1. Introduction

Until recently the adipose tissue had been considered an inert tissue, mainly devoted to energy storage. However, with the discovery of several adipocyte-derived factors, collectively known as adipokines, adipose tissue is currently regarded as an active player in the regulation of metabolism.

Adipose tissue is a complex, and highly active metabolic and endocrin organ. Responds to afferent signals from hormone systems and the central nervous system, and also expresses and secretes factors with important endocrine functions. The important endocrin function of adipose tissue is emphasized by the adverse metabolic consequences of both adipose tissue excess and deficiency.

2. Objectives

The aims of our examinations were to clear up whether there are changes in the serum levels of ghrelin, adipokines and insulin in children with acute viral hepatitis and in l-carnitine supplemented patients with end-stage renal disease. Therefore:

1. we evaluated the adiponectin, leptin, resistin, ghrelin and insulin levels in children with acute hepatitis and the association of these hormones with the severity of this condition.
2. a study was undertaken to determine plasma levels of leptin, adiponectin and resistin, as well as insulin and ghrelin in patients with end-stage renal disease (ESRD) before, during, and after l-carnitine supplementation. Attempts were also made to reveal the possible interactions between these hormons in well-defined study population and clinical settings.
3. we attempted to explore the influence of l-carnitine supplementation on asymmetric dimethylarginine (ADMA). Moreover, we evaluated the relationship of ADMA to insulin, resistin, leptin, adiponectin and ghrelin in ESRD patients before, during and after l-carnitine supplementation.

3. Materials and methods

3.1. Serum ghrelin, insulin and adipokine levels in children with acute hepatitis

Twenty five patients were enrolled in the study, all of them suffered from acute viral hepatitis. The diagnosis of acute hepatitis was based on the physical examination (hepatomegaly, jaundice etc.), the biochemical parameters and the serological detection of acute hepatotropic virus infection. The age of the patients ranged from 2.2 to 17.2 years (mean: 10.4 years), 10 of them were male and 15 female. For statistical evaluation we used age- and gender-specific body mass index (BMI) matched percentile values. The fasting state(nausea, vomiting, diarrhoea) before admittance lasted for 2-7 days (mean 4.16 days). The causes of the hepatitis were as follows: in 20 patients (80%) Hepatitis A virus (HAV) and in 5 patients (20%) Epstein-Barr virus (EBV) infection. We evaluated the two different hepatitis entities together because no differences were noted when the parameters of the two groups were analysed separately.

Serum samples for adiponectin, leptin, resistin, ghrelin and insulin were drawn at 08:00-09:00 am after an overnight fast and were stored at -20°C until analysis. We collected the first blood samples on the morning after the day of admission to hospital and the second samples were collected after 2 months of recovery. At the same time liver function was analysed as well. Recovery was defined as normalisation of liver function tests.

The aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), serum albumin and total bilirubin were measured by routine laboratory tests. Ghrelin was analyzed with a radioimmunoassay kit from Mediagnost, Reutlingen, Germany, and leptin, adiponectin ,resistin and insulin by enzyme linked immunosorbent assay (ELISA) kit from Mediagnost, Reutlingen, Germany.

Epstein-Barr virus infection was verified by Trinity Biotech Captia Epstein-Barr Virus Viral Capsid Antigen (VCA) IgM and IgG kits.

Hepatitis A infection was tested by using anti-Hepatitis A virus IgM antibodies detecting enzyme-immunoassay (EIA) kit (Dia. Pro, Diagnostic Bioprobes, Italy).

Statistical evaluations were performed by using Wilcoxon matched pairs test and by linear and multiple regression analyses.

3.2. L-carnitine supplementation and adipokines in patients with end-stage renal disease

Ten consecutive non-diabetic patients (6 males, 4 females) with ESRD on maintenance HD for at least 6 months were selected for this longitudinal study. Their age was 63.4±16.7 years (mean±SD). None of them had acute illness, heart failure or previous carnitine supplementation. The underlying renal pathologies that progressed to ESRD were the following: nephrocalcinosis (2), chronic pyelonephritis (4) and chronic glomerulonephritis (4). Most of the patients received antihypertensive therapy (ACE inhibitors 4, calcium channel blockers 4, beta-blockers 6, others 2), but none of them was on lipid-lowering drugs. Additional drugs for all individual patients were calcitriol, calcium carbonate and iron, as well

as erythropoietin. The average weeky dose of erythropoietin was 3400±3405 IU at week 0, 3000±3018 IU at week 12, 3100±2998 IU at week 28.

