Evaluation of NOGGIN and ASPORIN as new biomarkers for the diagnosis of nonalcoholic fatty liver disease (NAFLD)

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Background

NOGGIN is a secreted homodimeric glycoprotein known to inhibit some of the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-beta (TGF)-β superfamily. ASPORIN is a secreted dimeric extracellular matrix protein, and, like decorin and biglycan, belongs to the Small Leucine- Rich Proteins (SLRP) class I family. The main biological function of ASPORIN consists in the regulation of TGF-β1 activity to which it binds directly. A pilot study ([1] Polyzos et al.) has recently shown that NOGGIN is lower in patients with nonalcoholic fatty liver disease (NAFLD) which is becoming a major burden for the health system in developed countries. Since the regulatory functions of NOGGIN and ASPORIN do overlap in being both related to the TGF-β superfamily, we speculated that also ASPORIN serum measurements may be implicated in the pathophysiology of NAFLD and performed a small pilot study in the same population. ASPORIN and NOGGIN levels were measured with a metal enhanced direct fluorescent immunoassay (FIA) based on a recently developed high sensitivity fluorescence enhancement technology (FluoBolt™).

Methods

FluoBolt™-Technology & Metal Enhanced Fluorescence:

MEF is based on the electromagnetic interaction of excitation light with nano-meter-sized metal structures, which dramatically increases the quantum yield of fluorescent molecules in the immediate vicinity of the metal structures (Fig. 1).

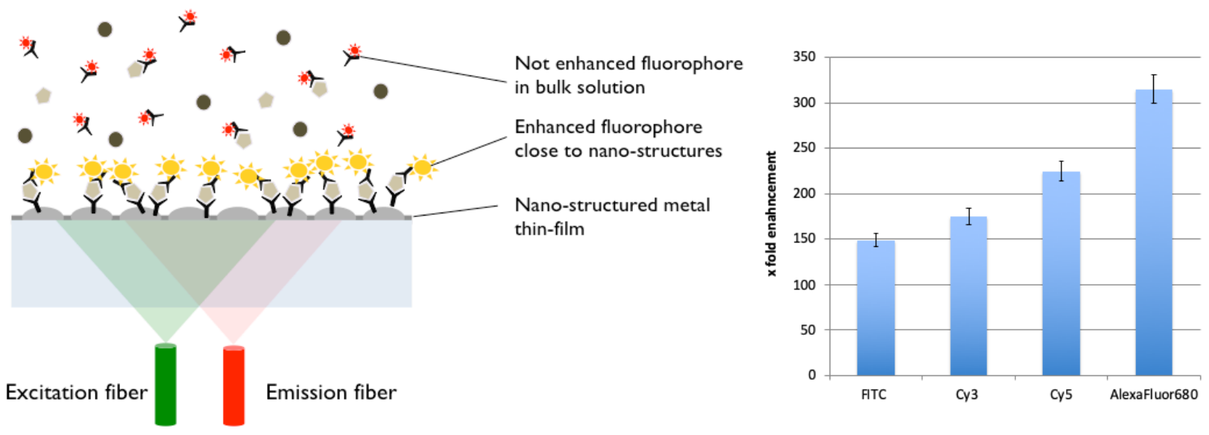

Fig. 1, Schematic representation of metal-enhanced fluorescence and achieved enhancement of various fluorescent dyes

This increase in light yield can be used to generate **highly sensitive** biomarker tests. In addition the suppression of bulk fluorescence allows **immuno-assays without any washing steps.** So far, the commercialisation of MEF for clinical tests has been unsuccessful due to the inadequate reproducibility of the required nano-metal structures and the non-availability suitable biomarker fluorescence tests. In cooperation with STRATEC Consumables, we solved these problems by combining highly reproducible nano-structuring technologies, originally developed for Blu-Ray and DVD manufacturing (Fig 2), with long-term experience in biomarker development thus creating a **fully MTP-compatible MEF-assay platform** ([2] Hawa et al**.)** that can be also be easily adapted to other type of assay format (e.g. microfluidic chips, arrays or lateral flow devices).

