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Potential role of afamin in the assessment and follow-up of the cardiometabolic risk in patients with adult growth hormone deficiency and morbid obesity

by Balázs Ratku

Supervisor:
Sándor Somodi MD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF HEALTH SCIENCES

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POTENTIAL ROLE OF AFAMIN IN THE ASSESSMENT AND FOLLOW-UP OF THE CARDIOMETABOLIC RISK IN PATIENTS WITH ADULT GROWTH HORMONE DEFICIENCY AND MORBID OBESITY

by Balázs Ratku MSc

Supervisor: Sándor Somodi PhD

Doctoral School of Health Sciences, University of Debrecen

Head of the **Defense Committee**:	Margit Balázs PhD, DSc
Reviewers:	László Márk PhD
	Attila Jakab PhD
Members of the Defense Committee:	Nikolette Szűcs PhD
	Tamás Deli PhD

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1. INTRODUCTION

Based on a current World Health Organization (WHO) report, obesity accounts for more than 13% of total deaths in the European region. Although, obesity causes a serious public health issue throughout the whole European Union, the situation in Hungary is particularly serious: the proportion of people who are considered overweight or obese is much higher than the EU average. Unlike obesity, adult growth hormone deficiency (AGHD) is a rare endocrine disease affecting approximately 1750 patients in our country. While it is obvious that these conditions are different regarding their public health significance, both conditions are characterized by a clustering of traditional and novel cardiometabolic risk factors leading to an increased cardiovascular mortality.

It is well-documented that both conditions are frequently complicated by metabolic syndrome, which aggravates the already existing cardiovascular risk. To improve cardiovascular mortality, identification of patients with the most adverse cardiometabolic risk profile would be essential, however the utility of classical metabolic syndrome criteria to determine cardiometabolic risk is highly debatable in these conditions.

By definition, biological marker (biomarker) is a characteristic, which is measured as a sensitive indicator of normal biological processes, pathological processes, or responses to therapeutical interventions. In AGHD and obesity, there is an unmet need for a biomarker with the capacity to reflect the cardiometabolic risk and it's changes to the adequate treatment. In the absence of ideal biomarker, the researchers' attention has shifted to the evaluation of organokines, which owing to their fine-tuning role in metabolic processes, can detect even subclinical changes, and as biomarkers may improve the risk assessment in these patients. Afamin, a liver-derived glycoprotein also belongs to these organokines. Based on literature data, afamin shows strong association with metabolic syndrome and can predict the development of type 2 diabetes mellitus (T2DM).

Despite the high risk of metabolic syndrome and T2DM in obesity and AGHD, there is only limited information on the regulation of afamin in obesity, while in AGHD

concentrations of the afamin and it's potential role as a biomarker has not been studied so far.

2. Review of the literature

2.1. Afamin

Human afamin is a liver-derived glycoprotein discovered in 1994 as the fourth member of the human albumin gene family. Even though afamin has been studied intensively in the past 2 decades, our knowledge regarding its physiological role is still limited. As afamin is demonstrated to be a binding protein for Vitamin E, it has been suggested to have a role as a Vitamin E carrier in the plasma and other bodily fluids. Later studies could find significant associations between the Vitamin E and afamin concentrations in the ovarian and cerebrospinal fluid but not in the plasma. In preclinical studies, afamin has been suggested to have neuroprotective effects, therefore, several studies have investigated potential biomarker properties of afamin in various neurological disorders, including Parkinson's disease and multiple sclerosis. In addition, afamin has been investigated as a potential biomarker for several types of malignancies, including ovarian and colorectal cancer and found to be a promising predictive marker for preeclampsia.

In 2014 Kronenberg et al. demonstrated increased body weight and higher plasma concentrations of lipids and glucose in transgenic mice overexpressing the human afamin gene. To investigate the observed findings in humans, they initiated a large-scale population-based study, in which they found strong associations between the afamin concentrations and the prevalence of metabolic syndrome. Furthermore, afamin strongly related to each individual component of the metabolic syndrome. In another multicenter study, afamin levels demonstrated strong associations with prediabetes, insulin resistance and the prevalence of T2DM. More recently, higher afamin levels have been found in non-alcoholic fatty liver disease (NAFLD), which is considered the hepatic manifestation of metabolic syndrome. In obesity, compared to healthy controls, higher and similar afamin levels have also been reported.

2.2. ADULT GROWTH HORMONE DEFICIENCY (AGHD)

AGHD is a distinct endocrine disorder characterized by decreased bone mineral density, reduced physical performance, decreased skeletal muscle mass, reduced quality of life, increased fat mass and adverse cardiometabolic risk profile. Due to the several possible etiologies as well as the coexistence of other pituitary hormone deficiencies, AGHD is considered a heterogenous disease. In most cases AGHD develops in the adulthood constituting the subtype of adult-onset growth hormone deficiency (AoGHD), while the less frequent subtype, the childhood-onset growth hormone deficiency (CoGHD) develops during childhood and continues to persist in the adulthood. Based on large international surveillance databases, pituitary adenomas and craniopharyngiomas account for more than half of the cases of AGHD, although recent findings suggest that certain non-classical causes (e.g., traumatic brain injury, stroke) might be more frequent than previously thought.

Due to the pulsatile GH secretion, low GH levels do not prove the presence of AGHD, therefore dynamic testing (e.g., insulin tolerance test) is generally required for the diagnosis. Diagnostic value of low insulin-like growth factor -1 (IGF-1) level is also very limited, because patients with AGHD might have IGF-1 levels within normal range, and low IGF-1 levels might also have other, more frequent causes than AGHD.

Since AGHD is associated with a clustering of cardiovascular risk factors, including visceral obesity, atherogenic dyslipidemia and insulin resistance, it results in a clinical picture similar to the metabolic syndrome. Based on findings of the last two decades, besides traditional cardiovascular risk factors, endothelial dysfunction, low-grade inflammation, impaired adipokine profile, oxidative stress, subclinical systolic dysfunction, decreased fibrinolytic activity may also contribute to the increased cardiovascular morbidity and mortality in AGHD. Growth hormone replacement therapy (GHRT) is considered to have a favourable effect on cardiovascular risk factors.

