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N-glycan based biomarker distinguishing non-alcoholic steatohepatitis from steatosis independently of fibrosis

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ABSTRACT

Background: Non-alcoholic fatty liver disease is a spectrum of disorders ranging from steatosis to non-alcoholic steatohepatitis (NASH). Steatosis of the liver is benign, whereas NASH can progress to cirrhosis or even hepatocellular carcinoma. Currently, a liver biopsy is the only validated method to distinct NASH from steatosis.
Aim: The objective of this study was to identify a biomarker specific for NASH based on the *N*-glycosylation of serum proteins.
Methods: *N*-glycosylation patterns were assessed using DNA sequencer-assisted fluorophore-assisted capillary electrophoresis and compared with histology.
Results: Initially, a glycomarker (log[NGA2F]/[NA2]) was developed based on the results obtained in 51 obese non-alcoholic patients scheduled for bariatric surgery. Multivariate analysis showed that our glycomarker had the lowest *P*-value of all biomarkers in distinguishing NASH from steatosis ($P = 0.069$). The glycomarker was validated in a cohort of 224 non-alcoholic fatty liver disease patients. In both pilot and validation study, glycomarker score increased in ascending amount of lobular inflammation (single-factor ANOVA, $P \leq 0.001$ and $P = 0.012$, respectively). The *N*-glycan profile of immunoglobulin G in the NASH population confirmed the significantly increased undergalactosylation present in these patients.
Conclusion: Our glycomarker specifically recognises liver inflammation in obese individuals which is the main trigger for the development of steatohepatitis and can differentiate between steatosis and NASH.

1. Introduction

The prevalence of obesity is increasing dramatically in Western countries. Overnutrition and physical inactivity has led to an overweight population that reaches epidemic proportions. In the United States alone, one third of the population is obese (body mass index (BMI) > 30) [1]. Obese patients are prone to develop steatosis or steatohepatitis of the liver. Steatosis is a rather benign condition, but non-alcoholic steatohepatitis (NASH) can develop into cirrhosis and hepatocellular carcinoma (HCC) in 10–15% of the patients [2].

In this respect, it is of crucial importance to distinguish both liver conditions in the obese population. A liver biopsy is still the golden standard despite the well-known complications. Non-invasive alternatives including ultrasonography or biochemical markers can give an indication of the degree of steatosis, but they cannot make the distinction between NASH and simple steatosis [3]. Several biomarkers have been proposed to make this distinction. These biomarkers can be divided into anthropomorphic

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variables such as BMI and waist-to-hip ratio (WHR) and biochemical variables including homeostatic model assessment (HOMA) index, serum adiponectin level, alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), serum cholesterol level, serum high density lipoproteins (HDL) level, serum triglycerides (TG) level and serum uric acid level [4–12]. The current best serological marker for NASH is caspase-cleaved cytokeratin-18 (K18F) [13]. However, K18F is not specific for NASH. Caspase-generated CK-18 fragments are released during hepatocyte apoptosis, a fundamental process in virtually all acute and chronic liver diseases that are characterised by inflammation, steatosis, or fibrosis [14]. One study reported very high sensitivity and specificity (91.7% and 95.8%, respectively) with an AUC of 0.956 in differentiating NASH from fatty liver using tissue polypeptide-specific antigen, a serological mirror of total keratin 18 [15].

Glycomics aims at determining the abundance of the different glycan structures in a quantitative way in a particular biofluid, mostly serum or plasma. It has previously shown its diagnostic value in cirrhotic and HCC patients [16,17]. The GlycoFibroTest is a similar marker to monitor fibrotic patients in which agalactosylated glycans play an important role. These glycans lack one or both galactoses in the glycan structure, they can only be found on Immunoglobulin G (IgG) and gradually augment with increasing METAVIR-stages [18]. In fact, IgG is the glycoprotein on which the main glycomic alterations occur in human chronic liver disease [19].

The mechanism of NASH is poorly understood. It is thought to be immune-mediated and inflammatory stress runs as a second hit in the pathogenesis of NASH [20]. Previous research has confirmed the strong association between inflammatory factors, notably interleukin-6 and spleen enlargement, and the presence of NASH in obese patients [21]. Therefore, as the distinction between NASH and steatosis is mainly inflammation-related, we investigated whether the agalacto-IgG component could be used to distinguish NASH from steatosis.

