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Please cite this article as: Santilli, Francesca, Blardi, Patrizia, Scapellato, Carlo, Bocchia, Monica, Guazzi, Gianni, Terzuoli, Lucia, Tabucchi, Antonella, Silvietti, Antonella, Lucani, Benedetta, Gioffrè, Walter Renato, Scarpini, Francesca, Fazio, Franc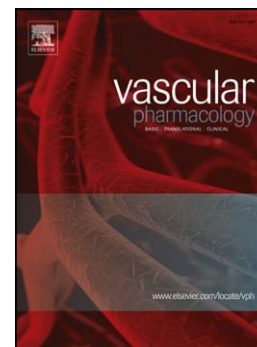esca, Davì, Giovanni, Puccetti, Luca, Decreased plasma endogenous soluble RAGE, and enhanced adipokine secretion, oxidative stress and platelet/coagulative activation identify non-alcoholic fatty liver disease among patients with Familial Combined Hyperlipidemia and/or Metabolic syndrome, *Vascular Pharmacology* (2015), doi: 10.1016/j.vph.2015.04.004

Decreased plasma endogenous soluble RAGE, and enhanced adipokine secretion, oxidative stress and platelet/coagulative activation identify non-alcoholic fatty liver disease among patients with Familial Combined Hyperlipidemia and/or Metabolic syndrome

Francesca Santilli, MD, PhD[1], Patrizia Blardi, MD[2], Carlo Scapellato, MD[3], Monica Bocchia, MD[4], Gianni Guazzi, MD[5], Lucia Terzuoli, PhD[3], Antonella Tabucchi, PhD[3], Antonella Silvietti, BSc[3], Benedetta Lucani, MD[4], Walter Renato Gioffrè, MD[6], Francesca Scarpini, MD[7], Francesca Fazio, MD[1], Giovanni Davì, MD[1] and Luca Puccetti, MD, PhD[4,7]

[1] Department of Medicine and Aging, University of Chieti, Italy; [2] Clinical Pharmacology, University of Siena, Italy; [3] Division of Clinical Pathology, University Teaching Hospital of Siena, Italy; [4] Division of Hematology, University of Siena, Italy; [5] Unit of Emergency Radiology, University Teaching Hospital of Siena, Italy; [6] Preventive Medicine CIRAS, University of Siena, Italy; [7] Atherothrombosis Centre, University of Siena, Italy

Corresponding Author:

Luca Puccetti, MD

Division of Hematology, "Policlinico Santa Maria alle Scotte"

v.le Bracci 18, 53100, Siena, Italy

Tel: +39-0577-585744; Fax: +39-0577-586185; e-mail: luca.puccetti@unisi.it

Abbreviations: FCHL, familial combined hyperlipidemia; MS, metabolic syndrome; esRAGE, endogenous secretory receptor for advanced glycation-end-products; NAFLD, non-alcoholic fatty liver disease; LOX-1, lectin-like oxidized LDL receptor 1.

Abstract

Objective. In patients with familial combined hyperlipidemia (FCHL), without metabolic syndrome (MS), occurrence of NAFLD is related to a specific pro-inflammatory profile, influenced by genetic traits, involved in oxidative stress and adipokine secretion.

Among FCHL or MS patients, hyperactivity of the ligand-receptor for advanced glycation-end-products (RAGE) pathway, as reflected by inadequate protective response by the endogenous secretory (es)RAGE, in concert with genetic predisposition, may identify those with NAFLD even before and regardless of MS.

Methods. We cross-sectionally compared 60 patients with vs. 50 without NAFLD. Each group included patients with FCHL alone, MS alone, and FCHL plus MS.

Results. NAFLD patients had significantly lower plasma esRAGE, IL-10 and adiponectin, and higher CD40 ligand, endogenous thrombin potential and oxidized LDL. The effects of MS plus FCHL were additive. The genotypic cluster including LOX-1 IVS4-14A plus ADIPO 45GG and 256 GT/GG plus IL-10 10-1082G, together with higher esRAGE levels highly discriminate FCHL and MS patients not developing NAFLD.

Conclusions. Among FCHL or MS patients, noncarriers of the protective genotypic cluster, with lower esRAGE and higher degree of atherothrombotic abnormalities coincide with the diagnosis of NAFLD. This suggests an interplay between genotype, adipokine secretion, oxidative stress and platelet/coagulative activation, accelerating NAFLD occurrence as a proxy for cardiovascular disease.

Keywords: esRAGE; FCHL; metabolic syndrome; NAFLD; atherotrombosis; CD40/CD40L.