2.2.1. CARDIOMETABOLIC RISK FACTORS IN AGHD

Adult growth hormone deficiency is generally associated with dyslipidemia, which is considered the most significant contributor to the unfavourable cardiometabolic risk profile in these patients. C-reactive protein (CRP) levels in GH-unsubstituted AGHD patients show approximately a fourfold to fivefold increase, which indicates the presence of a proinflammatory state and along with the dyslipidemia improve with adequate GH-substitution. AGHD typically causes alterations in the body compositions involving a 7-10% increase in fat mass, decreased skeletal muscle mass, decreased bone mineral content, and reduced total body water content. Besides accumulation of fat tissue and visceral obesity, the endocrine function of the adipose tissue is also disturbed in AGHD resulting in an adverse adipokine profile.

GH/IGF-1 axis plays an essential role in maintaining the physiologic function of the cardiovascular system. Normal function of GH/IGF-1 axis is believed to counteract atherosclerosis and contribute to maintain normal left ventricular mass and systolic function. Consequently, reduced left ventricular mass, subclinical systolic dysfunction and reduced coronary flow reserve are frequently experienced in patients with AGHD, and these abnormalities are reported to improve during GH-substitution.

GH-substitution has a complex effect on insulin sensitivity. Several, mainly short-term studies reported negative effect of growth hormone substitution on insulin resistance, however, this side effect does not seem to be significant during long-term (> 1 year) GH replacement therapy. On the other hand, visceral obesity, reduced skeletal muscle mass, decreased IGF-1, and lack of physical activity related to unsubstituted AGHD usually leads to impaired insulin sensitivity and increases the risk of T2DM.

The prevalence of metabolic syndrome is about 20–30% in the general population while it was found considerably higher in AGHD affecting about half of the patients. Although growth hormone substitution has been reported to decrease the cardiometabolic risk, it does not reduce the prevalence of metabolic syndrome in patients with AGHD. However, the use of metabolic syndrome criteria in AGHD is

highly debatable. Indeed, it is not clear whether the prognostic significance of the metabolic syndrome in AGHD patients is the same as in the general population. This question is still unanswered, but it is obvious that AGHD patients with metabolic syndrome has higher risk of developing T2DM, cardiovascular and cerebrovascular diseases compared to those AGHD patients with no metabolic syndrome. Since the routinely used IGF-1 is not always related to the severity of cardiometabolic dysregulation in AGHD, there is a growing need for biomarkers that reflect the patients' cardiometabolic risk and its changes during GH-substitution.

2.3. CARDIOMETABOLIC RISK FACTORS IN OBESITY

Obesity is strongly linked to a wide spectrum of cardiovascular diseases and frequently leads to obesity hypoventilation syndrome and obstructive sleep apnea, which also have a devastating impact on the function of the cardiovascular system.

With current scientific knowledge, obesity is considered one of the most important factors causing the rapid rise in the prevalence of T2DM. Although, the relationship between obesity and diabetes mellitus has not been fully understood, in obesity structural and functional alterations of the adipose tissue have been suggested to have a crucial role in the development of diabetes. The accumulation of fat tissue and hypertrophy of adipocytes as well as the simultaneous decrease in the oxygen supply resulting in the development of a dysfunctional adipose tissue, which secretes proinflammatory cytokines and causes an abnormal generation of reactive oxygen species. Infiltration of macrophages and proinflammatory cytokines secreted by the macrophages deepen the metabolic inflammation, impairs insulin signaling and eventually causes insulin resistance. Ectopic fat deposition induced lipotoxicity along with impairment of certain cell organelles (mitochondrial dysfunction, endoplasmic reticulum stress) lead to dedifferentiation and rapid loss of pancreatic β-cells and manifest diabetes mellitus. Based on several studies, microbiome dysfunction may also have a role in the development of insulin resistance and diabetes mellitus. In obesity,

increase in the gut permeability allows elevated levels of lipopolysaccharides enter the circulation causing systemic inflammation and impaired insulin signaling.

Dyslipidaemia involving higher triglyceride levels, decreased HDL-C levels, HDL dysfunction, normal or slightly increased LDL-C levels and higher small dense LDL concentrations, is a typical finding in obesity and contributes to the development of atherosclerosis. Besides higher risk of arterial thrombosis, obesity also increases the risk of venous thromboembolism due to the presence of systemic inflammation, higher concentration of prothrombotic molecules (e.g., PAI-1), decreased fibrinolytic activity and increased activity of thrombocytes.

Obesity induces adverse hemodynamic effects and several maladaptive alterations in the structure and function of the cardiovascular system. The increased circulating blood volume, the higher heart rate due to sympathetic activation as well as the elevated peripheral resistance result in an elevated cardiac output. Elevated cardiac output and increased wall stress lead to a progressive left ventricular dilation and hypertrophy, which if persistent, cause diastolic and systolic dysfunction. Left atrial enlargement also frequently develops, raising the risk of atrial fibrillation, stroke, and heart failure. In addition to hemodynamic changes, myocardial fat infiltration, increased fibrosis and conduction heterogeneity also have a role in the development of obesity-associated cardiomyopathy, which is also associated with a higher risk of arrhythmias.

As a result of the obesity-associated cardiomyopathy, the cardiovascular mortality increases by 7% for every additional 2 years of being obese. Furthermore, in obesity, heart failure develops about 10 years earlier than in those with normal body weight. Electrical remodeling, modification in the ion channel function and autonomous nervous system dysfunction also contribute to the higher rates of ventricular arrhythmias, which may explain that the incidence of sudden cardiac death can be up to 40 times higher in patients with severe obesity compared to the general population.