For routine laboratory examinations samples were taken before HD treatment while the patients were in stable clinical conditions. Routine tests included assessment of hemoglobin, iron status, plasma creatinine and urea nitrogen concentrations, glucose, protein and lipid profiles, CRP, as well as electrolyte status.

The patients underwent three HD sessions per week in the morning shift, each having a duration of 4 hours. On-line HD was carried out by using Fresenius 5008 B equipment with Helixone/Fresenius polysulfone high-flux dialyzer membranes (FX 60 and FX 80 dialyzers). No sodium or ultrafiltration profiles have been used. The same dialysis fluid was used in all patients with temperature of 36.5 °C, sodium concentration: 138 mmol/l, calcium concentration 1.5 mmol/l and conductivity: 14 mS/cm. The blood flow, substitution fluid volume and other HD parameters were not changed during the study period. The efficiency of HD was indicated by the Kt/V>1.2 in all cases.

Carnitine supplementation protocol

The study was performed over 28 weeks and included three different observational periods. During the baseline peroid no carnitine supplement was given (week 0). The next 12 weeks comprised the carnitine treatment period (weeks 1-12) followed by the post-carnitine period (weeks 13-28). In this latter no further carnitine was provided; the body carnitine pool of the patients was regarded to be replenished. The patients in the 12 weeks active treatment period were supplemented after each HD session with 1 g l-carnitine intravenously (Sigma-Tau Arzneimittel GmbH Düsseldorf, Germany). After the iv administration of l-carnitine the venous line was flushed with saline over about 2 min.

Simultaneously with routine laboratory tests blood samples were taken for carnitine profiles (heparinized tubes) and for hormones controlling energy balance (tubes containing EDTA). Sample collection was performed following an overnight fast immediately before the respective HD session was staarted. Plasma samples were separated in a refrigerated centrifuge and stored at -70°C until analysis. For carnitine and hormone measurements blood was obtained at weeks 0 (baseline) and 2, 4, 8, 12 (active treatment period), as well as 24, 25, 26, 27 and 28 (post-treatment period).

Routine biochemical parameters were measured by standard laboratory methods. Free carnitine and acylcarnitine plasma levels were determined by using electron spray ionization tandem-mass spectrometry. Plasma insulin, leptin, adiponectin and resistin concentrations were measured by ELISA using tracers obtained from Mediagnost, Reutlingen, Germany. Ghrelin was analyzed with radioimmunoassay using commercially available kits (Mediagnost, Reutlingen, Germany). The coefficient of variation was <5% within assays and <15% between assays for all hormone measurements. Age, gender and BMI matched healthy subjects served as controls for the hormone parameters measured.

All statistical analyses were performed with SPSS, version 11.5 (SPSS Inc. Chicago, Ill. USA). Normality of data was evaluated by the Kolmogorov-Smirnov test. Variables are presented as mean±SD. To compare differences between groups repeated measures ANOVA were used. Correlations between variables were assessed applying univariate regression analysis. $p<0.05$ was considered as statistically significant.

3.3. Endothel dysfunction and adipokines in l-carnitine supplemented patients with end-stage renal disease

In this survey the study population, the laboratory measurements and the statistical analysis were the same as mentioned in the previous chapter. We used asymmetric dimethylarginine (ADMA) as a marker of endothel dysfunction. Plasma samples were separated in a refrigerated centrifuge and stored at -20°C until analysis. ADMA levels were determined with liquid chromatography-tandem mass spectrometry (LC-MS-MS).

4. **Results**

4.1. Serum ghrelin, insulin and adipokine levels in children with acute hepatitis

Serum ghrelin and adiponectin levels were significantly increased during hepatitis when compared with those after recovery (831.4 ± 276.44 *vs.* 736.21 ± 274.91 pg/ml, $p<0.0001$; and 22,91 ±12,93 *vs.* 15,16;±8,81 µg/ml, $p<0,001$, respectively). Serum adiponectin levels correlated significantly with age- and gender-specific BMI matched percentile values ($p=0.0062$, $r=-0.53$, CI: -0.392 to -0.073). The correlation between BMI and serum adiponectin levels is a well documented phenomenon in the literature, it is not associated with the hepatitis, but it could be detected in our patients as well.

We defined the severity of hepatitis in the degree of elevation in serum alanine aminotransferase (ALAT) and bilirubin values. Clinically all cases proved to be mild to moderate, the liver enzyme elevation, however, was arbitrarily used as a marker of disease severity.