Chemical compounds studied in this article:

Acetaminophen (PubChem CID: 1983); Insulin (PubChem CID: 70678557); Tryglycerides (PubChem CID: 5460048); Cholesterol (PubChem CID: 5997)

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses several pathologic conditions, ranging from simple steatosis to nonalcoholic steatohepatitis and cirrhosis, and is characterized by an excessive amount of intrahepatic triglyceride, reflecting an imbalance between complex metabolic events [1]. However, it is not clear whether NAFLD causes metabolic dysfunction or if metabolic dysfunction is responsible for intrahepatic triglyceride accumulation [2]. Approximately 20 to 30% of adults in the general population in Western countries develop NAFLD, and its prevalence increases up to 70-90% among obese and/or diabetic subjects [3]. Thus, NAFLD is increasingly prevalent and considered as a feature of the metabolic syndrome (MS) [4]. Consistently, in patients with NAFLD, cardiovascular disease accounts for the outcomes more frequently and to a greater extent than does the progression of liver disease [5,6], raising the relevant question whether fat in the liver is a simple epiphenomenon for metabolic disturbances or poses an independent risk above and beyond known cardiovascular risk factors. In a large study examining 768 nonobese, nondiabetic adults, the prevalence of NAFLD was 23.4% [7]. Thus, despite the close correlations among NAFLD, abdominal obesity, and insulin resistance, at least at certain stages of the natural history of NAFLD, obesity/insulin resistance and liver fat accumulation may be dissociated.

In patients with familial combined hyperlipidemia (FCHL), the most common atherogenic lipid disorder [8], without insulin resistance or MS, we have recently reported that the occurrence of NAFLD is related to a specific pro-atherogenic profile of inflammatory mediators, at least partially influenced by genetic traits involved in oxidative stress and adipokine secretion [9]. We showed that the concurrent presence of lectin-like oxidized LDL receptor 1 (LOX-1) IVS4-14G allele variant plus Adiponectin 45TT and 256 GT/TT plus interleukin (IL)10 10-1082A, is able to identify to a large and significant extent the NAFLD phenotype and is associated with a more aggressive biochemical profile, in comparison to FCHL patients not carrying this genotypic cluster. This pattern included low circulating levels of the anti-inflammatory adipokines adiponectin and IL-10, high concentrations of oxidized LDL and soluble CD40 ligand (CD40L), as well as increased

endogenous thrombin potential (ETP), reflecting increased platelet-coagulative activation, whereas leptin, IL-1 and IL-6, tumour necrosis factor (TNF)-α and C-reactive protein were not significantly affected, although they were previously implicated in the pathogenesis of NAFLD [9]. Thus, in patients with FCHL, even in the absence of MS, the NAFLD phenotype appears to be the result of the interaction between a high-risk genotype and an unfavourable milieu associated with increased free fatty acids and chronic, low-grade inflammation.

The multiligand receptor for advanced glycation end products (RAGE) is expressed in a wide variety of tissues, including the liver [10]. Interactions between AGEs and RAGE can promote a number of adverse physiologic pathways implicated in the development of NAFLD, such as oxidative stress, inflammation, and insulin resistance [11,12], as well as liver fibrosis, inflammation, and regeneration [13]. Plasma levels of soluble RAGE (sRAGE), arising from both receptor ectodomain shedding and splice variant secretion (endogenous secretory RAGE, esRAGE), may counteract RAGE-mediated pathogenesis, by acting as a decoy [11]. Lower circulating levels of sRAGE are independently associated with the risk of diabetes, coronary artery disease, hypertension, metabolic syndrome [14,15], and other chronic diseases, such as NAFLD, where sRAGE is inversely correlated with ALT and AST [16].

We addressed the hypothesis that ligand-RAGE pathway hyperactivity and inadequate endogenous protective response by esRAGE, in concert with a number of pro-inflammatory and pro-atherothrombotic biochemical abnormalities and possibly genetic polymorphisms, may be associated with the diagnosis of NAFLD, even before or regardless of the presence of MS.

2. Materials and methods

The present study was an observational, longitudinal, single-time evaluation of patients with FCHL and/or MS, with or without NAFLD, as diagnosed on ultrasonography. This non-invasive method has now proven to be a reliable tool in the diagnosis of liver steatosis in patients with metabolic disturbances and increased cardiovascular risk. Indeed, high sensitivity and specificity has been reported in the diagnosis of fatty liver disease, thus avoiding use of invasive techniques such as liver biopsy [5].

FCHL was diagnosed according to previously reported criteria [17, 18] with probands required to have all the following criteria: TC and/or TG levels and apoB concentrations greater than or equal to the age- and sex-specific 90th percentiles in the Italian populations in at least three controls over a two-year period; a similar lipid profile including apoB levels in at least two family members and alternatively a proven coronary artery disease (CAD) event before the age of 60 in a first-degree relative.