2.3.1. OBESITY PHENOTYPES AND THEIR RELATIONSHIP WITH THE CARDIOMETABOLIC RISK

Coexistence of obesity and metabolic syndrome constitute an obesity phenotype with an adverse cardiometabolic risk profile. This subgroup of obesity is frequently identified as metabolically unhealthy obesity (MUO), although, due to the lack of universally accepted definition, our research group defines this phenotype as non-diabetic obesity (NDO). Another obesity phenotype is the so-called metabolically healthy obesity (MHO), which involves obese patients with no apparent cardiovascular and metabolic complications. The clinical significance of the MHO concept is highly debatable, because currently there is no universally accepted criteria for identifying MHO.

In 2014 Rey-López et al. identified 30 different definitions of metabolic health. Interestingly BMI>30 kg/m^2 was the only one component, which was used in all definitions. According to the different definitions, the prevalence of MHO among obese individuals changed between 10 and 51%. Regardless of the definition, MHO is not considered a rare condition. The prevalence is higher in women compared to men and decreases with age in both sexes. Furthermore, according to literature data, MHO is neither a stable nor a benign condition. In a prospective study, over 10 years of follow-up 65% of MHO individuals converted to MUO, in addition, compared to healthy individuals MHO patients have been reported to have higher risk of atherosclerotic cardiovascular disease, type 2 diabetes mellitus as well as cerebrovascular diseases.

The clinical applicability of the concept of MHO is still unclear. However, given the magnitude of the obesity as a health problem as well as the availability of certain treatment strategies (e.g., obesity surgery), identification of the patient with the highest cardiometabolic risk and their prioritization may be necessary. Considering the abovementioned controversies, metabolic syndrome criteria are possibly not suitable for the risk assessment. Studies in the past decades showed that concentration of certain organokines are different in the different obesity phenotypes. Therefore, it can be

hypothesized that levels of certain organokines or the pattern of these bioactive molecules may promote the risk stratification in obese patients.

3. AIMS

In GH-substituted (GHS) and GH-unsubstituted (GHN) AGHD patients and the healthy control group we aimed at:

- determining the serum afamin concentrations,
- evaluating the associations between afamin concentrations and anthropometric data as well as parameters of carbohydrate- and lipid-metabolism.

In GHS patients we aimed at:

- determining and comparing the serum afamin concentrations, anthropometric data, lipid- and body composition parameters during continuous GH-substitution, after two months of GH-withdrawal and then one month GH-reinstitution,
- evaluating associations between the changes of serum afamin concentrations and the changes of anthropometric data and body composition parameters as well as parameters of carbohydrate- and lipid-metabolism.

In non-diabetic obese (NDO) patients, obese T2DM patients and the healthy control group we aimed at:

- determining the serum afamin concentrations,
- evaluating the associations between afamin concentrations and anthropometric data, parameters of carbohydrate-metabolism and lipid-parameters, including LDL and HDL subfractions,
- determining serum RBP4 and PAI-1 concentrations and evaluating associations between serum afamin and RBP4 and PAI-1 concentrations.

4. MATERIALS AND METHODS

4.1. STUDY POPULATION

4.1.1. AGHD PATIENTS AND CONTROL SUBJECTS

From May 2021 to May 2023, a total of 20 AGHD patients (11 GHS and 9 GHU patients) with an established diagnosis of AGHD were recruited form the outpatient clinic of our Endocrinology Unit. The diagnosis of AGHD was based on a peak serum GH response to insulin tolerance test less than 3 μg/L when adequate hypoglycemia (blood glucose lower than 2.2 mmol/L) was achieved. At the time of enrolment, each GHS patient received stable GH replacement for at least a year. GHU subjects were either GH naive or received no GH substitution for at least 2 years before study entry. In two GHU subjects GHRT was not initiated because of safety concerns (e.g., risk of tumor recurrence or progression), while seven patients stopped replacement therapy due to side effects (n=2) or lack of perceived positive effects (n=5). Exclusion criteria included active malignancy, heart failure, kidney failure, liver cirrhosis, pregnancy, breastfeeding, and inability to comply with the study protocol. Each enrolled patient had multiple pituitary hormone deficiencies. At study entry all concomitant pituitary deficiencies were adequately substituted. Gender, age, the proportion of childhood-onset GH-deficient and T2DM patients did not differ significantly between the GHS and GHU groups. In the cross-sectional study, both AGHD patients (n=20) and controls (n=37) underwent anthropometric measurements, body composition analysis, measurement of routine laboratory parameters as well as measurement of serum afamin concentrations.

GHS patients (n=11) were invited to participate in the prospective GH-withdrawal study. After detailed explanation, all 11 GHS patients consented to participate in the study. GH-substitution of the 11 enrolled GHS patients was discontinued on the day of the baseline visit (Visit 1). All other hormone replacement therapies were continued unchanged during the study. After two months of GH-withdrawal, patients underwent the same examinations (Visit 2). Then, their pre-study

GH doses were reinstituted, and the patients progressed to a 1-month GH-reinstitution period. One month later, while on GHRT (Visit 3), the same tests were repeated as in Visits 1 and 2, to assess the reversibility of changes induced by GH-withdrawal.

Ethical approval was obtained from the Regional Ethics Committee of the University of Debrecen (registration number: RKIB/IKEB 5576-2020). All participants provided written informed consent and the study was conducted in accordance with the Declaration of Helsinki.

4.1.2. NDO PATIENTS, OBESE T2DM PATIENTS AND CONTROL SUBJECTS

106 NDO subjects and 62 obese patients with T2DM were enrolled from our obesity and diabetes outpatient clinics at the Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Hungary. 49 healthy, lean volunteers were also enrolled as controls in our study. All three groups were matched for gender and age. Obesity was defined as a body mass index (BMI) ≥30 kg/m^2. Patients with endocrine, liver, kidney, pulmonary, neurological, gastrointestinal, acute infection, autoimmune disease, or malignancies were excluded. Further exclusion criteria were pregnancy, lactation, smoking, and regular alcohol consumption. Besides anthropometric and laboratory data, we also assessed the medications of the enrolled subjects. Patients with T2DM were treated either with antidiabetics (mostly metformin and glucagon-like peptide-1 receptor agonists) or with insulin. The participants were referred to scheduled medical appointments from 08:00–10:00 am, and we asked the patients to arrive after an overnight fast. All participants provided written, informed consent.

