higher in obese non-diabetic patients; however, they were still in normal range. Despite this, BMI, waist circumference, fasting glucose, Tg, hsCRP and LDL-C levels did correlate well with afamin concentrations in non-diabetic obese patients, similar to diabetics. This suggests that afamin may contribute to the development of insulin resistance at an early stage. Previous studies did not examine the relationship between afamin levels and lipid subfractions in obese patients. We found significant correlations between the percentage and amount of HDL subfractions and afamin levels. The binding of afamin to small, dense HDL subfractions and the shift to these HDL fractions in obesity have been described previously. After the multiple regression analysis, the amount of small HDL subfraction turned out to be one major determinant of afamin. The strong correlation observed between HDL subfractions and afamin levels is a

remarkable finding and may help us to better understand the function of HDL subfractions. It is well known that several antiatherogenic proteins—e.g., human paraoxonase-1—are also bound to small, dense HDL subfractions. However, the increased amount and percentage of small, dense HDL subfractions alone—as small HDL levels were only 18.1% higher in obese non-diabetic patients—does not explain the significant increase in afamin levels. The role of fat-soluble, herbal molecules, frequently referred to as vitamin E, has been studied for decades. It is widely established that free radicals—produced during increased oxidative stress in inflammatory conditions—cause lipid peroxidation and thus contribute to atherosclerosis. The antioxidant properties of α- and γ-tocopherols—the two most common and widely consumed vitamin E components—have been well described. At the same time, results from studies focusing on the effects of vitamin E on atherosclerosis are incomprehensive due to different characteristics and cardiovascular risk between study populations. Currently, we think that vitamin E supplementation could exert beneficial effects on atherosclerosis in some high-risk patients. The levels of α- and γ-tocopherol were slightly, but significantly, higher in obese, non-diabetic patients. The α- and γ-tocopherol/total cholesterol ratios were also significantly higher in obese patients. Our study was the first to examine α- and γ-tocopherol levels in obese, non-diabetic patients, although the relationship between vitamin E and lipid levels has been previously described in various patient groups. We did not find any correlation between α- and γ-tocopherol and afamin levels. This suggests that afamin—despite the fact that it features specific α- and γ-tocopherol binding sites—does not play a crucial role in the regulation of vitamin E levels. As oxidized LDL levels are higher in obese, non-diabetic patients, increased α- and γ-tocopherol concentrations could be beneficial in reducing endogen oxidative stress in these patients. However, further studies are needed to evaluate this hypothesis.

Our goal was to better understand the role of afamin in the regulation of lipoprotein metabolism and in oxidative stress in morbidly obese, non-diabetic subjects in order to define the effect of obesity on these parameters without the concomitant effect of carbohydrate disturbances. This information could be useful to estimate obesity-associated cardiovascular risk and may help us to come up with personalized therapeutic strategies in obese subjects.

### 5.2. Diabetic, septic patients

As diabetes mellitus is a major risk factor of sepsis, we aimed to evaluate the possible effects of diabetes mellitus and poor glycemic control on the diagnosis and prognosis of sepsis. This is the first study to evaluate the potential role of HbA1c in diabetic, septic patients. In SIRS 24 h patients, we found a significant positive correlation between glucose and HbA1c levels, while significant negative correlations were observed between white blood cell count and glucose, white blood cell count and HbA1c. These correlations were observed in most cases even if patients were divided based on gender, anti-diabetic therapy (oral anti-diabetic agents vs. insulin therapy), age (<65 yrs vs.≥65 yrs) and hospitalization in

