Environment and genetics in the pathogenesis and influence on therapeutic outcome of non-alcoholic fatty liver disease (NAFLD)

EXTENDED ABSTRACT

Background: In Western-lifestyle countries non-alcoholic fatty liver disease (NAFLD) currently represents one of the predominant hepatopathies, rapidly destined to become the most common cause of chronic liver damage, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), as well as the first indication for the organ transplantation worldwide (1). Its global prevalence is currently estimated approximately 25% of the general population and its spread is proceeding like wildfire.
NAFLD affects up to 15-25% of the general population (with values ranging from 20% to 40% in the United States and from 10% to 20% in Asian countries) (2,3) with considerable prevalence also among the young population: it is, in fact, the most common liver disease among adolescents in North America (4).
To date, there is still no curative therapy for this disease; therefore, analysing and understanding the mechanisms underlying its development and progression appears to be the only viable route to devising an effective treatment.
Endocrine disruptors are chemicals capable of affecting the biology of the endocrine system (5), mainly concentrated in food packaging or food crop insecticide residues.
In particular, bisphenol A (BPA) is a chemical whose hyperaccumulation has been correlated with the development of type 2 diabetes mellitus (T2DM) and cardiovascular diseases (6, 7).
Thus, the work of the doctoral thesis has been articulated for several years from the exploration of a new pathogenetic theory to the search for a possible therapeutic strategy, focusing, moreover, on the analysis of new disease-related genetic polymorphisms with a high predictive impact on the therapeutic outcome.

Objectives:
Cohort I: Observational

- Clinical study: evaluation of plasma and urinary bisphenol A levels in NAFLD patients compared to healthy subjects and correlate it with the extent of histologically documented liver necroinflammation and fibrosis.
- In vitro study: evaluation of the effects of exposure to different concentrations of bisphenol A in human HepG2 cells on both oxidative stress induction and cell proliferation.

Cohort II: clinical trial

- To evaluate the effect of six-month administration of silibin complexed with vitamin D and vitamin E (RealSIL 100D®) on metabolic markers, oxidative stress, endothelial dysfunction and worsening of disease in patients exposed to BPA.
- To evaluate the effect of genetics (with particular reference to the following single nucleotide polymorphisms: PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738) on the therapeutic response to the administration of silibin complexed with vitamin D and vitamin E (RealSIL 100D®).

Methods: Cohort I consisted of 60 patients with a histological diagnosis of NAFLD with or without T2DM and 60 healthy subjects. The exposure of NAFLD patients to BPA was assessed by high-performance liquid chromatography (HPLC) and mass spectrometry on both plasma and urine samples. The peroxidative potential of the endocrine disruptor was

assessed by quantification of thiobarbituric acid reactive substances (TBARS) with a special kit. A cell proliferation assay was also performed on the HepG2 cell model exposed to increasing doses of BPA using the MTT assay.

Cohort II consisted of 60 NAFLD patients receiving 303 mg silibin-phospholipid complex, 10 ug vitamin D and 15 mg vitamin E twice daily for six months and 30 NAFLD patients in the placebo arm. All patients in cohort II were followed up for a further six months after the end of treatment in order to assess whether the beneficial effect of taking the drug was perpetuated also after it was unsteady.

During the study period, the patients remained on a free diet based on their pre-enrolment eating habits and no exercise was prescribed. Furthermore, we performed at baseline (T0), at the end of the treatment period (T6) and after six months of follow-up (T12) a nutritional assessment (WinFood, Medimatica s.r.l., Martinsicuro, Italy), clinical parameters, routine haematochemical tests, the assessment of the homeostatic pattern of insulin resistance, NAFLD fibrosis score (NFS), FIB-4, TBARS, as well as the determination of tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β interleukin (IL)-18 and IL-22, metalloproteinase 2, epidermal growht factor receptor (EGFR), insulin growht factor (IGF)-II, differentiation cluster (CD)-44, high mobility group box-1, Endocan, fibroscan and controlled attenuation parameter (CAP).

A sub-analysis was then performed on patients found to be non-responders to treatment. After appropriate genetic screening, 32 patients showed at least one mutation between PNPLA3 I148I/M, I148M/M, TM6SF2 167E/K, 167K/K and MBOAT7 TMC4C/T or TMC4T/T and were compared in terms of treatment outcome to 60 wild type patients (30 treated as described above and 30 untreated control).

