

## Fetuin-A as a Possible Marker of Nonalcoholic Fatty Liver Disease in Patients with Non-Surgical Weight Loss

Groener JB<sup>1</sup>, Lehnhoff D<sup>2</sup>, Roeder E<sup>3</sup>, Piel D<sup>1</sup>, Kender Z<sup>1</sup>, Wolfrum C<sup>3</sup> and Rudofsky G<sup>1,4</sup>

<sup>1</sup>Department of Endocrinology and Clinical Chemistry, Department of Medicine, Im Neuenheimer Feld, Heidelberg, Germany

<sup>2</sup>Department of Rheumatology and Clinical Immunology, Rheumatologikum Hamburg, Schon Klinik Hamburg Eilbek, Dehnhaide, Hamburg, Germany

<sup>3</sup>The Swiss Federal Institute of Technology, ETH Zurich, Institute of Food Nutrition and Health, Schorenstrasse, Schwerzenbach, Switzerland

<sup>4</sup>Department of Endocrinology and Diabetes, Department of Medicine, Kantonsspital Olten, Baslerstrasse, Olten, Switzerland

\*Corresponding author: Jan Benedikt Groener, MD, University of Heidelberg, Division of Endocrinology and Metabolism, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany, Tel: 0049-6221-56-37639, Fax: 0049-6221-56-4233; E-mail: [jan.groener@med.uni-heidelberg.de](mailto:jan.groener@med.uni-heidelberg.de)

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### Abstract

**Objective:** Overweight and obesity are ranked as the fifth leading cause for global deaths and significantly contribute to nonalcoholic fatty liver disease (NAFLD), a common comorbidity finally leading to end-stage liver disease. The diagnostic tool for confirming NAFLD is liver biopsy, an invasive procedure known to cause complications. Results from transient elastography are not reliable in obese patients. Therefore, alternative diagnostic approaches are necessary. One putative biomarker is fetuin-A, also known as  $\alpha^2$ -Heremans-Schmid Glycoprotein (AHSG), which has been proposed for the diagnosis of NAFLD. We therefore aimed to examine the role of fetuin-A in severely obese patients and its value in predicting NAFLD.

**Methods:** 62 obese patients with a body mass index of at least 30 kg/m<sup>2</sup> undergoing a medically supervised weight loss program were analysed at time points T0 (before weight loss), T1 (after non-surgical weight loss within 12 weeks), and T2 (after 52 weeks). Anthropometrical parameters, laboratory values for NAFLD, fetuin-A, "Fatty Liver-index" (FLI), and NASH-Score were determined.

**Results:** 38 out of 62 patients completed the program. These patients showed a significant decrease in BMI (T0: 41.5 ± 7.1 kg/m<sup>2</sup>; T2: 33.5 ± 7.0 kg/m<sup>2</sup>; p<0.001 each). While markers of NAFLD improved from T0 to T2, no significant change was observed in fetuin-A (p=0.36). FLI and NASH-Score significantly improved over time (p ≤ 0.001; p=0.03). Both scores failed to show a correlation with fetuin-A at baseline or during the program.

**Conclusion:** Fetuin-A does not appear to be suitable for diagnosis and follow-up of NAFLD in patients with severe obesity.

**Keywords:**  $\alpha^2$ -Heremans-Schmid glycoprotein; Steatohepatitis; Obesity

**Abbreviations:** AAR; AST/ALT Ratio; AHSG;  $\alpha^2$ -Heremans-Schmid Glycoprotein; ALT; Alanine Amino Transferase; BMI; Body Mass Index; EDTA; Ethylene Diamine Tetra Acetic Acid; FLI; Fatty Liver Index; GGT; Gamma-Glutamyl Transferase; HOMA-IR; Homeostasis Model Assessment Insulin Resistance Index; IL-33; Interleukin-33; kcal; Kilo Calories; kD; Kilo Dalton; kg; Kilo Gramms; m<sup>2</sup>; Square Meter; NAFLD; Non-Alcoholic Fatty Liver Disease; NASH; Non-Alcoholic Steato Hepatitis; WHO; World Health Organization; T2DM; Type 2 Diabetes Mellitus

### Introduction

According to the World Health Organization (WHO) overweight and obesity are the fifth leading cause for global deaths [1]. Worldwide obesity has more than doubled over the last thirty years [1]. In 2012, about 65% of the world's population lived in countries where overweight and obesity killed more people than underweight [1].