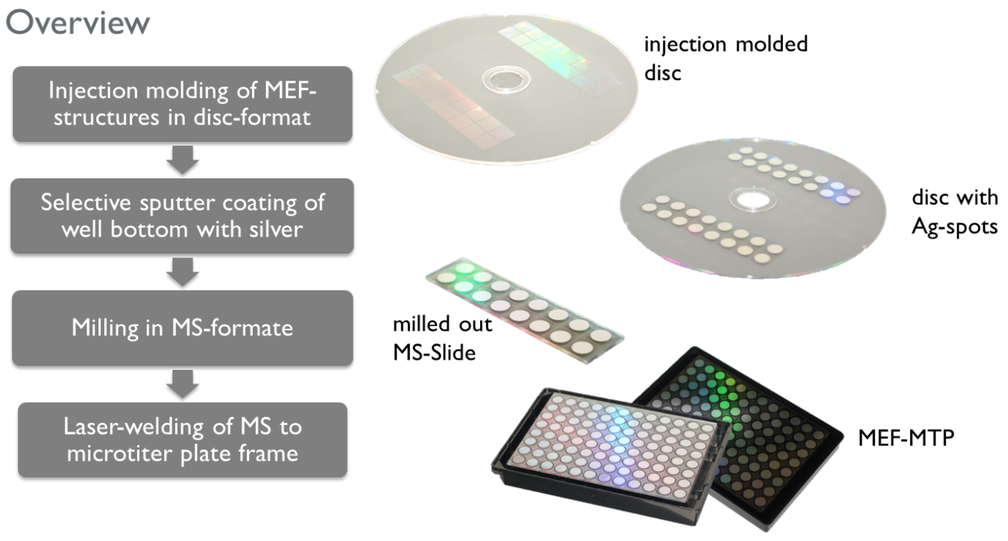

Fig. 2, Manufacturing process of a metal enhanced fluorescence micro titer plate (MEF–MTP)

MEF-MTPs show fluorescence enhancement with fluorescent dyes **from 480 to 720 nm** and can be read **with any** commercial available **fluorescent microplate reader.**

NAFLD patient serum samples:

We included 15 patients with simple **steatosis (SS),** 16 with nonalcoholic steatohepatitis **(NASH)** and 24 controls without NAFLD into this pilot **case control study**. We also determined the influence of vitamin E (400 IU/day) or spironolactone (25 mg/day) plus vitamin E (400 IU/day) for 52 weeks on serum levels of NOGGIN and ASPORIN. Inclusion criteria for **NAFLD patients** were age > 18 years, **ultrasound imaging** detecting fatty liver and abnormal liver function tests for at least 6 months before liver biopsy. **Liver biopsy** was performed in all NAFLD patients by
one experienced radiologist under computed tomography guidance and was interpreted by two experienced pathologists. Inclusion criteria for the **controls** were age > 18 years, no history of and currently **normal liver ultrasound** imaging and **normal liver function tests**.

FluoBolt™- MEF-FIA assay procedure for NOGGIN and ASPORIN:

- 25 µl / 50 µl of AlexaFlour680 labeled anti-NOG/ASP detection antibody and 20/10 µl serum samples were added to the MEF-MTP precoated with anti-NOG/ASP capture antibody
- Plates were sealed with an adhesive microplate sealing film and incubated over night at room temperature in the dark
- Results were read using a TECAN F200pro microplate reader with a 680/720 nm excitation/emission filter. Data were analysed in Microsoft Excel.