Hypertension was defined as a blood pressure >140/90 mm Hg or the use of agents for the treatment of hypertension. Blood pressure has been measured according to the recommendations for indirect measurement of arterial blood pressure of the American Heart Association and the Eight Report of the Joint National Committee on the Prevention, Detection, Evaluation and Treatment of High Blood Pressure [19]. MS was diagnosed according to the ATPIII criteria [20]. Patients with type 1 or type 2 diabetes were excluded. Furthermore, secondary causes of steatosis, including alcohol abuse (≥30 g alcohol daily for men and ≥20 g for women), total parenteral nutrition, hepatitis B and hepatitis C virus infection, and the use of drugs known to favour steatosis were excluded. Other forms of liver disease were likewise ruled out in all patients by clinical and laboratory evaluation. Patients with cancer, recent infectious diseases, autoimmune disorders, renal insufficiency or proteinuria (by serum creatinine levels and urinalysis), were also excluded. Cardiovascular disease was ruled out (negative clinical history of stroke, ischemic heart disease or peripheral arterial occlusive disease; negative stress electrocardiogram and arterial Doppler scanning). Finally,

patients were excluded if users in the previous month of anti-hypertensive, anti-thrombotic, lipid-lowering, anti-inflammatory, drugs, except for acetaminophen, and immunosuppressant/immunomodulant drugs.

We performed a cross-sectional comparison between 60 patients with NAFLD vs. 50 without,, as diagnosed on ultrasonography. Each group included FCHL alone (n=24 and n=23); MS alone (n=24 and n=15) and FCHL plus MS (n=12 and n=12). Patients were enrolled among those referring to all the clinical participating centres between June 2012 and February 2013. Sixty of these patients had mild to severe liver steatosis (35 male and 25 female, mean age 55±2.1 yrs, body mass index (BMI) 26.9±2.9 kg/m^2) on ultrasonography and fifty patients did not have liver steatosis (26 male and 24 female, mean age 56.3±3.2 yrs, BMI 26.5±2.8 kg/m^2). The time course of lipid profile compatible with FCHL (including specific subjective changes) was 2.1±0.9 years for NAFLD- subjects and 2.0±0.8 years for NAFLD+ patients.

Patients and controls were compared for complete lipid profile, transaminases, homeostasis model assessment (HOMA)-index, as well as for circulating levels of esRAGE, soluble CD40 ligand (sCD40L), Endogenous Thrombin Potential (ETP), tumor-necrosis factor-α, interleukin (IL)-6 and -10, adiponectin, leptin, and high sensitivity C-reactive protein (hs-CRP). Moreover, to investigate whether genetic polymorphisms related to these biochemical abnormalities may predispose or protect from the risk of developing NAFLD, each patient was genotyped for polymorphisms related to inflammation (Adiponectin 45TT and 256 GT/TT, IL10 10-1082A) and oxidative stress (LOX-1 IVS4-14 A/G) using specific primers and polymerase chain reaction (PCR) technique [9].

The protocol was approved by the Local Ethics Committee and each enrolled subject gave written informed consent to participate in the study.

2.1 *Measurements*

Liver ultrasonography. Liver ultrasonography was performed by a single blinded operator with

Mylab™70 (Esaote, Genoa, Italy) and the degree of liver steatosis severity was assessed and classified as absent, mild and severe on the basis of normal, abnormally intense, high-level echoes arising from the hepatic parenchyma, liver–kidney differences in echo amplitude, echo penetration into the deep portion of the liver and clarity of the blood vessel structure in the liver [21].

HOMA-index and Fatty liver index (FLI) were calculated as previously described [22,23].

Lipid profile detection: TC, HDL-C, TG (each test, both interassay and intra-assay CVs < 3%), Apo-A and Apo-B were determined by immunonephelometry (Siemens BNII Nephelometer, Marburg, Germany), with calibration traceable to the International Federation of Clinical Chemistry primary standards. Using two different controls, the between-assay variation for repeated measurements was 5.2% and 5.8% for Apo A-I, and 2.5% and 3.2% for Apo B, respectively [24]. LDL-C was calculated by the Friedewald's formula (each subject had TG below 4.52 mmol/L). oxLDL levels in serum were determined using ox-LDL Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) Kit (Immundiagnostik AG - Bensheim, Germany). The detection limit of the assay was 4.13 ng/ml; the overall intra-assay and inter-assay coefficients of variation were 4.8% and 6%, respectively. Furthermore, ox-LDL were analysed within one week after collection and immediate storage at -80°C, in order to avoid any putative further in-vitro oxidation, as previously stated [24].

esRAGE. Plasma esRAGE was measured using the B-Bridge ELISA (B-Bridge International, Cupertino, CA) as previously described [25]. This assay specifically measures the esRAGE/RAGEv1 protein only, due to the use of an antibody directed against the unique COOH-terminal sequence of RAGE-v1 and does not cross-react with other potential forms of sRAGE. The overall intra-assay and inter-assay coefficients of variation were both 6%.