For this sub-analysis, patients were divided into three different groups: wild type control NAFLD group, treated wild type NAFLD group and treated mutated NAFLD group, consisting of patients carrying at least one of the above mutations. The entire study protocol is available at https://www.Clinicaltrials.gov (NCT04640324).

Results:

NAFLD patients, particularly those with steatohepatitis (p: 0.04), showed significantly higher levels of BPA in plasma and urine than control patients ($p < 0.0001$), confirming a direct association between BPA exposure, peroxidative damage and histological stage of disease (p: 0.03).

After a BPA-free diet for 1 month, patients with NAFLD showed a significant reduction in its circulating levels ($p<0.02$), with no significant reduction in urinary levels.

In contrast, the specially constructed cell model showed the proliferative ($p<0.0001$) and oxidative ($p<0.0001$) potential possessed by BPA at concentrations of 0.05 μM maintained in culture for 48 hours.

Concerning cohort II, six months after baseline the percentage of treated NAFLD patients who showed a statistically significant improvement in alanine aminotransferase (ALT) and gamma glutamyltranspeptidase (γGT) was higher than untreated NAFLD patients (p= 0:046 and p= 0:032, respectively). On the other hand, no significant change in AST was found in the two patient groups at six months from baseline (T6) and at the end of the observation period (T12). At the end of the observation period (T12), the proportion of treated NAFLD patients who maintained ALT and γGT improvement showed a significant reduction, becoming similar to that of the untreated (p= 0:143; p= 0:091). With regard to metabolic parameters, the percentage of patients who showed a statistically significant improvement in insulin, HOMA-IR, vitamin D and degree of steatosis assessed at CAP at six months from baseline was higher in treated patients than in untreated patients, although complete normalisation was not found for the latter parameter (p= 0:032, p= 0:044, p= 0:038 and p= 0:042, respectively). This

difference remained statistically significant at T12 (p= 0:04, p= 0:04, p= 0:03 and p= 0.04, respectively).
Among the systemic inflammation parameters, the percentage of patients who improved at T6 compared to baseline was higher in the treated group than in the untreated group with regard to CRP and TNF-α (p= 0.03 and p= 0.037, respectively). These parameters in the treated group became similar to those of the untreated patients at T12 (p= 0.112 and p= 0.657, respectively). There were no significant changes in ferritin at the three observation times for both study groups.
Among the markers of disease worsening/progression, the proportion of patients who showed a significant improvement at T6 compared to baseline in EGFR, IL-18, IGF-II, TGF-β and MMP-2 was higher in treated patients than in untreated patients (p= 0.04, p= 0.04, p= 0.03, and p= 0.02, respectively). At T12, the improvement was maintained (p= 0.04, p= 0.04, p= 0.04, and p= 0.03, respectively). In contrast, no significant changes were found at the three observation times for CD-44, IL-22, FIB- 4, NFS and stiffness in the two patient groups.
Finally, regarding the sub-analysis for genetics, in our clinical setting, only the treated wild type group showed a statistically significant improvement in glycaemia, insulinaemia, HOMA-IR, ALT, CRP, and TBARS after six months of treatment. Logistic regression analysis confirmed that, for all improved parameters, genotype played a crucial role in obtaining a useful treatment effect, independent of other confounding variables: age, gender, comorbidities, medication, liver stiffness and CAP.
Importantly, our results highlighted the relative independence of the specific genotype for the lack of therapeutic effect. In other words, the presence of at least one of the above-mentioned mutations was able to result in the loss of therapeutic effect to the prescribed regimen.

Conclusions: The results of our study highlight the role of BPA as an environmental factor involved in the progression of NAFLD.
We demonstrated the increased exposure to BPA of patients with NAFLD compared to controls. In particular, patients with active tissue necroinflammation, and directly proportional to it, had higher levels of plasma BPA than those with simple steatosis. Although potentially eliminable from the body through the maintenance of an efficient renal excretory system, BPA requires some time before it can be completely removed, mainly due to its high lipophilicity, which results in tissue accumulation.
This is precisely the reason for the choice of therapeutic strategy used in this clinical context, given, moreover, the demonstration of the pro-oxidant and proliferative effect of this environmental contaminant on Hep-G2. The strongly antioxidant activity of the drug complex used in the clinical trial for certain parameters proved to be intake-dependent. How much and how this therapeutic strategy may impact on patients' prognosis is not yet known, but the identification of predictive factors of treatment response in the era of personalised precision medicine certainly represents a milestone for the evolution of this interesting field of research.

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