2. Patients and methods

2.1. Patients

275 Non-alcoholic fatty liver disease (NAFLD) patients from three Belgian academic centres (Ghent University Hospital, Antwerp University Hospital and Erasmus Hospital Brussels) were included in this retrospective study. First, a pilot study (Ghent) was conducted on 51 chronically obese patients that were scheduled for bariatric surgery. Subsequently, the results found in this cohort of patients were validated in two independent cohorts of 50 (Brussels) and 174 (Antwerp) NAFLD patients. The validation study also consisted of patients that were scheduled for bariatric surgery ($n = 51$; 22.8%), but the largest part were patients that were diagnosed with NAFLD through a percutaneous liver biopsy without surgery ($n = 173$; 77.2%). Patients were selected based on clinical data such as BMI (>30 kg/m^2) and the absence of high amount of alcohol consumption (<200 g per week for men and <100 g for women) and were also tested and found negative for viral hepatitis, auto-immune and cholestatic conditions. All patients signed an informed consent. The Ethical Committee of Ghent University Hospital approved the protocols.

In the pilot study, anthropomorphic data (BMI, WHR) and biochemical markers (serum adiponectin level, ALT, GGT, HDL, TG, serum cholesterol level, serum uric acid level, serum ferritin level, HOMA-index, C-reactive protein (CRP) and K18F) were determined in every patient. In the validation study, we additionally determined aspartate aminotransferase (AST), alkaline phosphatase (ALP) and low density lipoprotein (LDL) concentration in every patient. The HOMA-index was calculated as: [fasting insulin concentration (μU/ml) X fasting glucose concentration (mmol/l)/22.5]. ALT, AST, ALP, GGT, HDL, LDL, TG, cholesterol, uric acid, glucose and CRP were determined in serum using routine photometric tests on a Modular P800 (Roche Diagnostics GmbH, Mannheim, Germany). Insulin and ferritin were determined in serum on a Modular E170 (Roche Diagnostics GmbH, Mannheim, Germany). Two Enzyme-Linked Immuno Sorbant Assays were used to determine the serum concentration of K18F (PEVIVA AB, Bromma, Sweden) and adiponectin (R&D Systems, Minneapolis, MN, USA). An aliquot of serum was also immediately frozen for glycomic analysis and stored at −20 °C.

2.2. Histological analysis

In the pilot study, a wedge liver biopsy was carried out during the planned bariatric procedure (mean diameter 86.2 mm (±50)), whereas a tru-cut liver biopsy was performed on bariatric surgery patients in the validation study. A routine Menghini liver biopsy was carried out in the validation study on the NAFLD patients that were not operated (mean diameter 18.0 mm (±7)). Slides were stained with haematoxylin–eosin and picrosirius red. Patients were diagnosed blindly by a pathologist (L.L.) based on classical histopathological features. The NAFLD activity score (NAS) was used to classify the NAFLD patients in a steatosis (NAS < 3), borderline NASH (NAS of 3 or 4) and NASH group (NAS ≥ 5) and scoring of the histological features was performed as determined by Kleiner et al. [22].

2.3. IgG depletion

IgG depletion was performed as previously described with modifications (protein A/G was used instead of protein A) [23].

2.4. Glycomic analysis

Blood was collected on the day the biopsy was performed. For an elaborate description of the protocol, we refer to Laroy et al. [24]. Thirteen peaks were present in the total serum protein electropherogram, whereas only seven peaks were present in the IgG electropherogram (Fig. 1). The height of every peak was quantified to obtain a numerical description of the profiles and these were normalised to the total intensity of the measured peaks (represented as a percentage of the total peak height). These data were analysed with SPSS 16.0 software (SPSS, Chicago, IL, USA). Structural analysis was performed as previously described [16].

2.5. Statistical analysis

Variables uniformly distributed were expressed as mean ± standard deviation. Those that were not normally distributed were expressed as median and range. Statistical analysis was performed by a single-factor ANOVA in three groups (steatosis, borderline NASH and NASH) for normally distributed variables and Kruskal–Wallis for not normally distributed data or otherwise indicated (independent samples *t*-test). The GGT variable displayed many outliners in the three groups and was log-transformed. *Post hoc* tests were performed using Tukey HSD tests. In pilot and validation study, a multiple linear regression analysis was performed including all variables that were significant in the univariate analysis. The degree of linear relationship between two variables was measured with the Pearson correlation test (normally distributed data). *P*-values <0.05 were considered significant in all analyses.