THE ROLE AND INTERACTIONS OF FETUIN-A IN HEPATIC AND CARDIOVASCULAR DISEASES

PhD thesis

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Introduction

Cardiovascular diseases are leading cause of morbidity and mortality in most countries. Investigation of new biomarkers may facilitate our understanding of atherosclerosis, improveprediction of mortality and represent new therapeutic targets.The predictive value of these molecules is often limited, which can be improved by combining them and using their ratio.

Fetuin-A correlates positively with various risk factors of atherosclerosis, e.g., obesity, diabetes and the metabolic syndrome. The special properties of the glycoprotein lie behind these associations. Fetuin-A inhibits the activation of the insulin receptor. Elevated level of the glycoprotein leads to weight gain and steatosis of the liver.Fetuin-A binds free fatty acids and these complexes cause the insulin resistance and inflammation of the adipose tissue. Fetuin-A also inhibits adiponectin production. However, fetuin-A is a negative acute phase reactant and the natural inhibitor of ectopic calcification. Thus, the subclinical inflammation present is atherosclerosis may decrease its level, or the low level of the protein may lead to accelerated vascular calcification. These contradictory biological effects of fetuin-A hinder the evaluation of its role in cardiovascular diseases. Studies investigating fetuin-A level in atherosclerotic patients yielded inconsistent results, especially when patients with advanced atherosclerosis were enrolled. Previous myocardial infarction may be considered a hard endpoint for atherosclerosis. Thus, investigation of post-infarction patients helps to clarify the role of fetuin-A in atherosclerosis.

Ghrelin has numerous cardioprotective effects. However, the evaluation of the serum concentration of this peptide yielded contradictory results. The exact role of ghrelin in carbohydrate metabolism is still obscure. The relationship of ghrelin and other adipokines have not been investigated in the presence of diabetes and cardiovascular disease.

Data is limited regarding adipokine ratios. The selection of molecules used in these combinationswas often based on the scope of the research team, and not on careful consideration and statistical analysis.

Fetuin-A is a liver secreted glycoprotein which predisposes it as a biomarker for liver diseases. Previously we reported decreased levels of the glycoprotein in several hepatic diseases. Fetuin-A proved to be an independent predictor of short term mortality in alcoholic cirrhosis. The predictive value of the glycoprotein for long term survival and its relationship with mortality prediction scores have not been investigated.

Hepatic involvement is frequent in Wilson's disease but cirrhosis is less common compared to alcoholic liver disease. When cirrhosis develops in Wilson's diseasethough, it is often insidious. The change of fetuin-A level in Wilson's disease, the alterations of the glycoprotein in the presence of liver involvement and cirrhosis have not been investigated.

Aims

1.In advanced atherosclerosis both elevated and decreased fetuin-A concentrations were reported. Fetuin-A level decreases with age. Our aim was to determine whether patients who were older than 60 years, had impaired metabolism and had survived myocardial infarction had elevated serum fetuin-A level.

2. Fetuin-A is a negative acute phase reactant and it is involved in a number of pathological metabolic processes. We aimed to evaluate whether the glycoprotein had tighter relationship with adipokines regulating metabolism or rather with markers of subclinical inflammation. Therefore we investigated the relationship between fetuin-A and adiponectin, leptin, CRP, TNF-α and resistin.

3. Alterations of serum ghrelin level in cardiovascular diseases is not fully understood. We intended to determine whether patients surviving myocardial infarction had lower or elevated ghrelin concentration.

4. The relationship between ghrelin and molecules influencing its level has not been investigated in post-myocardial infarctions patients. To identify the possible determinants of acyl-ghrelin, we also intended to evaluate its associations with molecules characteristic of different domains ofmetabolism and subclinical inflammation: insulin (carbohydrate metabolism), leptin (energy balance), adiponectin andfetuin-A (fatty liver disease, glucose and lipid metabolism)and TNF-α and resistin (inflammatory domain). Weincluded patients with type 2 diabetes mellitus to clarify the relationship between ghrelin and serum levels of insulin and glucose in coronary atherosclerosis.