Endogenous Thrombin Potential (ETP). ETP was measured in platelet-poor plasma using a commercially available assay (for research use only; Siemens, Marburg, Germany) in a BCS-XP System (Siemens, Marburg, Germany) according to manufacturer's instructions. Coagulation activation was initiated by incubation of plasma with phospholipids, human recombinant tissue factor (Innovin; Siemens, Marburg, Germany), and calcium ions in the absence of thrombomodulin.

The concentrations of phospholipids and tissue factor are confidential to the manufacturer. Thrombin generation and subsequent inactivation was recorded by monitoring conversion of a specific slow reacting chromogenic substrate at a wavelength of 405 nm over time. A mathematical algorithm was applied to correct the substrate conversion curve for the activity of 2-macroglobulin–bound thrombin, which has no known biological activity but is still capable of cleaving small chromogenic substrates. The ETP value was calculated as the area under the thrombin generation curve. Evaluation of reaction curves as well as computer-assisted calculation of thrombin generation over time were performed through the curve evaluation software Curves version 1.0 with specification 3.2 (for research use only; Dade Behring). ETP values are given as percent of normal. Standardization was performed by measuring the ETP standard (Siemens) daily, in parallel to the patient samples. Value assignment of the ETP standard (percent of normal) was performed against a normal plasma pool of 50 healthy donors which was defined to have 100% ETP [26].

Soluble CD40 ligand: sCD40L was evaluated, according to manufacturer instructions and previously reported specific recommendations regarding the appropriate specimen and preparation for laboratory evaluation [27], by Platinum ELISA kits from eBioscience, Ltd. (Hatfield, Ireland, United Kingdom). sCD40 assay exhibits the following performance characteristics: the limit of detection, defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations), was 1.3 pg/ml, while the overall intra-assay and inter-assay coefficients of variation were 5.5% and 7.0 % respectively.

High-sensitivity C-reactive Protein: hs-CRP was detected by a specific ELISA kit (Siemens, Marburg, Germany).

Leptin and Adiponectin. Plasma leptin and adiponectin levels were determined by ELISA using ready kits of Quantikine® human Leptin Immunoassay (R&D Systems, Minneapolis, Minnesota) according to manufacturer instructions. Sensitivity of undiluted samples for leptin was 7.8 pg/ml. Inter- and intra-assay coefficients of variation were 5.4% and 3.3%, respectively. Sensitivity of undiluted samples for adiponectin was 0.246 ng/ml. Inter- and intra-assay coefficients

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of variation were 6.9% and 4.7%, respectively.

IL-6, TNF alpha, IL-10. Cytokines (IL-6, TNF-α, IL-10) were determined in the serum samples, collected as described above, using the high sensitivity Quantikine ELISA assay for IL-6 and TNF-α (R&D systems, Minneapolis, Minnesota) according to the manufacturer's specifications. Samples were assayed in duplicate. The sensitivity of the text was 0.6 pg/ml and 0.5 pg/ml respectively for TNF-α and IL-6. Inter- and intra-assay coefficients of variation were in the range between 5.9-6.4% and 4.7-5.1%, respectively for each variable. IL-10 was evaluated according to manufacturer instructions by Platinum ELISA kits from eBioscience, Ltd. (Hatfield, Ireland, United Kingdom). The limit of detection, defined as above, was 0.1 pg/ml, and the overall intra-assay coefficient of variation was 3.2%, while the overall inter-assay coefficient of variation was 5.6%.

ELISA equipment. Each ELISA test was performed on a Biorad iMark Microplate Absorbance Reader (Biorad, Hercules, CA) except for sCD40, IL-10 and ox-LDL assays that were automatized on DSX processor, elaborating a specific software for performing the entire analytical processes, according to the manufacturers' protocols. The DSX analytical processor (DSX™, Bouty-Technogenetics Milan, Italy) is an automated 4-plate ELISA processing system capable of performing the entire complement of analytical steps required for ELISA immunoassays: adding samples and reagents, washing, incubation, and absorbance detection.

2.2 *Genetics*

Genomic DNA from peripheral blood leukocytes was extracted by the QIAmp Blood Kit (QIAGEN, Hilden, Germany).

LOX-1. Genotyping for IVS4-14 A/G polymorphism (rs3736235) of OLR1 (Genbank Accession no. NM002543) human gene encoding LOX-1 receptor was performed with the Loxin Test® (Technogenetics, Sesto San Giovanni, Italy) through polymerase chain reaction (PCR) followed by electrophoresis on agarose gel. Unless otherwise indicated by the manufacturer, the experimental conditions were those previously employed for the LOX-1 3'UTR C/T polymorphism (rs1050283) [28], that is in complete linkage disequilibrium with IVS4-14 A/G polymorphism [29,30] .