Permission to carry out this study was granted by the Regional Ethics Committee of the University of Debrecen and the Medical Research Council (registration numbers: DE RKEB/IKEB 5513B-2020 and IV/7989-1/2020/EKU, respectively).

4.2. ANTHROPOMETRIC MEASUREMENTS

Measurements of height, weight, and waist circumference were obtained for anthropometric analysis. Waist circumference was also measured, and BMI was calculated for each subject.

4.3. BODY COMPOSITION ANALYSIS IN AGHD PATIENTS AND CONTROL SUBJECTS

Body composition was analyzed using multi-frequency bioelectrical impedance analysis (BIA) (InBody720, Inbody Co., LTD, Seoul, Korea). Testing was performed according to the manufacturer's instructions. The following body composition parameters were measured: body fat mass, percent body fat, fat free mass, skeletal muscle mass, visceral fat area, total body water, extracellular water content, intracellular water content and bone mineral content.

4.4. MEASUREMENT OF ROUTINE LABORATORY PARAMETERS

After an overnight fast, venous blood samples will be collected into Vacutainer® tubes (Becton Dickinson, San Jose, CA, USA). Sera and EDTA-anticoagulated plasmas were separated at 3500 g for 15 min 4 °C. Routine laboratory parameters, including high sensitivity C-reactive protein (hsCRP), fasting glucose, hemoglobin A1C (HbA1C), glomerular filtration rate (GFR), liver enzymes, supersensitive thyroid stimulating hormone (sTSH), thyroxine, triglyceride, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) were performed with a Cobas c600 autoanalyzer (Roche Ltd., Mannheim, Germany) at the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary. In AGHD patients, serum IGF-1, testosterone and cortisol levels were also measured. HOMA-IR was calculated by (fasting insulin x fasting glucose)/22.5.

4.5. MEASUREMENT OF SERUM AFAMIN CONCENTRATIONS

Serum afamin levels were measured by a commercially available ELISA kit (Afamin Human ELISA, cat. number: RD194428100R, BioVendor, Brno, Czech

Republic) according to the manufacturer's instructions. The intra- and inter assay variation coefficients were <3.61 % and <3.4 %, respectively.

4.6. MEASUREMENT OF SERUM PAI-1 AND RBP4

Serum RBP4 level was determined by ELISA (Human RBP4 Quantikine ELISA Kit, cat. number: DRB400, R&D Systems, Abingdon, UK) with 5.7-8.1% intra-assay and 5.8-8.6% inter-assay coefficients according to the manufacturer's instructions. Human plasma serpin E1/PAI-1 level was measured by a commercially available DuoSet ELISA (cat. number: DY1786, R&D Systems, Abingdon, UK).

4.7. LDL SUBFRACTION ANALYSIS

Up to seven LDL subfractions were distributed based on their size using the Lipoprint System (Quantimetrix Corporation, Redondo Beach, CA, USA) according to the manufacturer's instructions. 25 L of the sample were mixed in polyacrylamide gel tubes with 200 L of Sudan Black containing a loading gel. Tubes were photopolymerized for 30 min and then electrophorized at 3 mA/tubes for 64 min. Each electrophoresis chamber involved a quality control provided by Quantimetrix (Liposure Serum Lipoprotein Control, Quantimetrix Corp., Redondo Beach, CA, USA). Subfraction bands were scanned with an ArtixScan M1 digital scanner (Microtek International Inc., CA, USA) and analyzed with the Lipoware Software (Quantimetrix Corp., Redondo Beach, CA, USA). After the VLDL peak, the percentage of midbands C through A mainly corresponded to the intermediate density lipoprotein (IDL) subfraction in the densitogram. The percentage of large LDL (large LDL %) was defined as the summed percentages of LDL1 and LDL2, whereas the 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 area under the curve (AUC) of subfractions by the total cholesterol concentration. The intra-assay precisions were 0.58-7.28% for VLDL, 3.85-11.14% for midbands and 1.05–1.52% for LDL, respectively. The inter-assay precisions were 7.12-9.40% for VLDL, 7.47-10.90% for midbands and 1.26-1.57% for LDL, respectively.

4.8. HDL SUBFRACTION ANALYSIS

Up to ten HDL subfractions were distributed based on their size using the Lipoprint System (Quantimetrix Corp., Redondo Beach, CA, USA) according to the manufacturer's instructions. Briefly, 25 L of the sample were mixed in polyacrylamide gel tubes with 300 L of Sudan Black containing a loading gel. Tubes were photopolymerized for 30 min and then electrophorized at 3 mA/tubes for 54 min. The remaining steps of assay were identical to those of the LDL subfraction test. During HDL subfraction analyses, large (HDL-1 to HDL-3), intermediate (HDL-4 to HDL-7), and small (HDL-8 to HDL-10) HDL subfractions were distributed between VLDL + LDL, and albumin peaks. The cholesterol content of HDL subfractions was calculated by multiplying the HDL-C of the sample. The intra- and inter-assay precisions were 0.90-1.47% and 2.49-4.75%, respectively.

4.9. STATISTICAL ANALYSIS

Data were expressed as mean ± SD or median ± interquartile range unless otherwise specified. Statistical analyses were performed using Statistica 13.5.0.17 software (TIBCO Software Inc., Tulsa, OK, USA). Graphs were prepared using GraphPad Prism 9.4.1 (GraphPad Prism Software Inc., San Diego, CA, USA). The normality of data was checked using Kolmogorov-Smirnov and Shapiro-Wilk tests. Nonnormally distributed data were transformed logarithmically before analysis. Comparison of baseline parameters between groups was performed using one-way ANOVA with Tukey's post hoc test. The effect of GH-withdrawal was analyzed using repeated measures ANOVA with Tukey's post hoc test. To avoid problems of non-sphericity, Greenhouse-Geisser correction was used for all variables. When normal distribution could not be reached by logarithmic transformation, Kruskal-Wallis test and Friedman-test were performed. Differences between continuous variables were calculated using Chi-square test and Fisher's exact test. Pearson's and Spearman's correlation were used to explore associations between selected variables. $P \leq 0.05$ was considered statistically significant.