5. The role of new biomarkers in cardiovascular mortality prediction is limited, which can be improved by combining them and using their ratio. We intended to identify a cytokine ratio that could effectively differentiate between patients and referent subjects.

6. In patients with alcoholic liver cirrhosis our primary aim was to determine whether fetuin-A was eligible to predict long-term mortality.

7. We intended to compare the predictive value of fetuin-A to the currently used scores (Child-Pugh (CP) and MELD scores). We aimed to evaluate whether combining these scores with the measurement of the glycoprotein might improve mortality prediction.
8. Fetuin-A is a negative acute phase reactant and its level correlates with liver function. We intended to clarify which property of the glycoprotein was dominant in alcoholic liver cirrhosis.
9. We aimed to investigate whether serum fetuin-A level was decreased in patients with Wilson's disease, particularly if liver involvement was present. We planned to evaluate the possible association between fetuin-A level and the disease severity index, Nazar score. We also intended to evaluate if fetuin-A was eligible for identifying patients with cirrhosis. Our aim was to investigate the possible association of the H1069Q mutation with serum fetuin-A level.

Patient and methods

Patients

Post-myocardial infarction patients

We included patients with history of myocardial infarction 6-24 months prior to the start of the study (120 males, 51 females, mean age 62 ± 6 years, mean ± SD). Only STEMI cases were included, diagnosed by ECG and troponin elevation. Exclusion criteria were as follows: clinical or laboratory signs of acute infection, malignant tumor (propagation or oncologic treatment within the previous 5 years), hepatic diseases (apart from non-alcoholic fatty liver disease), renal failure (eGFR<60 ml/min), immune suppression, severe medical or surgical conditions, i.e. myocardial infarction within 6 months, stroke (at any time), trauma, surgical procedure. Diabetes was diagnosed according to current EASD criteria (fasting plasma glucose ≥ 7.1 mmol/l or 2-hour oral glucose tolerance test ≥ 11.1 mmol/l). Only type 2 diabetic patients treated with diet and oral antidiabetic drugs or bedtime insulin were enrolled. Patients with BMI above 30 kg/m^2 were considered obese. Sixty-five per cent of patients with previous myocardial infarction took statins and 70% of them were on aspirin treatment. Eighty-one age-matched persons, free of known cardiovascular diseases served as control group.

Patients with liver diseases

Ninety-three patients with alcoholic liver cirrhosis (52 men, 41 women, mean age: 54 ± 13 years, mean ± SD) were tested. Besides history and appropriate clinical symptoms the diagnosis was established by abdominal ultrasonography, and liver CT scan. For ethical reasons liver biopsy was not performed. Exclusion criteria were as follows: cirrhosis of viral origin (HBsAg and anti-HCV positivity), or autoimmune etiology (antinuclear and anti-smooth muscle antibodies), liver cancer (based on history and findings during routine clinical follow-up), and treatment with hepatotoxic drugs (hepatotoxicity noted as very frequent, frequent, or not frequent adverse reaction inprescribing information). Blood samples were taken at the time of enrolment and in the 1st, 3rd, 6th and 12th months thereafter. Patients with alcoholic liver cirrhosis were only on supportive therapy (diuretics, beta-blockers).
The Child-Pugh and MELD scores were calculated, the latter according to the formula of Kamath et al..
Fifty patients with Wilson's disease (29 men, 21 women, age: 33.6 ± 12.4 years, mean ± SD, duration of disease: 11.4 ± 7.1) were tested. Biopsy was performed when liver involvement was suspected. Patient were followed-up at the Hepatologyoutpatient clinic of the 1st Department of Internal Medicine and were on d-penicillamine treatment. Patients with cirrhosis of viral origin (HBsAg and anti-HCV positivity), or autoimmune etiology (antinuclear and anti-smooth muscle antibodies), liver cancer, and treatment with hepatotoxic drugs were excluded. Fifty-one healthy, age-matched subjects (26 males and 25 females) served as a control group (mean age: 35 ± 8 years).