Linear regression analysis confirmed that there was a significant association of changes in serum ghrelin and resistin levels and the severity of hepatitis ($p=0.0053$, $r=-0.54$, CI: -3392 to -664.4; and $p=0.011$, $r=-0.507$, CI: 0.0021 to 0.0003, respectively). These correlations

appeared to be much more pronounced if the logarithm of ALAT values were considered (p=0.0018, r=0.592, CI: -1.827 to -0.475; and p=0.0015, r=-0.6, CI: -3.46 to -0.93, respectively), indicating an exponential correlation of the increase in ghrelin and resistin levels and the severity of hepatic demage. Serum resistin levels showed significant correlation with total bilirubin levels as well (p=0.042, r=-0.418, CI: -0.043 to -0.0009). Furthermore we could also demonstrate an association between the changes of serum leptin levels and the severity of the disease, but it just fell short of statistical significance (p=0.0646, r=0.383, CI:-12.89 to 402.4). The ghrelin/leptin-imbalance, also failed to reach statistical significance (p=0.261, r=0.239, CI: -4.978 to 1.421). There was a slight decrease in serum leptin levels during the illness which did not reach statistical significance (2.25±2.08 *vs.* 3.1±4.24 ng/ml, p=0.269). No changes in serum insulin levels during and after the hepatitis could be detected. Total bilirubin (66.6±126.23 vs. 7.3±5.25 μmol/L), ALAT (1445.2±1038.35 vs. 33.6±25.95 IU/L) and aspartate aminotransferase (ASAT) (1127.6±920.65 vs. 40.1±22.14 IU/L) values normalized in each patient.

4.2. L-carnitine supplementation and adipokines in patients with end-stage renal disease

L-carnitine supplementation did not cause alterations in routine laboratory parameters. However, 12-16 weeks after completion of l-carnitine administration there was a moderate, but statistically significant increase in triglycerides ($p<0.05$) and decrease in total protein and albumin plasma concentrations ($p<0.05$).

Plasma free-, acyl-, and total carnitine concentrations are markedly reduced in patients with ESRD on regular HD when compared with those in healthy control subjects (baseline values). In response to l-carnitine supplementation there was a progressive increase in plasma carnitine levels and their peak values were reached at the completion of l-carnitine administration (week: 12). After cessation of l-carnitine therapy plasma carnitine profiles declined significantly, however, they remained higher than baseline values even in weeks 24-28. These results indicate the long-lasting replenishement of depleted body carnitine store in patients with ESRD on maintenance HD when supplemental l-carnitine was given for a period of 12 weeks.

Plasma levels of all hormones studied are greatly elevated in our patients (baseline values) as compared to those in healthy subjects. Plasma insulin levels responded to l-carnitine with a slight but substained increase from 15.5±11.1 μU/ml in week 0 to 19.9±18.8 μU/ml in week 12 that persisted after l-carnitine therapy was discontinued (26.2±17.2 μU/ml in week 26). Plasma resistin concentrations tended to be depressed by l-carnitine (16.8±14.6 ng/ml in week 0 vs 11.2±4.7 ng/ml in week 2 and 9.3±3.8 ng/ml in week 12, respectively). No consistent changes in plasma resistin levels could be observed in the post-carnitine period (11.8±7.9 ng/ml in week 28). Plasma leptin concentrations in our patients exceeded much the control values (93.7±89.1 μg/ml in week 0) and they remained practically unaffected by l-carnitine supplementation (112.6±133.5 μg/ml in week 12) or by discontinuation of l-carnitine administration (118.9±118.1 μg/ml in week 28).

It is of note however, that in response to l-carnitine therapy circulating adiponectin increased significantly from the baseline value of 20.2±12.7 μg/ml to 32.7±20.2 μg/ml in week 2 and 35.4±19.6 μg/ml in week 12, respectively ($p<0.03$). During the post-carnitine period no consistent changes could be demonstrated. Plasma ghrelin concentrations proved to be similar during the pre-carnitine-, active carnitine,- and post-carnitine treatment periods.

With respect to the complex interactions between hormones controlling energy metabolism and the possible influence of carnitine on these hormones attempts were made to reveal the relationship between the individual hormones studied under these particular clinical conditions. Plasma insulin levels correlated positively with leptin ($r=0.525$, $p<0.0001$) and resistin ($r=0.284$, $p<0.005$), whereas adiponectin levels correlated inversely with leptin ($r=-0.255$, $p<0.02$) and resistin ($r=-0.213$, $p<0.04$) irrespective of carnitine status.