Obesity comorbidities include multiple diseases, for example type 2 diabetes mellitus (T2DM), coronary heart disease, and dyslipidemia [2]. Another disease strongly correlated with obesity is "Nonalcoholic Fatty Liver Disease" (NAFLD). The prevalence of NAFLD is 10 to 24% in various countries and it increases up to 74% in obese persons [3-6]. In return, prevalence of obesity in patients with NAFLD varied between 30 and 100% [3,7-12]. Moreover, patients with NAFLD show an increased risk of cardiovascular disease [13,14].

NAFLD includes different stages of liver damage such as steatosis, NASH, and advanced fibrosis, which may progress to cirrhosis and end-stage liver disease [3,15]. NAFLD has gained importance over the last years in the donor selection process for liver transplantation and in the post-transplant period given the high recurrence rate of disease [16]. However, prevalence of NAFLD is underestimated as diagnosis is difficult, especially in patients without elevated liver function tests.

NAFLD is usually diagnosed in patients with persisting asymptomatic elevation of aminotransferase levels after exclusion of other causes, or by radiologic signs of fatty liver disease found incidentally [17-19]. Lately, transient elastography has proven to be a well validated method for diagnosis of liver fibrosis of different causes,

and can be used for diagnosis and follow-up examinations in NAFLD as well [20]. However, in obese patients, results can still be unreliable [21]. Therefore, the gold standard in terms of sensitivity and specificity for confirming NAFLD remains liver biopsy, although it is an invasive procedure, which can cause complications [19,22].

Up to now, different approaches of non-invasive score-systems or biochemical markers to predict the probability of NAFLD or NASH have been proposed. Two of these are the "Fatty Liver-index" (FLI) and NASH-Score. FLI is an algorithm based on Body-Mass-Index (BMI), waist circumference, triglycerides, and gamma-glutamyltransferase (GGT) while the NASH-Score predicts NASH by total cholesterol, alanine aminotransferase (ALT), AST/ALT ratio (AAR), and GGT [23,24].

Furthermore, fetuin-A has been discussed as a possible biochemical marker for detection of NAFLD. Fetuin-A is a 60 kD plasma protein predominantly synthesized in the liver which is known for inducing insulin resistance by inhibiting the insulin stimulated insulin receptor kinase activity [25] and by mediating the lipid induced insulin resistance as a ligand of toll-like receptor 4 [26].

The fetuin-A gene is located at chromosome 3q27, a susceptibility locus for type 2 diabetes and metabolic syndrome [27,28]. The absence of fetuin-A in fetuin-null mice protects against obesity and insulin resistance associated with aging [29]. High plasma levels of fetuin-A are associated with high carotid intima-media thickness, an early marker of atherosclerosis, and with an increased risk for myocardial infarction as well as ischemic stroke [30,31]. Several studies have shown a significantly higher serum concentration of fetuin-A in moderately obese patients and its reduction after weight loss [4]. Our aim was to examine the role of fetuin-A in severely obese patients before and after non-surgical weight loss and its value in predicting NAFLD correlating it to the aforementioned scores.

## Materials and Methods

**Protocol and subjects:** The study had been approved by the Ethics Committee of the University of Heidelberg. All patients gave written informed consent. The study included 62 patients who participated in a low calorie weight loss program at the University of Heidelberg from July 2008 until March 2010. 24 participants did not complete the program. At the end of the study a total of 38 patients were included for further analysis. The low calorie weight loss program used is a medically lead interdisciplinary ambulant therapy program for obese patients with a BMI of at least 30 kg/m<sup>2</sup>.