Laboratory tests

Determination of serum fetuin-A concentration

We used radial immunodiffusion (RID) for the determination of serum fetuin-A levels. In brief, 5 µl of patient's sera diluted to 1:4 was applied in 11.5 ml of Litex agarose gel (Sigma). Serum samples (1:4 dilution) with known concentrations of fetuin-A served as standards. The incubation was done at room temperature for 48 hours. We used anti-fetuin-A (IgG fraction, Incstar, Cat No. 81931, 13.7 mg/ml, in a final concentration of 84 µl/11.5 ml gel) as antibody. The intra- and inter-assay coefficient variations of the test were 4.11% and 4.85%, respectively.

Other laboratory determinations

Serum ghrelin (Linco Research Inc., St. Charles, MO, USA, Ghrelin (active) RIA kit 125 tubes, Cat. No. GHRA-88HK, specific for octanoilated (acylated) on serine 3 variant, sensitivity: 10 pg/ml, specificity: human ghrelin 100%, intra-assay precision [IACV%]: 7.425%, interassay precision [IECV%]: 13.45%, according to manufacturer's instructions) and adiponectin levels (Linco Research, St. Charles, MO, USA, human adiponectin RIA kit 125 tubes (Cat. # HADP-61HK), sensitivity: 1 ng/ml, IACV: 3.86%, IECV: 8.47%) were measured by radioimmunoassay. Serum resistin (Linco Research Inc., Resistin ELISA kit, Cat. No. EZHR-95K, sensitivity: 0.16 ng/ml, specificity: human resistin 100%, IACV: 4.0%, IECV: 7.0%), TNF-α (Human TNF-α high sensitivity ELISA kit, Bender MedSystems GMBH, Vienna, Austria, Cat. No. BMS223HS, sensitivity: 0.13 pg/ml, IACV: 8.5%, IECV: 9.8 %), leptin (DRG International, Mountainside, NJ, USA, Cat. No. EIA-2395 sensitivity: 1 ng/ml IACV: 6.91%, IECV: 11.55 %) were measured by ELISA.C-reactive protein (CRP) concentration was determined by particle-enhanced immunoturbidimetric assay (Roche Cobas Integra 4000). The detection limit of the assay was 0.07 mg/l, the coefficient of variation was 3.9% at 108 mg/l mean value. Serum α2-macroglobulin, transferrin, haptoglobin and orosomucoid were also measured by radial immunodiffusion. We used immunoturbidimetric assay (TinaquantCeruloplasmin; Roche, Indianapolis, IN, USA) to determine serum ceruloplasmin, and absorption photometry to measure serum copper (Merckotest, Diagnostica Merck, Darmstadt, Germany).All other measurements were routine laboratory tests.

Statistical analysis

Paired and unpaired data were analyzed by the Wilcoxonand Mann-Whitney U tests, respectively. Multiple samples were analyzed byKruskal-Wallis test. We used Spearman's rank correlation and partial correlation to evaluate association between variables. Contingency tables were analyzed by Fisher's exact test and chi square test. In multivariate analysis linear and logistic regressions were used. Patients' survival was calculated by the Kaplan-Meier test and life table analysis. Optimal cutoff points were detected by ROC analysis.

During the evaluation of adipokine combinations we used the above mentioned correlations and factor analysis to assess the strength of associations between different molecules. We conducted discriminant analysis to evaluate the predictive value of the investigated biomarkers for subjects belonging to the patient group. Members of anadipokine ratio should have a weak correlation and thus would belong to different groups in factor analysis. These

adipokines should differ markedly between the patient and control group and would have a strong discriminative value in discriminant analysis.

Statistical analyses were performed with SPSS software (SPSS Software, IBM, Armonk, New York, USA). The level of $p < 0.05$ was considered significant, except for patients with alcoholic liver cirrhosis, where $p < 0.01$ was used.