5. RESULTS

5.1. RESULTS OF THE STUDY ON PATIENTS WITH AGHD

5.1.1. AFAMIN LEVELS, ANTHROPOMETRIC AND LABORATORY PARAMETERS

GHU patients had higher BMI than GHS and control subjects (p=0.04 and p=0.01, respectively), whereas BMI was comparable between GHS and controls. WC, WHR and percent body fat were also found significantly higher in GHU compared to control subjects.

Mean afamin concentration was 31 % higher in GHU compared to controls (p=0.03), while no significant difference was detected between GHU and GHS. Serum IGF-1 levels were lower in GHU compared to both GHS and control group. As a result of adequate GH-substitution, IGF-1 was similar in GHS and control subjects. hsCRP was also found higher in GHU than in control subjects (p=0.03) but the difference between GHU and GHS did not reach significance. Both GHU and GHS demonstrated higher insulin (p=0.03; p=0.05, respectively) and HOMA-IR (p=0.05; p=0.05, respectively) compared to controls. Serum AST concentrations were higher in GHU compared to GHS and controls (p=0.01, p<0.01, respectively). Fasting glucose, C-peptide, HbA1C, eGFR, thyroxine, cortisol levels and lipid parameters (triglyceride, total cholesterol, HDL-C, LDL-C) were not different in the three groups.

5.1.2. ASSOCIATIONS OF SERUM AFAMIN CONCENTRATIONS

When correlations between afamin and selected variables were calculated, all AGHD patients, including GHU and GHS patients were considered as a single AGHD cohort.

In AGHD patients, but not in controls, afamin showed strong positive correlations with skeletal muscle mass, bone mineral content, total body water, extracellular- and intracellular water content (p<0.01). In AGHD, afamin was positively correlated with HOMA-IR (p=0.01), insulin (p<0.01) and C-peptide (p=0.03) levels, while in the control group afamin did not correlate with any of the parameters of glucose metabolism. Afamin correlated positively with triglyceride (p<0.01) levels, BMI, WHR

and fat mass in controls, but not in AGHD patients. Both in AGHD and control subjects, afamin showed positive correlations with AST levels ($p=0.04$ and $p=0.02$, respectively) and WC ($p<0.01$ and $p<0.01$, respectively). Serum IGF-1 concentrations did not correlate with afamin levels either in AGHD subjects or in controls.

5.1.3. Effects of GH-withdrawal and GH-reinstitution

Two-month of GH-withdrawal did not result in significant changes in the anthropometric parameters. Percent body fat showed a slight increase after 2-month of GH-withdrawal (mean difference: 1.73%, $p=0.04$) and did not return to baseline following 1-month reinstitution. GH-withdrawal resulted in a substantial decrease in the fat-free mass, skeletal muscle mass, total body water and bone mineral content, but all of them returned nearly to baseline after 1-month of GH-reinstitution. Visceral fat area, fat mass and extracellular water content did not change significantly following GH-withdrawal. Serum IGF-1 concentrations declined following GH-withdrawal ($p<0.01$) and then increased ($p<0.01$) after reinstituting GHRT. Interestingly, afamin levels also showed a significant decrease after 2-month of GH-withdrawal ($p=0.03$) and then returned to baseline following GH-reinstitution. C-peptide, insulin and HOMA-IR also decreased following GH-withdrawal, but they did not return to baseline after 1-month of retreatment. The rise in hsCRP after GH-withdrawal did not reach statistical significance, but reinstitution of GHRT resulted in a decrease ($p<0.01$) in the hsCRP levels. AST, eGFR, thyroxine, cortisol, hemoglobin, hematocrit levels and parameters of lipid-metabolism remained unchanged throughout the study. The change of afamin (Δafamin) = (afamin after GH reinstitution – afamin after withdrawal) was positively correlated with the change of HOMA-IR (ΔHOMA-IR; $r=0.80$; $p<0.01$) and the change of insulin (Δinsulin; $r=0.71$; $p=0.02$).

5.2. Results of the Study on Obese Patients

5.2.1. Afamin levels, anthropometric and laboratory parameters

Compared to controls, both the NDO group and the T2DM group had significantly higher BMI, waist circumference, hsCRP, fasting glucose, fasting insulin, ALT, γ-GTP, and triglyceride levels. In addition, fasting glucose, sTSH, and triglyceride levels were significantly higher in patients with T2DM compared to NDO subjects, while HDL-C was significantly lower in patients with T2DM compared to lean controls. The mean serum afamin concentration was found to be 32.2% higher in the NDO group and nearly two-fold higher in the T2DM group compared to the controls (controls: 56 ± 30.3 vs. NDO: 82.6 ± 19.7 μg/mL vs. T2DM: 109.2 ± 21.4 μg/mL, ANOVA: $p<0.001$). Furthermore, afamin concentrations were also significantly different ($p=0.001$) in the two obese groups (NDO and obese T2DM).

5.2.2. Lipoprotein subfractions

Analyzing the distribution of LDL subfractions, the percentages of VLDL, large LDL, and small dense LDL subfractions were significantly higher in NDO and T2DM compared to controls. The mean LDL particle size was found to be significantly lower in both NDO and T2DM subjects compared to lean ones. In line with literature data, there was a shift towards small-sized HDL subfractions in NDO and T2DM patients: the percentage and amount of large HDL subfractions were significantly lower, while the percentage of small HDL subfractions was significantly higher in these groups.