The goal is a significant weight loss and its stabilization by changing nutritional habits and physical activity patterns in a group therapy consisting of 10 to 15 participants as well as doctors, nurses, physical therapists, psychologists, and nutritionists on a regular basis [32]. Divided into four parts, the first week is spent with medical and psychological analysis (T0) while the next 12 weeks comprise an optimized diet with 800 kcal/die combined with sports and behaviour therapy (T1). Part three contains change from formula diet to fat-reduced food and finally, the fourth part concentrates on integrating the acquired skills into daily routine over 33 weeks to ensure a long lasting success (T2). Other underlying causes of liver disease than NAFLD were excluded.

**Analysis:** Data was collected at time points T0 (before weight loss), T1 (after weight loss within 12 weeks), and T2 (after 52 weeks). Clinical data included height, weight, waist circumference, ALT, AST,

GGT, total cholesterol, triglycerides, fasting insulin, and blood glucose levels. Routine laboratory tests were performed by the Department of Clinical Chemistry of the University of Heidelberg according to standardized protocols. For fetuin-A analysis, citrated plasma was used, whereas adiponectin levels were measured in EDTA-plasma. Samples were centrifuged and frozen at -20°C. Both fetuin-A and adiponectin concentrations were evaluated using commercially available ELISA kits (Human Fetuin-A ELISA Kit, BioVendor GmbH, Heidelberg, Adiponectin total ELISA Kit, Immundiagnostik AG, Bensheim).

**Statistical analysis:** Scoring systems used to detect NAFLD were the FLI =  $(e^{0.953 \times \ln(\text{triglycerides in mg/dl})} + 0.139 \times \text{BMI in kg/m}^2 + 0.718 \times \ln(\text{GGT in U/l}) + 0.053 \times \text{waist circumference in cm} - 15.745) / (1 + e^{0.953 \times \ln(\text{triglycerides in mg/dl})} + 0.139 \times \text{BMI in kg/m}^2 + 0.718 \times \ln(\text{GGT in U/l}) + 0.053 \times \text{waist circumference in cm} - 15.745) \times 100$  [23] and the NASH-Score by gaining one point each for triglycerides  $\geq 200$  mg/dl, AST/ALT  $\leq 1$ , ALT  $\geq 30$  IU/l and GGT  $\geq 30$  IU/l while NASH is probable at  $\geq 3$  points [24]. The Homeostasis model assessment insulin resistance index (HOMA-IR) was calculated by Insulin [ $\mu\text{U/ml}$ ] X Glucose [mmol/l] / 22.5 [33]. Statistical analysis was performed using Microsoft<sup>TM</sup> Office<sup>®</sup> Excel<sup>®</sup> 2003 (Microsoft<sup>TM</sup> Deutschland GmbH, Munich) and IBM<sup>TM</sup> SPSS Statistics 19 (IBM<sup>TM</sup> Deutschland GmbH, Ehningen). Changes from T0 to T1, from T0 to T2, and from T1 to T2 were analysed by Student's t-test. For correlation analysis of specific parameters at T0 and analysis of correlation of changes in parameters at T0 and T2, Pearson's correlation was used. P-values  $< 0.05$  were considered statistically significant.

## Results

In our study, 62 patients undergoing the weight loss program were included for analysis of possible improvements in NAFLD. Twenty-four patients suspended the program early. Clinical characteristics of the 38 remaining patients at T0 are shown in (Table 1) and portray a homogeneous group of 38 adults with  $43.4 \pm 12.6$  years of age and a BMI of  $41.5 \pm 7.1$  kg/m<sup>2</sup>. First, potential associations between studied parameters for NAFLD at baseline (T0) were analysed. Therefore, we examined the association of FLI and NASH-Score to BMI at T0.

Gender male / female	16/22
Age [y]	43.4 $\pm$ 12.6
Height [m]	1.74 $\pm$ 0.10
Waist circumference [cm]	121 $\pm$ 15
Arterial hypertension yes / no	23/15
Diabetes mellitus yes / no	May-33
LDL-cholesterol [mg/dl]	128 $\pm$ 32
HDL-cholesterol [mg/dl]	49 $\pm$ 10
Fasting blood glucose [mg/dl]	111 $\pm$ 34

**Table 1:** Patients clinical and laboratory parameters before weight loss at time point 0 (T0). y=years; m=meters; cm=centimeters; mg=milligrams; dl=deciliters.