Results

Serum fetuin-A concentration in patients with previous myocardial infarction

Serum level of fetuin-A was significantly higher in patients with previous myocardial infarction compared to healthy controls (619 ± 96 μg/ml vs. 673 ± 103 μg/ml, mean± SD., $p < 0.001$). There was no significant difference between the serum fetuin-A levels of males and females neither in the control ($p = 0.617$) nor in the patient group ($p = 0.328$).

Among post-myocardial infarction patients 49 were obese, and 53 of them had diabetes. Serum fetuin-A levels correlated positively with BMI ($r = 0.342$, $p = 0.035$).Obese patients had slightly elevated serum fetuin-A levels compared to nonobese subjects (686 ± 102 μg/ml, n= 49, vs. 668 ± 104 μg/ml, n = 122, $p = 0.109$). Compared to non-diabetic patients those with diabetes had higher fetuin-A levels (680 ± 108 μg/ml, n = 53 vs. 670 ± 101 μg/ml, n = 118) but the difference was not statistically significant ($p = 0.371$).

Since obesity and diabetes are known to be associated with elevated serum fetuin-A concentration we wanted to see if there is a difference between healthy controls' and nonobese, non-diabetic patients' serum fetuin-A levels. Overweight subjects were present in both groups. Serum fetuin-A level remained significantly higher in patients without diabetes and BMI under 30 kg/m^2 (667 ± 101 μg/ml, n= 88) compared to controls (619 ± 96 μg/ml, $p = 0.002$).

Post-myocardial infarction patients had significantly lower adiponectin level compared to healthy controls (12.77 ± 3.15 μg/ml vs. 9.04 ± 4.31 μg/ml, $p < 0.001$). Serum fetuin-A levels correlated with adiponectin level in patients negatively ($r = -0.236$, $p = 0.006$). Adiponectin correlated with BMI ($r = -0.181$, $p = 0.018$). Since fetuin-A also correlated with BMI we used partial correlation to assess the effect of obesity on the correlation between fetuin-A and adiponectin. After adjusting for BMI the significance of this correlation remained significant ($r = -0.177$, $p = 0.043$). Multivariate analysis showed that fetuin-A level and BMI independently determined serum adiponectin in a model containing age, BMI, fetuin-A,

leptin, TNF-α and resistin as predictors (R^2: 0.203, p < 0.001, BMI (β): -0.131, p = 0.001, fetuin-A: -0.094, p = 0.014).

Serum leptin concentration was also higher in patents with former myocardial infarctionbetegekben (12.17 ± 9.16ng/ml vs. 31.89 ± 18.36ng/ml, p < 0,001) and positively correlated with BMI (r = 0.265, p = 0.022). Fetuin-A correlated with leptin level positively, but the statistical significance of this correlation was lost following correction for BMI (r = 0.209, p = 0.072).

CRP and TNF-αlevels were increased in post-infarction patients compared to controls (4.07 ± 0.24 vs. 6.13 ± 1.77 pg/ml, p < 0.001, and 1.95 ± 1.43 vs. 4.04 ± 3.98 mg/l, p = <0.001), but neither cytokinecorrelated with serum fetuin-A concentration significantly.

TNF-α plays a central role in subclinical inflammation. We evaluated which variables determined the serum level of this cytokine. Multivariate analysis showed that BMI and resistin but not fetuin-A, age, leptin or adiponectin had significant effect on serum TNF-α level (R^2: 0.227, p < 0.001, BMI (β): 0.087, p = 0.005, resistin: 0.110, p < 0.001).

Serum ghrelin concentration in patients with previous myocardial infarction

Patients had significantly lower ghrelin level compared to referent subjects (240.55 ± 59.33 vs. 337.96 ± 30.75 pg/ml, mean ± SD, p < 0.001). There was no significant difference between the serum fetuin-A levels of males and females neither in the control (p = 0.911) nor in the patient group (p = 0.371).