Results

NOGGIN and ASPORIN Serum Levels:

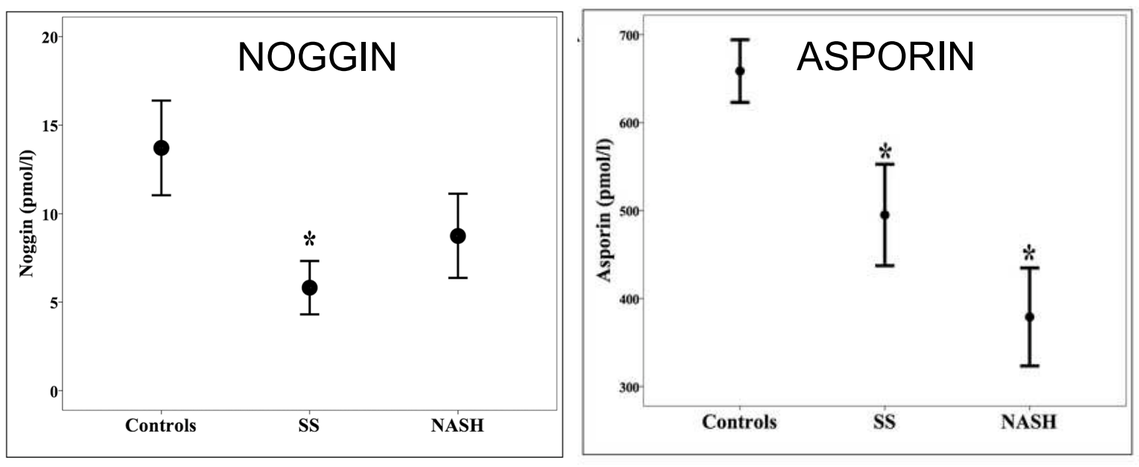

Fig. 4, Serum Levels of NOGGIN and ASPORIN in NAFLD patients and controls

NOGGIN levels have been found to be **lower in SS** (5.8±1.5 pmol/l) **and NASH** (8.7±2.4 pmol/l) patients than in controls (13.7±2.7 pmol/l; p for trend=0.040). **ASPORIN** levels were **also significantly lower** in SS (495±58 pmol/l) and NASH (379±56 pmol/l) patients than in controls (662±37 pmol/l; p-value for trend<0.001), without differing between SS and NASH patients.

Influence of Vitamin E with and without Spironolactone on NOGGIN and ASPORIN Serum Levels in NAFLD

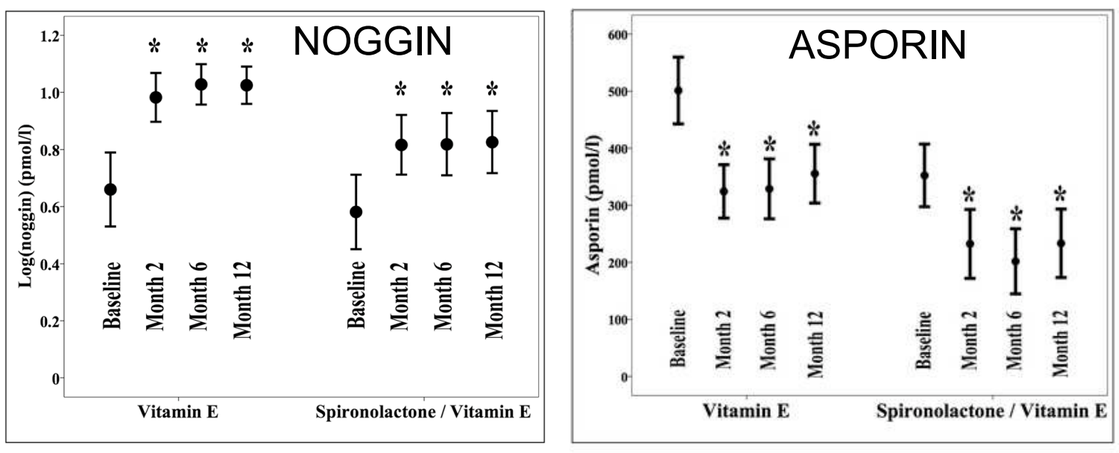

Fig. 5, Change of investigated biomarkers during 12 month Vitamin E / Spironolactone treatment

NOGGIN levels **increased** similarly 2 months post-treatment with vitamin E monotherapy or with the combination of spironolactone and vitamin E. In contrast **ASPORIN** levels significantly **decreased** during the course of therapy.