A significantly positive correlation could be detected between FLI and BMI (p=0.001) but not between NASH-Score and BMI at baseline

(p=0.24). Furthermore, we examined whether fetuin-A correlates to BMI at baseline. No association could be detected (p=0.20). We also explored possible associations between fetuin-A and FLI as well as between fetuin-A and NASH-Score at baseline. Again no association could be detected (p=0.71; p=0.28).

Second, we studied whether weight reduction is associated with a change in parameters. From T0 to T2 considerable weight loss ( $124.7 \pm 21.9$  kg to  $100.3 \pm 19.0$  kg; p<0.001) and decrease of BMI ( $41.5 \pm 7.1$

kg/m<sup>2</sup> to  $33.5 \pm 7.0$  kg/m<sup>2</sup>; p<0.001) were observed. ALT and triglycerides significantly decreased from T0 to T2 (p=0.02; p<0.001). Also, FLI (p<0.001) and NASH-Score (p=0.03) significantly improved from T0 to T2. However, fetuin-A did not change significantly from T0 to T2 (p=0.36). (Table 2) shows the analysed parameters at the different time points. In addition, as parameters for insulin sensitivity, adiponectin significantly increased from T0 to T2 (p<0.001), whereas HOMA-IR significantly decreased (p=0.001).

Parameters	T0	p-value	T1	p-value	T2	p-value
		T0 to T1		T1 to T2		T0 to T2
Weight [kg]	124.7 ± 21.9	<0.001	102.5 ± 19.2	0.21	100.3 ± 19.0	<0.001
BMI [kg/m <sup>2</sup> ]	41.5 ± 7.1	<0.001	34.2 ± 6.9	0.19	33.5 ± 7.0	<0.001
Fetuin-A [µg/ml]	289.2 ± 90.1	0.01	275.6 ± 95.7	0.19	284.2 ± 102.5	0.36
Adiponectin [µg/ml]	6.4 ± 2.6	0.01	7.3 ± 2.4	<0.001	9.3 ± 3.5	<0.001
ALT [U/l]	31 ± 11	0.44	30 ± 14	0.13	26 ± 11	0.02
Triglycerides [mg/dl]	146 ± 83	<0.001	92 ± 40	0.78	93 ± 50	<0.001
Fatty-Liver-Index	93.8 ± 7.9	<0.001	58.0 ± 29.6	0.14	57.1 ± 29.0	<0.001
NASH-Score	2.2 ± 1.0	0.05	1.6 ± 0.9	1	1.6 ± 1.3	0.03
HOMA-IR-Index	5.3 ± 3.7	<0.001	2.6 ± 1.6	0.34	3.4 ± 5.3	0.001

**Table 2:** Patients clinical and laboratory parameters in a longitudinal analysis over the 52 weeks of commercial weight loss program at time point 0 (T0) before weight loss, time point 1 (T1) after dramatic weight loss within 12 weeks and time point 2 (T2) after 52 weeks. BMI=Body mass index; ALT=Alanine aminotransferase; NASH=Nonalcoholic steatohepatitis; HOMA-IR=Homeostasis model assessment insulin resistance; kg=Kilograms; m<sup>2</sup>=Square meters; µg=Micrograms; ml=Milliliters; mg=Milligrams; dl=Deciliters.

Third, we studied changes in the analyzed parameters during the program. We observed a positive correlation between delta (Δ) FLI (Δ determinants as a term of change in the analysed parameters from T0 to T2) and ΔBMI (p=0.02) but not between ΔNASH-Score and ΔBMI (p=0.88). Next, we explored whether Δfetuin-A correlates to ΔBMI. No correlation could be detected (p=0.28). Finally, we could neither detect an association between Δfetuin-A and ΔFLI (p=0.3) nor between Δfetuin-A and ΔNASH-Score (p=0.82).

## Discussion and Conclusion

The weight loss program led to considerable weight reduction of 19.3%, with 81.6% of participants able to reduce weight at least 10% of their baseline weight, being in line with previously published weight loss programs including 8296 patients from 37 German centres [34].