ADIPONECTIN. Genotyping for 45TT (rs2241766) and 276GT/TT (rs1501299) in the adiponectin gene (Genbank Accession no. NM004797) was performed by PCR technique and agarose gel electrophoresis. Primers used to determine the genotypes were 5′-CTGCTATTAGCTCTGCCCGG-3′ for the SNP +45T/G polymorphism and 5′-ACCTCCTACACTGATATAAACTAT-3′ for the SNP +276G/T. The SNaPshot reaction was performed with mix containing 3.75 μl of TRIS-HCl, 1.25 μl of SNaPshot Multiplex Ready Reaction Mix (ABI Prism; Applied Biosystems), 0.15 μl of the primer for the SNP +45, 0.075 μl of the primer for the SNP +276, and 0.775 μl of dH2O. The mixture was incubated for 10 s in 94°C and conditions used for the PCR were 45 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. Reaction mixture was purified with 1 unit of shrimp alkaline phosphatase at 37°C for 60 min and at 75°C for 15 min.

IL-10. The polymorphism at position -1082 (rs 1800896) of the IL-10 gene (Genbank Accession no. X73536) was analysed by PCR technique. The following primers were used: IL-10 forward 5'-TCT GAA GAA GTC CTG ATG TC-3' and reverse 5'- CTC TTA CCT ATC CCT ACT TCC-3'. PCR was performed in a final volume of 50 μl containing 100 ng of DNA template, 200 μM of each dNTP, 200 nmol/μl of each specific primer, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 1.0 U taq DNA polymerase. The following primers were used: forward 5'-TCT GAA GAA GTC CTG ATG TC-3' and reverse 5'- CTC TTA CCT ATC CCT ACT TCC-3'. PCR conditions were as follows; 94 °C for 2 min, then 40 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. The 190 bp amplified product was subsequently incubated for 4 h with MnlI restriction enzyme and visualized in 4% agarose gel.

2.3 *Statistical analysis*

The sample size was calculated based on the primary hypothesis of the study, to detect a putative difference in esRAGE between patients with or without liver steatosis and associated metabolic features. Indeed, assuming a 25% difference in the levels of esRAGE among studied groups, based on the differences in a previously evaluated independent population [16], we estimated that 36

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subjects in each group were needed to detect a significant difference between NAFLD+ or NAFLD- subjects, with estimated pooled standard deviation of 300 pg/mL for both groups, with 90% power and with a significance level (α) of 5%.

Secondary biochemical endpoints were oxidized LDL, TNF-α, IL-6, IL-10, adiponectin, leptin, CD40L, ETP, for each of whom the estimated sample size also had a 90% power to detect a 25% difference between NAFLD+ or NAFLD-, with α=0.05, consistent with previous studies from our group [9, 31,32].

Furthermore, assuming a 20% difference in the frequency of different genotypes for a single polymorphism among studied sub-groups, based on the distribution in previously evaluated independent populations [33-35], we estimated that with 50 subjects in each group, the study had a 90% power to detect a significant difference between NAFLD+ or NAFLD- subjects, with α= 0.05, Thereafter, formal tests for interaction were employed to determine the relationship between subgroups for each single variable or polymorphism. Thus, with at least 12 patients recruited (due to the low frequency of NAFLD- in patients with MS and/or FCHL), the study had a 90% power to detect a 20% difference in the levels of each single evaluated cytokine in metabolic subgroups, and to detect a 10% difference in the frequency of each single polymorphism in metabolic subgroups with α=0.05.

ANOVA with adjustment for multiple comparisons post-hoc test (Scheffe's type), or the Kruskal Wallis (if non parametric variable) with specific post-hoc test, as well as Chi-square test (for categorical variables) were performed for comparisons among groups, as appropriate. The Kendall rank correlation coefficient was used to measure the association between the variables measured. To evaluate the relationship between plasma esRAGE and other factors, simple or stepwise regression analyses were performed. A multiple logistic regression analysis was employed to evaluate the putative relationships among the percent range transformed variables (5% difference steps) and categorical data such as genetic traits and/or the presence of liver steatosis. Furthermore, the statistical model consisting of a formal test for interaction was employed to determine the