5.2.3. Associations of serum afamin concentrations

Serum afamin showed significant positive correlations with age ($r = 0.17$; $p = 0.01$), BMI ($r = 0.39$; $p < 0.001$), and waist circumference ($r = 0.55$; $p < 0.001$) in overall participants. Furthermore, significant positive correlations were found between fasting glucose, HbA1C, fasting insulin, and afamin. The percentage of IDL subfractions and mean LDL size correlated negatively with serum afamin ($r = -0.29$; <0.001 and $r =$

-0.29; $p < 0.001$, respectively); while the percentage of large LDL and small dense LDL subfractions correlated positively with afamin ($r = 0.38$; $p < 0.001$ and $r = 0.19$; $p < 0.01$; respectively) in overall participants. The percentage of VLDL subfraction did not correlate with afamin.

Among HDL subfractions, HDL-1 to -5 subfractions, which correspond with large HDL and partially with intermediate HDL, showed strong, significant negative correlations with serum afamin in all subjects. While there were strong positive correlations between serum afamin and HDL-7 to -10 subfractions, which correspond partially with intermediate and mainly with small HDL subfractions in overall subjects.

5.2.4. RBP4 AND PAI-1 LEVELS AND THEIR ASSOCIATIONS WITH AFAMIN

Circulating RBP4 was significantly lower in the NDO and T2DM groups than normal weight individuals (controls: 41.4 ± 14.4 μg/mL vs. NDO: 32.3 ± 15 μg/mL vs. T2DM: 28.8 ± 12.3 μg/mL; one-way ANOVA: $p<0.001$). There was a negative correlation between RBP4 and afamin ($r = -0.22$; $p = 0.004$) (Figure 3b). Plasma PAI-1 was significantly higher in the NDO and T2DM groups (controls: 3.63 (1.99–7.29) ng/mL vs. NDO: 7.37 (4.94–10.42) ng/mL vs. T2DM: 6.62 (4.6–10.28) ng/mL; Kruskal-Wallis H test: $p<0.001$) and a significant positive correlation was detected between plasma PAI-1 and afamin in overall subjects ($r = 0.21$; $p = 0.002$). RBP4 and PAI levels did not differ between the NDO and obese T2DM groups (p=0,399 and p=0,804, respectively).

5.2.5. PREDICTORS OF AFAMIN

Since several correlations were observed between afamin and anthropometric/laboratory parameters, a backward stepwise multiple regression analysis was performed to determine the significant predictor(s) of afamin. The model included gender, age, BMI, mean LDL size, fasting glucose, large HDL (mmol/L), intermediate HDL (mmol/L), and small HDL (mmol/L). The analysis showed that BMI ($\beta = 0.214$; $p<0.001$), fasting glucose ($\beta = 0.291$; $p<0.001$), intermediate HDL (mmol/L)

(β = −0.36; p<0.001), and small HDL (mmol/L) (β = 0.446; p<0.001) were independent predictors of afamin.

5.3. SUMMARY OF THE NEW FINDINGS

In AGHD patients:

1. Afamin concentration was significantly higher in GH-unsubstituted AGHD patients compared to healthy controls, while no significant difference was detected between GH-unsubstituted and GH-substituted AGHD patients.
2. In the whole AGHD group afamin correlated positively with skeletal muscle mass, total body-, extracellular and intracellular water content, bone mineral content, waist circumference, HOMA-IR, insulin, C-peptide, and AST levels.
3. In healthy controls afamin positively correlated with BMI, waist-hip ratio, waist circumference, total body weight, AST, and triglyceride levels.
4. Two-month GH-withdrawal resulted in a significant decrease in skeletal muscle mass, bone mineral content, total body- and intracellular water content, however, these changes proved to be reversible after one month GH-reinstitution.
5. After two-month GH-withdrawal afamin concentration decreased significantly and then increased significantly after one month GH-reinstitution.
6. During GH-withdrawal and GH-reinstitution Δafamin showed strong positive correlation with ΔHOMA-IR and Δinsulin.

In NDO patients, obese T2DM patients and healthy controls:

7. Serum afamin concentration was significantly higher in obese T2DM patients compared to both NDO patients and healthy controls.
8. In the whole study population, the percentage of IDL subfractions and mean LDL size correlated negatively with serum afamin, while the percentage of large LDL and small-dense LDL subfractions correlated positively with afamin.

9. HDL-1 to -5 subfractions correlated negatively with serum afamin, while the HDL-7 to -10 subfractions correlated positively with serum afamin concentrations in overall subjects.
10. In the whole study population RBP4 levels correlated negatively, while PAI-1 levels correlated positively with the afamin concentrations.

6. DISCUSSION

As a finding of our cross-sectional study, we demonstrated for the first time that GH-unsubstituted AGHD patients have higher serum afamin concentrations than healthy controls. It should be mentioned that GHU patients and obese T2DM patients showed comparable serum afamin concentrations (105.2 vs. 109.2 μg/ml), which could reflect the severity of metabolic dysfunction associated with GH-unsubstituted AGHD. In the whole AGHD (GHU and GHS) group, afamin demonstrated strong positive correlations with skeletal muscle mass, bone mineral content, total body-, intracellular- and extracellular water content, while in healthy controls these associations were not detected. Previous studies found associations between afamin levels and parameters of carbohydrate-metabolism in patients with various insulin resistance-related conditions. In line with these findings, we also found significant positive correlations between serum afamin concentrations and insulin, C-peptide, and HOMA-IR in AGHD. On the contrary, afamin did not correlate with lipid-parameters in AGHD patients,

In our prospective study, 2-month GH-withdrawal did not result in significant alterations in the standard anthropometric parameters, however, there was a significant increase in the percent body fat as well as decrease in fat free mass, skeletal muscle mass, bone mineral content, intracellular- and total body water content. Substantial changes of body composition without changes of BMI agrees well with previous results indicating that BMI is a poor indicator in monitoring GHRT, because the shift from fat to lean mass is not necessarily reflected in the BMI. Besides significant changes in body composition induced by a short-term GH-withdrawal, our study also demonstrated that these changes are reversible after one month GH-reinstitution.