The achieved weight loss led to a reduction in ALT and triglyceride levels at the end of the study comparable to related studies showing a parallel reduction in triglyceride uptake and liver-storage after a 6 week period of calorie restriction [35]. These results suggest a reduction in liver fat content in our patients as well. Furthermore, longitudinal studies show a reduction in FLI and NASH-Scores at the end of the study period suggesting a decrease of NAFLD and NASH in various participants due to the achieved weight loss [23,24].

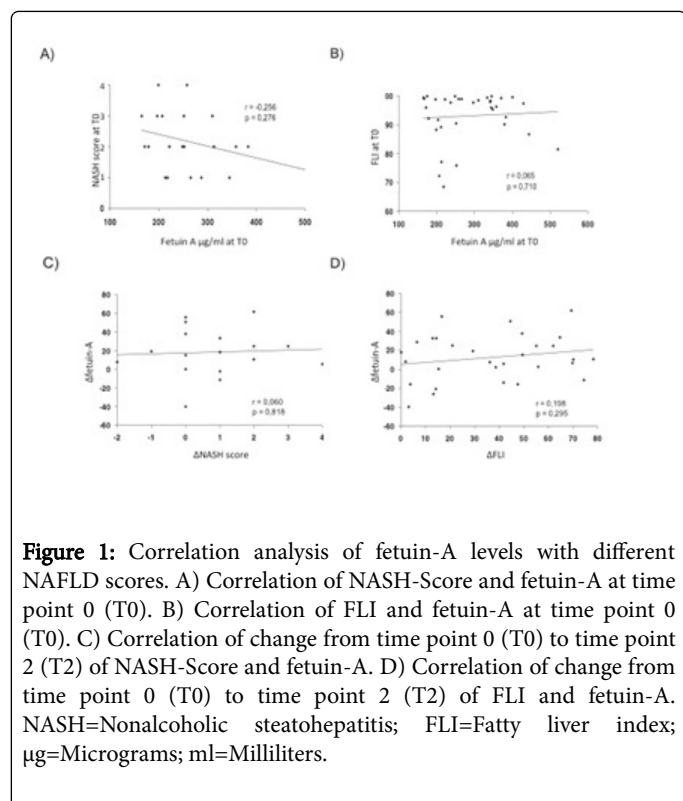
However, our results show that fetuin-A is not altered after 52 weeks although weight and single values as well as scores of NAFLD suggest that the disease has improved. Several studies have shown a link between serum concentrations of fetuin-A and weight loss. Our results

are not in line with previous studies as fetuin-A is only reduced after 12 weeks of formula diet but not at the end of the study although a marked weight loss was achieved. Elevated fetuin-A concentrations in obese patients compared with non-obese healthy control subjects and their reduction after weight loss by gastric bypass surgery was reported [4]. This might be explained by the fact that these studies were performed in less obese subjects. Despite considerable weight loss, subjects of our cohort remained obese at the end of the study due to the highly elevated baseline weight in most cases. Additionally, with respect to the surgical group, further metabolic effects, which might derive from the altered gastrointestinal tract after surgery needs to be discussed. These alterations are not present in a non-surgical approach. Moreover, reduction of fetuin-A in obese patients after non-surgical weight loss in adults and in children has been shown in former studies [36-38]. Comparing these results, the much lower initial weight ( $102 \pm 6$  kg) as well as BMI ( $33.4 \pm 1.4$  kg/m<sup>2</sup>) [36] and the fact that subjects were children of about 11 years of age [37] have to be taken into account. A 6-week aerobic exercise training in non-diabetic obese women did not affect serum fetuin-A levels [39].

In our cohort we observed a reduction of fetuin-A only after 12 weeks, which was not present at the end of the study although patients kept their weight stable and no major weight regain was documented. Possibly explaining the significant reduction of fetuin-A after 12 weeks but not after 52 weeks is the fact that weight loss within the first 12 weeks from  $124.7 \pm 21.9$  kg to  $102.5 \pm 19.2$  kg corresponds to an obvious weight reduction after formula diet with 800 kcal/day. Alimentary influence on fetuin-A needs to be discussed, as niacin

treatment has been shown to lower serum total- and phosphofetuin-A concentrations in individuals with metabolic syndrome [40]. Niacin levels in our formula diet were lower compared to the aforementioned studies but still 11-20% higher than the recommended minimum daily niacin intake. Therefore, the protein-based, niacin enriched diet might have caused this rather artificial effect on fetuin-A.