putative relationship for each single variable. Briefly, starting from logistic regression analysis in which Y was the analysed variable; the variable X1 and X2 were the presence or not (1 or 0) of polymorphisms or other non-parametric variables or categorized differences in other measurable parameters and X3 the combination. The simplified formula for calculation was: $Y = \beta 0 + \beta 1 X1 + \beta 2 X2 + \beta 3 X3$ and the null hypothesis was tested as H0: $\beta 3 = 0$. Final validation of data was assessed by a resampling technique (exact tests in SPSS 2003 module) and discrimination analysis by the Hosmer–Lemeshow method [$G^2_{HL} = \bullet^{10}_{J=1} (O_j - E_j)^2/E_j (1-E_j/n_j) < X^2_8$] where nj = number of observations in the jth group, Oj = $\bullet_1 yij$ = observed number of positive cases in the jth group, Ej = $\bullet$ pij = Expected number of positive cases in the jth group [36]. Finally, receiver operating characteristic curve (ROC) and training set area under the curve (AUC) were evaluated by parametric method using a maximum likelihood estimator to fit a smooth curve to the data points by a specific computed method (SPSS 2003 module). Each cluster of polymorphisms has been categorized with a different numeric value, in order to be evaluated in this analysis.

The Hanley-McNeil method and the z test were employed to determine the relationships and differences between generated AUCs (c indexes) for each parameter and the occurrence of NAFLD. All calculations, otherwise indicated, were performed using the SPSS library version 13 (SPSS Inc. Chicago, IL).

3. Results

Table 1 depicts the general characteristics of the six study groups. As expected, significant differences were seen across the study groups in BMI, waist circumference, systolic and diastolic blood pressure, lipid profile, including Apo-A and Apo-B, fasting plasma glucose, insulin and HOMA-IR, AST, ALT, gamma-GT, fatty liver index, NAFLD score and CRP levels (Table 1).

Circulating CD40L, ETP, oxLDL, were significantly lower and adiponectin and IL-10 significantly higher in both FCHL and MS patients without liver steatosis as compared to their counterpart with NAFLD (Figure 1). Moreover, within patients without NAFLD, no significant difference was observed in any biochemical variable in study between FCHL and MS patients, apart from adiponectin and ETP which were respectively lower and higher in the group with MS, indicating that MS *per se* exhibits a biochemical pattern roughly comparable to FCHL, and that a detrimental biochemical profile characterizes both FCHL and MS, only when NAFLD occurs.

Regardless of the presence of NAFLD, MS and FCHL have additive effects on the above mentioned biochemical abnormalities, which were further expressed in the subgroup with both FCHL and MS (Figure 1).

Merging groups according to the presence of MS and NAFLD, patients with MS without NAFLD showed comparable circulating levels of esRAGE, adiponectin and oxLDL and lower sCD40L and ETP *vs.* patients without MS but with NAFLD (data not shown).

ROC curve analysis for parameters fitting with the presence or absence of NAFLD, showed that a number of biochemical variables exhibit a significant predictive value in the diagnosis of NAFLD in patients with FCHL alone, MS alone, or FCHL and MS (Figure 2, panels A, B, C). In addition, as previously described in a distinct population with FCHL [9], the presence of the detrimental cluster LOX-1 IVS4-14G plus Adiponectin 45TT and 256 GT/TT plus IL-10 10-1082A, is able to discriminate subjects with NAFLD diagnosis (Figure 2, panels A, B, C).

Conversely, the genotypic cluster previously found to be "protective" from NAFLD in FCHL [9], including LOX-1 IVS4-14A plus ADIPO 45GG and 256 GT/GG plus IL-10 10-1082G, together

with higher circulating esRAGE levels, have the best discriminative power in distinguishing patients without vs. those with NAFLD (Figure 2, panels D, E, F). In particular, the "protective genotype" highly discriminated those with both FCHL and MS, not presenting NAFLD (O.R. 2.97, 95% CI 2.19-4.12, P< 0.01) (Figure 2, panel F). Finally, in patients with MS, no FCHL and no NAFLD, the OR for the same cluster is weaker but still slightly significant (OR 1.29 con 95% CI 0.98-2.06 p= 0.04997), (Figure 2, panel E). The concurrence of protective alleles for IL-10 and adiponectin (ADIPO 45GG and 256 GT/GG plus IL-10 10-1082G), without LOX-1 polymorphism, increases the discriminative power of the protective clustering from the diagnosis of NAFLD (OR 1.98 95% CI 1.26-3.01 p< 0.05).

esRAGE

Regardless of the underlying metabolic abnormality (FCHL or MS), circulating esRAGE was significantly lower in patients with NAFLD as compared to their counterpart without liver steatosis (P<0.0001), in association with higher levels of sCD40L, ETP, ox-LDL and lower levels of IL-10 and adiponectin (Figure 1).