As both diabetes and obesity are known to alter serum ghrelin levels, we compared the ghrelin level of nonobese, non-diabetic patients and referent people. Concentrations of ghrelin in patients remained significantly lower after the exclusion of obese and diabetic subjects (240.63 ± 54.08, n = 88 vs. 337.96 ± 30.75, n = 81, p<0.001). Among diabetic patients serum insulin levelwas elevated compared to non-diabetic patients (28.30 ± 16.30, n = 53 vs. 21.68 ± 14.80, n = 118, p = 0.008) but there was no significant difference in serum ghrelin level (231.06 ± 52.9 vs. 244.81 ± 61.7, p = 0.154).

We found no correlation between serum ghrelin level and fasting glucose concentration. In univariate analysis ghrelin had no effect on fasting glucose level (R^2 = 0.003, β= -0.057, p = 0.459).

The correlation between ghrelin and insulin levels was the strongest one, even after adjusting for BMI and gender (r = -0.369, p < 0.001; Part. corr. = -0.270, p = 0.001). However, there was no correlation between ghrelin and insulin levels among diabetic patients. The correlation between serum ghrelin and adiponectin concentrations weakened (r = 0.307, p <0.001; Part.

corr. = 0.280, p = 0.001), while that between ghrelin and resistin levels lost significance after correcting for BMI and gender (r = 0.178, p = 0.02; Part. corr. = -0.085, p = 0.317). There was no significant correlation between serum ghrelin levels and concentrations of fetuin-A, leptin, TNF-α or serum lipid levels.

In multivariate analysis we evaluated factors determining serum ghrelin level. Insulin and adiponectin levels proved to be independent predictors of ghrelin level in a model containing age, gender, BMI, fasting glucose, insulin, triglyceride, HDL-cholesterol, adiponectin, resistin, leptin, TNF-α and fetuin-A(R^2 = 0.199; p < 0.001, insulin (β): -0.327, p < 0.001, adiponectin: 0.301, p < 0.001).

Evaluation of adipokine combinations, the significance of the TNF-α / ghrelin ratio

We found the weakest correlation between ghrelin and TNF-α (r = 0.017, NS). In factor analysis we used principal component analysis with direct oblimin rotation. The KMO value did not reach 0.5 (0.486) probably due to low sample size and the heterogeneity of factors influencing the development of cardiovascular diseases.The Bartlett's test of sphericity was significant. The analysis yielded 5 groups, with biomarkers tightly correlated. We labeled the first group as “Inflammation factor” due to the high loading by the following items: TNF-α, CRP andresistin (0,861; 0,765; 0,649). In group 2 and 3 lipid fractions dominated, so we labeled it as “Lipid 1” and “Lipid 2factor”. In group 2 the loading of triglycerides, cholesterol and BMI were high, while in group 3 that of cholesterol, HDL-cholesterol and BMI (0.790; 0.660; 0.483; and 0.591; 0.817; 0.536). We labeled group 4 as “Metabolic factor” due to the high loading by fetuin-A, adiponectin and leptin (0.680; 0.609; 0.526). In group 5 ghrelin had the highest loading beside insulin and adiponectin (0.881; 0.600; 0.437), so we labeled this group as “Ghrelin factor”.

We conducted discriminant analysis to evaluate the predictive value of ghrelin, adiponectin, leptin, fetuin-A, resistin and TNF-α for previous myocardial infarction. These proteins accounted for 57.5% of between-group variability (canonical correlation: 0.758; Wilks’ lambda 0.426, p<0.001). The cross-validated classification showed that overall 90.1% (100% of referent subjects and 85.4% of patients) were correctly classified. Analysis of the structure matrix revealed ghrelin as the strongest predictor for belonging to the patient group (0.760), followed by TNF-α (-0.569). Using only these two parameters 89.7% of cases were correctly classified.