Correlation analysis of FLI, NASH-Score, and fetuin-A to BMI confirmed a positive correlation between BMI and FLI and of  $\Delta$ BMI and  $\Delta$ FLI at T0 while no correlation was found between NASH-Score and fetuin-A with BMI (Figure 1). Previous studies showing a decrease in fetuin-A after weight loss were not able to show a positive correlation between BMI and fetuin-A either [4,37]. However, the positive correlation between BMI and FLI might be caused by the reduction of BMI itself as one of the main variables in calculating FLI [23]. Our results neither show a positive correlation between FLI and NASH-Score to fetuin-A at T0 nor over time from T0 to T2 supporting the controversial results of former studies [36,41,42]. Few studies were able to show a significant correlation between serum fetuin-A levels to fat accumulation in the liver by magnetic resonance spectroscopy [41] while liver fat content was not associated with serum fetuin-A levels in other studies [36]. Additionally, an association of serum fetuin-A levels with liver fibrosis score index but not with histological NASH-Score was shown [42].



**Figure 1:** Correlation analysis of fetuin-A levels with different NAFLD scores. A) Correlation of NASH-Score and fetuin-A at time point 0 (T0). B) Correlation of FLI and fetuin-A at time point 0 (T0). C) Correlation of change from time point 0 (T0) to time point 2 (T2) of NASH-Score and fetuin-A. D) Correlation of change from time point 0 (T0) to time point 2 (T2) of FLI and fetuin-A. NASH=Nonalcoholic steatohepatitis; FLI=Fatty liver index; µg=Micrograms; ml=Milliliters.

Although fetuin-A has been shown to mediate lipid induced insulin resistance as an endogenous ligand of toll-like receptor 4, our data confirms this with a significant decrease in HOMA-IR from T0 to T2 independent of fetuin-A reduction, suggesting different mechanisms of insulin resistance [26].

However, our study has several limitations. Firstly, sample size is rather small. Therefore, potential effects of weight loss on fetuin-A levels could have been missed. Our main endpoint was the correlation

of fetuin-A levels with FLI during the weight loss program. Since fetuin-A levels only dropped by 1.7% from baseline, it is highly unlikely that a larger sample size would have led to clinically relevant outcomes. To rule out potential confounders on fetuin-A changes, a linear regression analysis correcting for age, gender, and changes in BMI and FLI was performed and showed no significant influence ( $R^2$  0.157). Furthermore, we did not confirm the presence of NAFLD or NASH by liver biopsy, magnetic resonance imaging, or even by transient elastography since results may still be unreliable in obese patients [21]. Due to broadly available evidence concerning the correlation of obesity and NAFLD, the first two methods did not seem ethically justified in our patients. Potential alternative markers for liver fibrosis could be IL-33 and its receptor ST2, which were found to be highly expressed in human liver fibrosis [43,44]. However, in the context of liver fibrosis, these markers have only been evaluated by immunohistochemistry in liver biopsies, not from blood [43]. Moreover, when measuring in serum, both IL-33 and ST2 seem to be elevated in rheumatic diseases like ankylosing spondylitis [44,45]. Therefore, these molecules do not seem to be specific for liver fibrosis, which does not make them feasible as specific routine markers in this context.

In conclusion we observed partially elevated levels of ALT, triglycerides, fetuin-A, FLI, and NASH-Score in extremely obese patients and their reduction after non-surgical weight loss urgently suggesting NAFLD and possibly even NASH in several patients and a partial recovery after weight loss. However, while other markers of NAFLD improved, fetuin-A did not change significantly. Therefore, our results suggest that fetuin-A is neither a suitable marker for NAFLD in extremely obese patients, nor a functional indicator for recovery due to therapeutic interventions.

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