Discussion

Nonalcoholic fatty liver disease (NAFLD) includes simple steatosis (SS) and nonalcoholic steatohepatitis (NASH), which may advance to cirrhosis and hepatocellular carcinoma [3]. This poses a **significant threat to the public health system** since it is estimated, that 30-40% of the general population in developed countries is affected and the need for its **noninvasive early diagnosis** and treatment **remains unmet** [4]. Current diagnostic means are either too expensive or time consuming to allow **screening of larger patient numbers** to identify those with the onset or the early phase of the disease who would profit from therapeutic intervention and/or life style change.
By using the recently developed FluoBolt™-Technology we were able to create assays for NOGGIN and ASPORIN wit high sensitivity. This allowed for the first time to measure the **decrease** of both markers in NAFLD, which would have been impossible with a less sensitive method. Serum levels of both markers reacted to treatment with vitamin although in opposite ways, which warrants further investigation. One path to do so, could be to follow for example the biological actions of TGF-ß and BMPs on liver tissue, that have been linked to fibrosis and regeneration [Fig. 6, Lit 5,6]

Summary

NOGGIN and ASPORIN may be **valuable biomarkers** for the **diagnosis of NAFLD patients** and they may also mediate the favour-able effect of vitamin E treatment, although mechanistic studies are needed. Further studies with higher patient numbers are also required to confirm these promising results.

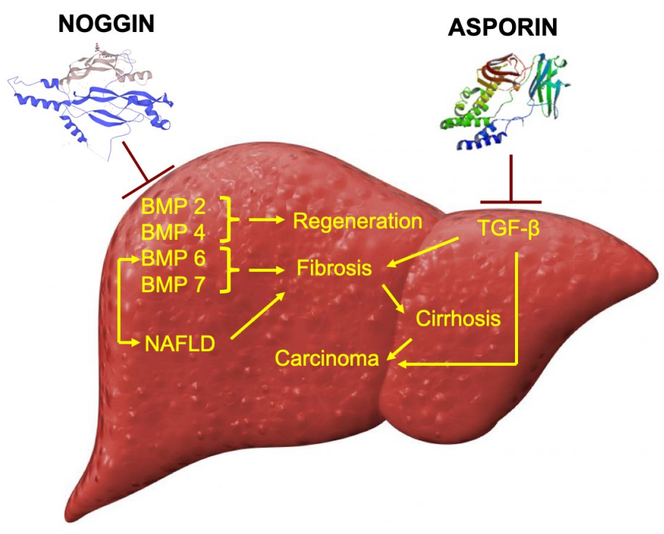

Fig. 6, Potential action of NOGGIN and ASPORIN on the liver

Literature

1) **Noggin levels in nonalcoholic fatty liver disease: the effect of vitamin E treatment.** Polyzos SA, et al Hormones (Athens). 2018 ;17(4):573-579
2) **Single step, direct fluorescence immunoassays based on metal enhanced fluorescence (MEF-FIA) applicable as micro plate-, array-, multiplexing- or point of care-format**. Hawa G et al., Anal Biochem. 2018;549:39-44
3) **Nonalcoholic fatty future disease**. Polyzos SA, Mantzoros CS (2016) Metabolism 65(8):1007–1016
4) **Epidemiology and Natural History of NAFLD.** Corte CD et al. ,J Med Biochem. 2015 ;34(1):13-17.
5) **The immunoreactivity of TGF-b1 in non-alcoholic fatty liver disease**. Kempinski et al., Folia Histochem Cytobiol. 2019;57(2):74-83
6) **BMP Signalling at the Crossroad of Liver Fibrosis and Regeneration**, Herrera B et al. Int J Mol Sci. 2017 Dec 23;19(1).

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