As in discriminant analysis ghrelin and TNF-α were best predictors and their correlation was the lowest among all proteins investigated and they belonged to different groups in factor analysis, we further analyzed discriminative value of the TNF-α/ghrelin ratio. Patients had significantly higher TNF-α/ghrelin ratio (Mann-Whitney test, $p<0.001$). We used the highest TNF-α/ghrelin ratio among controls as the cut-off point (0.014286), thus classifying all controls to the low TNF-α/ghrelin group. One-hundred and sixty-five of the 171 post-myocardial infarction patients (96.5%) had TNF-α/ghrelin ratio above the cut-off point, while only 6 of them fell in the group of controls with low TNF-α/ghrelin ratio.

The χ^2 test showed a significant relationship between TNF-α/ghrelin ratio and whether or not subjects belonged to the patient group ($\chi^2(1) = 215.6$, $p<0.001$). The effect size was 0.932. Risk estimation revealed that subjects having high TNF-α/ghrelin ratio had 11.25 times (CI 95% 5.80-21.80) higher chance for belonging to the patient group. Thus the TNF-α/ghrelin ratio was very effective in discriminating between post-infarction patients and controls.

The predictive role of fetuin-A concentration in alcoholic liver cirrhosis

During the one-year follow up 41 out of the 93 patients died, 37 of them due to liver failure, their data were analyzed further. Compared to survivors we found significantly lower baseline values of hematocrit, albumin, transferrin and fetuin-A concentrations and significantly higher creatinine, bilirubin, INR, CP and MELD score values in deceased patients. We observed significantly positive correlations of fetuin-A with hematocrit($r = 0.406$, $p= 0.001$), albumin ($r = 0.366$, $p = 0.002$), TF ($r = 0.529$, $p < 0.001$), and negative correlations with serum creatinine ($r = -0.312$, $p = 0.006$), INR ($r = -0.467$, $p < 0.001$), CRP ($r = -0.405$, $p = 0.001$), the MELD ($r = -0.342$, $p = 0.001$) and CP scores ($r = -0.522$, $p < 0.001$).

We used the ROC curve analysis to determine the optimal cutoff value and test the discrimination ability of serum fetuin-A concentration. Based on this curve, the fetuin-A concentration of 365 μg/ml has been identified as the optimal cutoff value for stratification of our patients. At this cutoff value the area under curve (AUC) was the greatest among all the parameters investigated (0.937 ± 0.025; $p < 0.001$; 95% CI.: 0.889 – 0.986).

The majority of patients with initial fetuin-A level below 365 μg/ml (32/35) died during the 1 year follow-up, while only 5 patients out of 54 with initial fetuin-A level above this point deceased. Patient with fetuin-A level below 365 μg/ml had a 9.874 higher relative risk to die within one year compared to patients with fetuin-A concentration above this level (95% C.I.: 4.258 – 22.898, $p < 0.001$).

Based on the Kaplan-Meier analysis the mean survival time of patients with fetuin-A levels below and above 365 μg/ml was 3 ± 1 months (mean ± S.E., 95% C.I.: 2 - 4) and 11 ± 1 months (95% C.I.: 11 - 12, $p < 0.001$).

A number of variables were significantly different between surviving and deceased patients, including fetuin-A. We used logistic regression analysis to evaluate which of these parameters might be considered as independent predictor of mortality. Variables that were significantly different in univariate analysis were included in the model (hematocrit, albumin, transferrin, creatinine, INR, MELD score and fetuin-A). As the MELD score includes creatinine and INR we excluded these variable from the final model. The model fit was significant (0.015). Serum fetuin-A level proved to be the only independent predictor of one year mortality (OR: 58.82, 95% C.I.: 3.703 – 950.8, $p = 0.004$).