Since even a short GH-withdrawal has been shown to adversely affect cardiometabolic risk factors, we expected higher afamin levels after GH-withdrawal. Surprisingly, afamin levels decreased significantly after GH-withdrawal and increased even more significantly during GH-reinstitution. As Δafamin showed strong positive correlations with ΔHOMA-IR and Δinsulin, it was revealed that the changes of afamin levels are largely attributable to the change of insulin sensitivity induced by GH-withdrawal and reinstitution. Previous studies have also linked the reduction of afamin levels to improved IR after bariatric surgery. Conversely, glucocorticoid therapy with its well-known negative effect on insulin sensitivity, has been found to increase afamin levels.

As an interesting finding, we demonstrated that long-term GH-deficiency and short-term GH-withdrawal result in opposite effects on afamin concentrations. Based on our results, in short-term GH-withdrawal afamin levels are predominantly influenced by the cessation of the diabetogenic actions of rhGH therapy. On the other hand, considering the strong association of afamin with WC, AST levels and measures of IR, higher afamin levels in long-term GH-deficiency are presumably associated with abdominal obesity, consequent insulin resistance and NAFLD.

Our results suggest that measuring afamin might be useful in the assessment of cardiometabolic risk in patients with AGHD. Considering the strong association of afamin with the body composition parameters, afamin measurement might also help to determine the efficacy of the growth hormone replacement therapy. Furthermore, during GH-substitution afamin can be used to monitor GH-associated changes of glucose-homeostasis, which is particularly important in patients with prediabetes.

Compared to healthy controls, previous studies also reported higher and comparable serum afamin levels in obese subjects. In our study, afamin levels were found to be 32.2% higher in the NDO group and nearly two-fold higher in the T2DM group. Unlike RBP4 and PAI concentrations, afamin levels significantly differed between the NDO and obese T2DM groups. Considering our findings and findings of earlier studies, afamin levels seem to be different in different obesity phenotypes (MHO,

NDO and obese T2DM), and it can also reflect the severity of metabolic dysfunction as well as the cardiovascular risk associated with the given phenotype.

In line with previous studies, HDL-subfraction analysis revealed a shift towards small-sized HDL subfractions in the NDO and obese T2DM groups compared to lean controls. In accordance with our finding, the accumulation of small HDL particles has also been reported in metabolically unhealthy overweight and obese subjects, furthermore in other studies lower large HDL-C and higher small HDL-C were independently related to the presence of T2DM.

In the whole study population as well as in the NDO and T2DM groups, afamin positively correlated with the BMI, waist circumference, HbA1C, fasting insulin and fasting glucose levels. In line with a previous study conducted on MHO obese subjects, we found strong associations between the afamin concentrations and the mean LDL size, large and small dense LDL subfractions. During Lipoprint electrophoresis, bidirectional associations were detected between HDL subfractions and afamin in overall subjects and in the NDO and T2DM groups: the large-sized HDL subfractions (HDL1-5) correlated negatively, while the small-sized HDL subfractions (HDL7-10) correlated positively with afamin. In former studies, higher amount, and percentage of small HDL subfractions were associated with higher risk of coronary heart disease. On the contrary, the amount and percentage of large HDL subfraction showed inverse association with the risk of myocardial infarction at a very young age. Consequently, in obese patients afamin shows positive correlation with HDL subfractions related to unfavourable cardiovascular risk, while it demonstrates negative correlation with those associated with favourable risk profile. Multiple regression analysis determined that in addition to BMI and fasting glucose, the levels of intermediate and small HDL were also significant predictors of afamin.

Potential role of organokines in the pathogenesis of obesity has been described in several studies. According to these findings, it seems that due to their dynamic interplay, it is not sufficient to investigate these bioactive molecules separately, but they require a more complex examination. Based on this hypothesis, in addition to measuring

afamin, we examined the serum concentrations of two other organokines, which also show strong association with the metabolic syndrome. In agreement with previous studies, compared to healthy controls PAI-1 levels were found significantly higher in the NDO and T2DM groups. As a novel finding of our study, we detected significant positive correlation between afamin and PAI-1 serum concentrations. Majority of the earlier studies found higher RBP4 levels in obesity and T2DM. Unlike these studies, we found lower RBP4 in both NDO and T2DM patients than in the controls. Neither serum RBP4 levels nor PAI levels differed significantly between NDO and obese T2DM patients. Afamin and RBP4 levels correlated negatively in the overall subjects. Concerning the lower levels of RBP4 found in NDO and T2DM patients, a recent review highlighted the controversies over the results of studies and pointed out the limitations of commercially available ELISA kits. On the other hands, a large prospective study on patients with prediabetes detected U-shaped relationship between RBP4 levels and the risk of T2DM, indicating that the risk is increased not only in the case of higher RBP4 but also in lower RBP4 levels. Although these finding as well as the former results of our research group also support the findings of the present study, due to the considerable controversies between studies, further research is required to clarify the role of RBP4 in the pathogenesis of obesity.

Based on our study, we concluded that because of the strong associations with anthropometric data, and parameters of carbohydrate- and lipid-metabolism, afamin measurement can promote cardiometabolic risk assessment in both obesity phenotypes and might help to identify patients with the highest risk.

The main strength of our AGHD study is the prospective self-control design, which, despite the small number of participants, enabled us to detect significant changes after GH-withdrawal and reinstitution. Considering that several other studies failed to find potential markers to monitor GH-substitution, it should be emphasized that our study identified a promising biomarker, which is significantly modified by GH-withdrawal and reinstitution. In connection with the second study, it has to be stressed that the afamin levels and its relationship with parameters of carbohydrate- and lipid-

metabolism have been investigated in two different obesity phenotypes. Furthermore, in NDO and obese T2DM patients, correlations of afamin levels with other organokines have also been studied.