Among patients with NAFLD, but not among their counterparts without NAFLD, plasma esRAGE levels were significantly lower in the MS vs the FCHL group (P<0.0001 and P=0.68, respectively). Patients with MS without NAFLD showed comparable circulating levels of esRAGE *vs.* patients without MS but with NAFLD (Figure 3 panel A). esRAGE was not further reduced by the concomitant diagnosis of FCHL and MS, when NAFLD has already occurred (Figure 1).

Plasma esRAGE was significantly and inversely related to CD40L, ETP, APO-B and IL-10, in the fifty patients without NAFLD. In NAFLD subjects, esRAGE was inversely related to fasting plasma glucose, CD40L, ETP, ox-LDL, TG, waist circumference, BMI, HOMA-IR, insulin, FLI, NAFLD score, leptin, ALT, gamma-GT, and directly related to adiponectin, IL-10 and HDL (Table 2 and Figure 3, panels B and C).

Carriers of the protective cluster exhibited significantly higher levels of esRAGE, as compared to non-carriers, both in the whole cohort (p<0.0001) and within subjects with and without MS

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(P<0.0001 and P=0.002, respectively, Figure 3, panel A).

4. Discussion

The main findings of our study are:

First, our data show that, both in FCHL and MS, circulating esRAGE is significantly lower in patients with NAFLD as compared to their counterpart without liver steatosis, in association with higher levels of ox-LDL, CD40L, and ETP and lower levels of IL-10 and adiponectin. Thus, regardless of the underlying metabolic abnormality (FCHL or MS), the highest degree of biochemical perturbation, in terms of lower esRAGE, IL-10 and adiponectin and higher ox-LDL, CD40L and ETP, characterizes and identifies the clinical diagnosis of NAFLD. Neither MS nor FCHL appear as the main drivers of such metabolic abnormalities, since the inflammatory and atherothrombotic biochemical pattern is far less evident in the absence of NAFLD, even in FCHL patients with MS or with MS alone.

Second, FCHL and MS synergize to accelerate these biochemical perturbations, regardless of the presence of NAFLD. This suggests that distinct mechanisms may concur to confer a pro-inflammatory and pro-atherothrombotic phenotype, confirming the hypothesis that NAFLD may be accelerated, but not necessarily triggered, by the occurrence of MS [37].

Third, the "protective" genotypic cluster from NAFLD, in FCHL, including LOX-1 IVS4-14A plus ADIPO 45GG and 256 GT/GG plus IL-10 10-1082G, highly discriminates those with FCHL and MS not presenting NAFLD. It appears that a genotype-phenotype interaction occurs, with a cluster of polymorphisms identifying those without NAFLD, and with MS only accelerating a process favoured by multiple mechanisms, acting on a perturbed metabolic soil.

Finally, we report that circulating esRAGE highly discriminates patients with NAFLD, independently of insulin resistance and MS, suggesting that activation of the AGE/RAGE axis may represent an additional mechanism favouring the progression of both liver and cardiovascular disease.

Interactions between AGEs and RAGE, expressed in a wide variety of tissues, including the liver, can promote oxidative stress, inflammation, and insulin resistance [11-13,

38,39], all implicated in NAFLD development, according to the "2-hits hypothesis" [40]. Circulating levels of sRAGE [16,41] and esRAGE [41] are highly reduced in adults and obese prepuberal children with NAFLD. In our study, plasma levels of this protective decoy receptor are preserved in subjects without NAFLD or MS, similarly impaired in individuals with MS without NAFLD or with NAFLD without MS (Figure 1, panel D), and further decreased in subjects with both NAFLD and MS. Consistent with our cross-sectional findings, high circulating esRAGE level has been associated with a low 2-year incidence of MS among Japanese adult men [42]. We also reported, in the group with NAFLD, an inverse correlation between esRAGE and oxidized LDL, a ligand for RAGE, known to enhance macrophage proliferation and oxidative stress in hyperlipidaemia [43]. In this setting, statin treatment can modulate the expression of AGEs and RAGE and is associated with increased esRAGE in parallel with decreased lipid peroxidation [44, 45].

Among FCHL patients, those with the lowest esRAGE and with highest degree of pro-inflammatory and atherothrombotic abnormalities, coincide with the clinical diagnosis of NAFLD, regardless of MS, suggesting an interplay between adipokine secretion, oxidative stress and platelet/coagulative activation, all accelerating NAFLD occurrence [46]. This is confirmed by the univariate correlations between esRAGE, CD40L and ETP, not only when NAFLD has already occurred, but also in the groups without NAFLD, suggesting that complex interactions between these mechanisms may precede and possibly contribute to NAFLD development.