We compared the predictive accuracies for one year mortality of fetuin-A concentration and the CP and MELD scores. Based on data from literature we used 20 and 10 points as cutoff values for the MELD and CP scores, and 365 μg/ml for fetuin-A. On ROC curve analysis the area under curve of serum fetuin-A exceeded those of the CP and MELD scores (AUC ± SD (95% C.I.): 0.937 ± 0.025 (0.889 – 0.986) vs. 0.739 ± 0.052 (0.637 – 0.871) vs. 0.865 ± 0.040 (0.787 – 0.943), $p < 0.001$ for all variables). The sensitivity and specificity of serum fetuin-A concentration were superior compared to those of MELD and CP scores (0.865 vs. 0.595 vs. 0.753 and 0.942 vs. 0.729 vs. 0.827). The relative risk of mortality was elevated among patients with MELD and CP scores above cutoff level (RR (95% C.I.) 2.205 (1.318 – 3.689), $p = 0.002$, and 3.878 (1.998 – 7.527), $p < 0.001$), but patients with low fetuin-A level had the highest risk (9.874 (4.258 – 22.898), $p < 0.001$).

The combination of the MELD score and fetuin-A concentration further improved the accuracy of mortality prediction. Patients with MELD score below 20 and fetuin-A concentration below 365 μg/ml had the worst prognosis, their mean survival was 2 ± 1 months (95% CI.: 1 – 3 month, log rank analysis: $p < 0.001$).

We have previously demonstrated that low serum fetuin-A level was associated with high mortality during one-month follow-up period. We evaluated the effect of deaths occurring during the first month on one year mortality prediction. Analyses showed that the predictive value of fetuin-A was not altered after the exclusion of patients who deceased during the first month.

There were 6 patients with concomitant alcoholic hepatitis (serum fetuin-A: 405 ± 85 μg/ml, mean ± S.D.) whose values did not differ significantly from those without it (434 ± μg/ml, $n = 83$, $p = 0.447$).

Four patients had intercurrent infection. There was no difference between their fetuin-A values prior and after the infection (445 ± 126 μg/ml vs. 475 ± 171 μg/ml, Wilcoxon test, p = 0.465).

Seven patients continued to consume alcohol. Their serum fetuin-A level (259 ± 97 μg/ml, mean ± S.D.) was significantly lower than those who were abstinent (418 ± 129 μg/ml, n = 82, p = 0.003). Six of them died during the observation period. The only one survivor had baseline fetuin-A concentration above 365 μg/ml.

Serum fetuin-A levels did not vary considerably throughout the entire observation period. Patients (n = 75) were divided to four quartiles according to their baseline fetuin-A levels. Patients in these four quartiles were followed and their final fetuin-A levels were compared to their baseline quartile values. Final fetuin-A values were slightly elevated as patients with lower serum level had higher risk of mortality. The change of fetuin-A level of patients in the lowest quartile was significant (Wilcoxon test, p = 0.022), but even in this quartile the final mean fetuin-A concentration was within the range of the baseline quartile.

The significance of fetuin-A determination in Wilson's disease

There was no significant difference between the serum fetuin-A level of patients with Wilson's disease and controls (605 ± 88 μg/ml vs. 598 ± 148, p = 0.419).We could not detect any significant difference between patients with and without liver involvement and Wilson's disease (622 ± 154, n = 16 vs. 562 ± 138, n = 34, p = 0.126). However patients with liver cirrhosis had significantly lower serum fetuin-A level compared to patients free of cirrhosis (491 ± 58, n = 10 vs. 631 ± 154, n = 40, p = 0.013).

We evaluated which parameters were helpful in identifying cirrhotic patients among subjects with Wilson's disease and liver involvement. Serum total protein (78 ± 5 vs. 73 ± 2 g/l; p = 0.023) and more pronouncedly fetuin-A concentration were significantly lower in cirrhotic patients than in those without it(676 ± 149 vs. 491 ± 58 μg/ml; p = 0.001).In patients with Wilson's disease we found significant positive correlation with serum total protein (r = 0.437; p = 0.01) and negative correlations between serum fetuin-A levels and the age of patients (r = -0.334; p = 0.018) and the duration of their disease (r = -0.390; p = 0.006). Among patient with liver involvement these correlations were stronger (total protein: r = 0.685; p < 0.001; age: r = -0.394; p = 0.02; duration of disease: r = -0.471; p = 0.005).