4.3. Endothel dysfunction and adipokines in l-carnitine supplemented patients with end-stage renal disease

In our patients serum ADMA concentrations correlated positively with ghrelin before, during and even after cessation of l-carnitine supplementation (r=0.464, p=0.003, CI: 0.047-0.216). Interestingly, the significant positive relationship of ADMA to ghrelin proved to be independent of body carnitine store.

We demonstrated an inverse relation of ADMA to leptin (r=-0.333, p=0.039, CI: -711.8—16.59) (r = -0.364, p = 0.023) and this relationship could be observed only during l-carnitine supplementation. Interestingly, we failed to demonstrate any association between ADMA and other adipokines or insulin, with or without l-carnitine supplementation. It is to be noted that during the post-supplemental period markedly depressed ADMA levels were observed ($p<0.05$), which can be regarded as a late onset effect of carnitine.

5. Discussion

In the first part of our study we wanted to explore whether there are changes in the serum levels of ghrelin, adipokines and insulin in children with acute viral hepatitis. No changes in insulin levels during and after hepatitis could be observed. We could verify a significant hyperghrelinemia, hyperadiponectinemia and a non-significant hypoleptinemia during acute hepatitis and an exponential correlation was found between the increase of ghrelin and resistin levels and the severity of hepatic failure. We suggest that both the decreased food intake and the severity of inflammation in acute viral hepatitis have a potential role in hyperghrelinaemia and hypoleptinaemia. The significantly higher adiponectin levels during acute hepatitis can be accounted for by its anti-inflammatory and hepatoprotective effect as well as by the decreased biliary clearance of this peptide.

In the second and third part of the study we carried out clinical investigations in l-carnitine supplemented patients with end-stage renal disease (ESRD).

Based on our present findings it can be concluded that plasma levels of adipokines and related hormones are greatly elevated in patients with ESRD. L-carnitine supplementation further augmented the plasma levels of adiponectin whereas plasma levels of the other hormones investigated (leptin, insulin, resistin, ghrelin) remained practically unchanged. Adiponectin has been reported to enhance mitochondrial fatty acid beta-oxidation in association with decreased lipogenesis. Based on these observations it can be postulated that l-carnitine and l-carnitine-induced adiponectin act in concert to slow down the progression of cardiovascular complications of ESRD. Interestingly, irrespective of l-carnitine administration close positive relationship could be established of insulin to leptin and resistin whereas adiponectin was found to correlate inversely with leptin and resisitin. The pathophysiological and clinical implications of these data are not apparent, one can assume however, that they may have a role in protecting the cardiovascular system and in preventing uremic complications.

An unexpected finding of our study is that there is a close, positive correlation beetween plasma ADMA and ghrelin levels in patients with ESRD irrespective of their carnitine status. Ghrelin and its receptors have been detected in cardiovascular tissues and it has been shown to inhibit pro-inflammatory cytokin production, mononuclear cell binding, and nuclear factor-KB activation in human endothelial cells. Accordingly, it is relevant to propose that ghrelin may act to protect the functional integrity of vascular endothelium and to attenuate accelerated atherosclerosis in uremia. The significant, positive relationship of ghrelin to ADMA, however, appears to argue against a vasculo-protective role of this peptide in our clinical setting. Nevertheless, activation of the ghrelin signaling pathway can be regarded as an adaptive response to endothelial dysfunction in order to re-establish/protect vascular function.

6. Summary

- We could verify significant changes of adipokines during acute hepatitis, and suggest that adipokines are active participants of acute inflammation.
- Changes of adiponectin, ghrelin and resistin levels during acute hepatitis can be regarded as markers of hepatic injury.
- Replenishment of the depleted body carnitine stores in patients with ESRD to prevent or correct the dialysis-related carnitine disorders interfere with serum adiponectin levels. It can be postulated that l-carnitine and l-carnitine-induced adiponectin act in concert to slow down the progression of cardiovascular complications of ESRD.
- The significant, positive relationship of ghrelin and significant, negativ relationship of leptin to ADMA, can be regarded as an adaptive response to endothelial dysfunction in order to re-establish/protect vascular function.

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The clinical and national health importance of the adipose tissue derived hormones

Ph.D. Thesis

Tóth Gergely M.D.

Departement of Pediatrics, Faculty of Medicine, University of Pécs

University of Pécs, Faculty of Health Sciences, Ph.D. School

Head of the Doctoral School: József Bódis, M.D., Ph.D., D.Sc., academician
Program Leader: Endre Sulyok, M.D., Ph.D., D.Sc.
Tutor: Endre Sulyok, M.D., Ph.D., D.Sc.

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