One limitation of our prospective GH-withdrawal study is the small sample size; therefore, our results are considered preliminary and require confirmation in future studies. However, it should be noted that AGHD is a rare endocrine disease and prospective studies especially GH-withdrawal studies often face recruitment difficulties. As a result, similarly small sample sizes are common among GH-withdrawal studies. Furthermore, in our cross-sectional study, when correlations of afamin were calculated, GHS and GHU patients were considered as a single AGHD cohort due to the small sample sizes. Thus, these results should be interpreted with caution. In our second study, the relatively small portion of male subjects is considered a limitation. Furthermore, to clarify the role of the studied organokines in the pathogenesis of obesity and obesity-related diseases, long-term prospective studies are also needed.

7. MAIN CONCLUSIONS

Main conclusions of the AGHD study:

1. In unsubstituted AGHD high afamin concentrations probably indicate adverse cardiometabolic risk profile.
2. In GH-substituted AGHD patients 2-month GH-withdrawal result in improved insulin sensitivity and unfavourable changes of body composition with unaltered anthropometric parameters (BMI, total body weight, waist circumference). Improvement in insulin sensitivity is presumably caused by the cessation of the diabetogenic effect of GH.
3. In GH-substituted AGHD patients, GH-withdrawal induced changes of body composition parameters quickly return to their baseline levels after GH-reinstitution.
4. In GH-substituted AGHD patients, short-term GH-withdrawal result in a significant decrease in the serum afamin concentrations, which is primarily

attributable to the improving insulin sensitivity. Comparable to the changes in body composition, afamin levels return to their baseline levels after GH-reinstitution.

5. In AGHD patients, due to the strong associations between afamin levels and body composition and insulin sensitivity, afamin can be a promising biomarker to monitor the efficacy of GH replacement therapy and the GH-induced changes of glucose-homeostasis.

Main conclusions of the obesity study:

1. Based on the strong associations detected between afamin levels and parameters of glucose metabolism, anthropometric data, lipid subfractions and PAI-1 levels, afamin concentrations can reflect the severity of metabolic dysregulation in obesity.
2. In NDO and obese T2DM patients, higher afamin concentrations also refer to the unfavourable changes in the distribution of lipoprotein subfractions.
3. Despite the insignificant differences in TC, LDL-C and HDL-C levels among study groups, analyzing lipoprotein subfractions revealed significant differences (lower mean LDL size, higher small dense LDL%, lower large HDL, higher small HDL%) in the NDO and T2DM patients compared to the controls. Abnormalities in the distribution of lipoprotein subfractions are more pronounced in the T2DM group compared to the NDO patients. Based on these findings, it can be stated that analyzing lipoprotein subfractions might have an important role in the cardiovascular risk assessment in obese subjects.

8. SUMMARY

Afamin is a hepatokine, which shows strong association with the metabolic syndrome and T2DM. In AGHD afamin concentrations have not been studied, while in obesity only limited information is available in connection with the afamin. In our first study, we examined afamin concentrations in GH-substituted (n=11) and GH-unsubstituted AGHD (n=9) patients as well as in healthy controls (n=37). In the GHS

group, afamin concentrations along with other laboratory and body composition parameters have been measured after a 2-month GH-withdrawal and then following one month GH-reinstitution. In the second study, we investigated afamin serum concentrations in a non-diabetic obese (NDO, n=106) and an obese T2DM (n=62) group as well as a healthy control group (n=49). We also studied the associations between afamin concentrations and parameters of carbohydrate- and lipid-metabolism, lipoprotein subfractions, serum PAI-1 and RBP4 levels.

Serum afamin concentrations were significantly higher in GHU patients than in healthy controls, although it did not differ between GHU and GHS subjects. In the whole AGHD group afamin correlated positively with skeletal muscle mass, total body-, extracellular and intracellular water content, bone mineral content, waist circumference, HOMA-IR, insulin, C-peptide, and AST levels. Two-month GH-withdrawal resulted in a significant decrease in skeletal muscle mass, bone mineral content, total body- and intracellular water content, however, these changes proved to be reversible after one month GH-reinstitution. After two-month GH-withdrawal afamin concentration decreased significantly and then increased significantly following one month GH-reinstitution. During GH-withdrawal and GH-reinstitution Δafamin showed strong positive correlation with ΔHOMA-IR and Δinsulin. Serum afamin concentration was significantly higher in obese T2DM patients compared to both NDO patients and healthy controls. In the whole study population, the percentage of IDL subfraction and mean LDL size correlated negatively with serum afamin, while the percentage of large LDL and small-dense LDL subfractions correlated positively with afamin. HDL-1 to -5 subfractions correlated negatively with serum afamin, while the HDL-7 to -10 subfractions correlated positively with serum afamin concentrations in overall subjects. In the whole study population RBP4 levels correlated negatively, while PAI-1 levels correlated positively with the afamin concentrations.

Based on our findings, in AGHD afamin could be a promising biomarker in the cardiometabolic risk assessment and can be used to monitor GH-associated changes of glucose-homeostasis. In NDO and obese T2DM patients afamin levels reflect the

severity of metabolic dysfunction and therefore can help to estimate cardiometabolic risk. To confirm our result further studies with larger sample of AGHD patients are needed. In obesity, prospective studies are also required to further investigate the role of afamin.

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List of publications related to the dissertation

1. **Ratku, B.**, Lőrincz, H., Csiha, S., Borbásné Sebestyén, V., Berta, E., Bodor, M., Nagy, E. V., Szabó, Z., Harangi, M., Somodi, S.: Serum afamin and its implications in adult growth hormone deficiency: a prospective GH-withdrawal study.
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2. Lőrincz, H., **Ratku, B.**, Csiha, S., Seres, I., Szabó, Z., Paragh, G., Harangi, M., Somodi, S.: Impaired Organokine Regulation in Non-Diabetic Obese Subjects: halfway to the Cardiometabolic Danger Zone.
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4. Lőrincz, H., Csiha, S., **Ratku, B.**, Somodi, S., Sztanek, F., Paragh, G., Harangi, M.: Associations between Serum Kallistatin Levels and Markers of Glucose Homeostasis, Inflammation, and Lipoprotein Metabolism in Patients with Type 2 Diabetes and Nondiabetic Obesity.
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