the previous 90 days. One possible explanation behind the observed negative correlations between white blood cell count and glucose, white blood cell count and HbA1c is glucose toxicity, a phenomenon previously described in pancreatic beta cells. According to previous studies, hyperglycemia in diabetic, septic patients increases oxidative stress and induces glucose-induced apoptosis mainly in metabolically active cells (e.g., white blood cells in sepsis), resulting in cell death. There are some diabetic, septic patients - in whom sepsis is diagnosed based on the SIRS criteria - whose white blood cell count is normal. These diabetic, septic patients with normal white blood cell counts (WBC count between 4–12 $\times 10^9$/l) have higher HbA1c levels. This observation is crucial as white blood cell count is an important part of the SIRS criteria (positive criterium: white blood cell count < 4,000/$mm^3$ or > 12,000/$mm^3$ or >10% bands). It may occur in diabetic, septic patients - in whom the diagnosis is based on the SIRS criteria - that white blood cell count is normal (between 4–12$\times 10^9$/l), and there is only one other positive SIRS criterium (heart rate >90/min, temperature < 36°C or > 38° C, respiratory rate >20/min or $PaCO_2$ < 32 mmHg). According to the definition of sepsis - based on the SIRS criteria - these patients are not septic; however, the potential life-threatening immune processes might have already started. HbA1c - based on the negative correlation found between white blood cell count and HbA1c levels - can be a useful tool in finding these patients: in diabetic, septic patients, normal white blood cell count (4–12 $\times 10^9$/l) with elevated HbA1c levels should be considered a positive SIRS criterium. Therefore, HbA1c - measured within 24 hours after admission (preferably upon arrival) - could turn out to be an efficient way to identify these diabetic, septic patients early and initiate sepsis treatment accordingly. Further, large, multicentric studies are needed to confirm our hypothesis.

In the SOFA 24 h group, we found a significant positive correlation between glucose and HbA1c levels, significant negative correlations between white blood cell count and glucose, white blood cell count and HbA1c. We also found a significant positive correlation between length of hospital stay and HbA1c levels in survivors. A significant negative correlation was observed between white blood cell count and HbA1c in SOFA 24 h diabetic, septic patients similarly to the SIRS 24 h group. It must be noted that white blood cell count is not a SOFA criterium. Therefore, its correlation with HbA1c and consequently the possible early diagnostic potential of HbA1c is not that significant in SOFA patients. On the other hand - as there was a significant positive correlation between length of hospital and HbA1c levels in survivors - HbA1c may be a significant prognostic tool in diabetic, septic patients in whom the diagnosis is based on the SOFA criteria.

We did not find any significant correlation in SIRS 30 d patients. However previous studies found no significant difference between HbA1c levels measured on admission and 30 days earlier in critically ill patients. According to this the same correlations we found in SIRS 24 h patients should have been observed in SIRS 30 d patients. A possible explanation for this difference is that HbA1c in our study was measured within 30 days prior to these patients' ED admission, and not 30 days prior exactly, and HbA1c measured on admission correlates better with a glucose concentration of the previous weeks.

We did not find any significant correlation in the SOFA 30 d group either.

## 5.3. New results

### Non-diabetic, obese patients:

- Serum afamin and oxidized LDL levels were significantly higher in obese patients.
- The amount and percentage of large and small LDL subfractions were higher, while LDL size was significantly smaller in obese, non-diabetics.
- The amount and percentage of large HDL subfraction was lower, while the amount and percentage of small HDL subfraction was significantly higher in obese patients.
- α- and γ-tocopherol levels - as well as α-tokoferol/cholesterol and γ-tokoferol/cholesterol ratios - were significantly higher in non-diabetic, obese patients, and did not correlate with afamin concentrations.
- There was a positive correlation between afamin and oxidized LDL and a negative correlation between afamin and average LDL size.
- There was a negative correlation between the amount and percentage of large HDL and afamin, and a positive correlation between the amount and percentage of small HDL and afamin.
- Waist circumference, HbA1c and the amount of small HDL subfraction are the main independent predictors of afamin.