The Nazar score is a helpful tool in evaluating liver damage and prognosis. We investigated the relationship between fetuin-A and the Nazar score to indirectly assess the possible prognostic value of serum fetuin-A concentration. The number of patients with severe liver

damage was too low for statistical analysis, so we compared patients with moderate and severe liver damage (Nazar score 1-4) to patients with liver involvement, but without liver damage (Nazar score 0). Elevated Nazar score was significantly associated with lower serum fetuin-A level (661 ± 148 vs. 485 ± 28 μg/ml; p = 0.006).

Thus, serum fetuin-A concentration proved to be a useful parameter in assessing liver damage and the presence of cirrhosis. We further analyzed the sensitivity and specificity of the glycoprotein for cirrhosis among the 33 patients with liver involvement. The area under curve was 0.869 ± 0.061 (95% C.I.: 0.749 – 0.988, p = 0.001). At the cutoff point of 510 μg/ml the sensitivity was 70.0% and the specificity was 83.3%. Serum fetuin-A concentration below this cutoff point was associated with elevated risk for having cirrhosis (relative risk: 3.807 (95% C.I.: 1.117 – 12.973), p = 0.030).

The genes of ceruloplasmin and fetuin-A have been mapped to the 3q21-3qter region of the 3rd chromosome. However we found no relationship between the two proteins neither among patients with Wilson's disease (r -0.80; p = 0.618), nor among those with liver involvement (r = -0.13; p = 0.519).

We investigated the association between fetuin-A concentration and the most common ATP7B mutation in Eastern Europe (H1069Q). Serum fetuin-A level did not differ between patients with homozygous, heterozygous or no mutation (556 ± 115 vs. 621 ± 161 vs. 597± 152 μg/ml, Kruskal-Wallis test, p = 0.142)

Conclusion

1. Patients with previous myocardial infarction have elevated serum fetuin-A level. The concentration of the glycoprotein is – non-significantly – elevated in diabetic and obese persons and correlates with adiponectin and leptin levels. Fetuin-A has the strongest association with adiponectin concentration, it is an independent determinant of the latter protein.

2. In patients with cardiovascular disease fetuin-A levels decrease with age and with the progression of vascular calcification. Contrary to the previously presumed 55-56 years, fetuin-A might remain elevated above the age of 60, if metabolic disorders, diabetes, obesity is present.

3. Although fetuin-A is known to be a negative acute phase reactant, the concentration of the glycoprotein is not associated with inflammatory cytokine levels.

4. Serum desacyl-ghrelin level is decreased in patients with previous myocardial infarction and it correlates tightly with insulin level.
5. Ghrelin is not associated with subclinical inflammation.
6. TNF-αand ghrelin levels differ markedly between control subjects and post-myocardial infarction patients and the two molecules are not associated. The TNF-α / ghrelin ratio effectively discriminates between control subjects and post-infarction patients.
7. In alcoholic cirrhosis low fetuin-A concentration is an accurate, reliable and independent predictor of one year mortality.
8. The low serum concentration of fetuin-A in these patients is a consequence of impaired liver function and not of inflammatory processes.
9. In alcoholic liver disease the sensitivity, specificity and predictive value for long-term mortality of fetuin-A is superior to those of the Child-Pugh and MELD scores. Combining the determination of fetuin-A concentration and the calculation of the MELD score is beneficial in identifying cirrhotic patients with high mortality risk.
10. Fetuin-A level is not decreased in Wilson's disease, not even if liver involvement is present. The presence of the H1069Q mutation is not associated with fetuin-A serum concentration. Low fetuin-A level in Wilson's disease is useful in identifying patients with liver cirrhosis.

List of publications

Publications related to the dissertation

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