### Diabetic, septic patients:

- In the SIRS 24h group there was a significant positive correlation between glucose and HbA1c levels. We found significant negative correlations between white blood cell count and glucose, white blood cell count and HbA1c levels in the SIRS 24h group. Correlations were observed in most cases even if patients were divided based on gender, anti-diabetic therapy (oral anti-diabetic agents vs. insulin therapy), age (<65 yrs vs.≥65 yrs) and hospitalization in the previous 90 days.
- In the SOFA 24h group there was a significant positive correlation between glucose and HbA1c levels. Additionally, there was a significant positive correlation between HbA1c levels and length of hospital stay in survivors. We also found significant negative correlations between white blood cell count and glucose and white blood cell count and HbA1c levels in SOFA 24h patients. The previous correlations in the SOFA 24h group were observed in most cases even if patients were divided based on gender, anti-diabetic therapy (oral anti-diabetic agents vs. insulin therapy), age (<65 yrs vs.≥65 yrs) and hospitalization in the previous 90 days.
- Glucose levels and insulin usage turned out to be independent predictors of HbA1c.

## 5.4. Limitations

When examining obese, non-diabetics we had no detailed information on the eating habits and vitamin E consumption of our patients. Data are also limited due to the small number of patients involved. Based on our results, it is not clear whether increased afamin levels in obesity contribute to the development of insulin resistance or are simply unrelated consequences of obesity. Thus, further examinations on larger number of obese individuals and enrolment of prediabetic and type 2 diabetic patients may help to enhance the statistical power of the study.

In diabetic, septic patients data was also limited due to the small number of patients. Therefore, we are planning to carry out a prospective study with more patients involved.

# 6. Summary

Afamin levels were elevated in obese, non-diabetic patients and concentrations did not correlate with α- and γ-tocopherol levels. Strong correlations were found between large and small HDL subfraction levels, oxLDL, mean LDL size, the components of metabolic syndrome and serum afamin concentrations in obese non-diabetics. Elevated concentrations of afamin and their association with lipoprotein subfractions might be useful when assessing obesity-associated cardiovascular risk. Based on our findings, afamin may play a role in the development of early carbohydrate and lipid abnormalities and oxidative stress in obese, non-diabetic patients. Further studies are needed to clarify the role of afamin in obesity and the development of insulin resistance.

Based on our results, we can conclude that even normal white blood cell count could be abnormal in diabetic, septic patients in whom the diagnosis is based on the SIRS criteria if an elevated HbA1c level is measured within 24 hours after admission (preferably upon arrival). Therefore, in these patients, normal white blood cell count ($4\text{-}12\times10^9$/l) with elevated HbA1c levels could be considered a positive SIRS criterium. Poor glycemic control - and hence elevated HbA1c - results in altered white blood cell response in case of an acute infection, and this has to be considered when diagnosing sepsis, especially when the SIRS criteria are used. In diabetic, septic patients, in whom the diagnosis of sepsis is based on the SOFA score and HbA1c is measured within 24 hours after admission (preferably upon arrival), HbA1c could be an important prognostic tool as there is a significant positive correlation between HbA1c levels and length of hospital stay in survivors. Based on our findings, HbA1c could turn on to be far more than a simple parameter of glycemic control, and it could also be a marker for the diagnosis of sepsis and may have values regarding hospital stay and mortality in septic diabetic patients. Further, multicenter studies focusing on the possible diagnostic and prognostic role of HbA1c in diabetic, septic patients are needed to verify our data.

## 7. Acknowledgement

I would like to express my gratitude to my supervisor, Professor György Paragh, M.D. who has supported my progression in the medical field since I was a medical student. Without his advice and support this work could not have been created. I would also like to thank Professor Zoltán Szabó, M.D. for his support.

Dr. Hajnalka Lőrincz provided indispensable help with my measurements and also took a huge part in the creation of this work. I am also thakful for the help and support provided by Professor Mariann Harangi, M.D., Dr. Ildikó Seres, Andrea Hrabovszky, my colleagues at the Department of Internal Medicine and the Department of Emergency Medicine.