Assessing the cause-and effect relationship between the biochemical abnormalities observed in our patients and NAFLD occurrence, was beyond the scope of our cross-sectional study. We acknowledge the small sample size, largely related to the difficulty in the recruitment of patients satisfying the tight inclusion and exclusion criteria, with particular reference to the subgroups with either FCHL or MS and no evidence of NAFLD. In an attempt to obtain meaningful results from less numerous subgroups, formal tests for interaction and post-hoc tests, to adjust for multiple comparisons, were employed. Moreover, measurement of total sRAGE besides esRAGE would

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have provided a balanced view on the total soluble RAGE axis, although esRAGE has received much attention in recent years, especially in the setting of MS [42] and NAFLD [41]. Nevertheless, it is tempting to show that a number of polymorphisms concur to prevent the development of NAFLD and associated pro-atherothrombotic profile, thus highlighting the contribution of a genotype-phenotype interaction in the development of NAFLD. Carriers of the "protective" genotype and those with the highest circulating level of esRAGE are not likely to develop NAFLD, independently of the coexistence of insulin resistance and MS diagnosis. These findings are consistent with data indicating that hepatic, rather than peripheral insulin resistance, is predictive of intra-hepatocellular lipid accumulation [47].

FCHL patients represent 10-20% of premature myocardial infarction survivors [48], but the predictors of their diverse outcome are still to be unravelled. In this regard, NAFLD occurrence, as a result of a detrimental interaction between genotype and metabolic milieu, may be an intermediate step in the natural history of FCHL, mirroring a more aggressive cardiovascular phenotype. Identifying NAFLD as a crucial indicator of progression, both on the liver and cardiovascular side, may help identifying FCHL patients at increased risk of atherothrombotic events, even in the absence of clinical features of the MS.

4.1 Conclusions

In conclusion, regardless of the underlying metabolic abnormality (FCHL or MS), circulating esRAGE, in concert with a number of pro-inflammatory and pro-atherothrombotic biochemical abnormalities and genetic polymorphisms, discriminates patients with NAFLD, suggesting that activation of the AGE/RAGE pathway may contribute to the progression of both liver and cardiovascular disease.

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Figure legends

Figure 1. Plasma CD40L (panel A), ETP (Panel B), ox-LDL (Panel C), esRAGE (Panel D), adiponectin (Panel E), IL-10 (Panel F), in patients with familial combined hyperlipoproteinemia (FCHL) alone, metabolic syndrome (MS) alone, and FCHL plus MS, with or without non-alcoholic fatty liver disease (NAFLD).

Figure 2. Receiver Operating Characteristic curve (ROC curve) and training set Area Under Curve (AUCs) for parameters fitting with the presence or absence of liver steatosis in the setting of non-alcoholic fatty liver disease (NAFLD+, NAFLD-) in patients with familial combined hyperlipoproteinemia (FCHL), metabolic syndrome (MS) or both. ROC and AUCs were evaluated by parametric method using a maximum likelihood estimator to fit a smooth curve to the data points by a specific computed method (SPSS 2003 module). Data reported are those showing a z test > 1.96 ($p < 0.05$) for true positive association with NAFLD+ or NAFLD- with respect to each other variable.

Panel A: GTA cluster (including LOX-1 IVS4-14G plus ADIPO 45TT and 256 GT/TT plus IL-10 10-1082A) (c-statistic 0.788, $p < 0.001$), oxidized (ox)-LDL (c-statistic 0.706, $p < 0.01$), endogenous soluble receptor for advanced glycation end products (esRAGE) (c-statistic 0.611, $p < 0.05$), adiponectin (c-statistic 0.521, $p < 0.05$).

Panel B: Homeostasis model assessment of insulin resistance (HOMA-IR) (c-statistic 0.751, $p < 0.001$), esRAGE (c-statistic 0.730, $p < 0.001$), adiponectin (c-statistic 0.561, $p < 0.05$), HDL (c-statistic 0.520, $p < 0.05$), GTA cluster (c-statistic 0.517, $p < 0.05$).

Panel C: HOMA-IR (c-statistic 0.740, $p < 0.001$), esRAGE (c-statistic 0.711, $p < 0.01$), adiponectin (c-statistic 0.636, $p < 0.01$), GTA cluster (c-statistic 0.568, $p < 0.05$), HDL (c-statistic 0.535, $p < 0.05$), ox-LDL (c-statistic 0.526, $p < 0.01$).

Panel D: AGG cluster (ADIPO 45GG and 256 GT/GG plus IL-10 10-1082G) (c-statistic 0.832, $p < 0.001$), esRAGE (c-statistic 0.705, $p < 0.01$), Interleukin (IL)-10 (c-statistic 0.549, $p < 0.05$).

Panel E: AGG cluster (c-statistic 0.720, $p < 0.01$), esRAGE (c-statistic 0.709, $p < 0.01$), IL-10 (c-statistic 0.585, $p < 0.05$).

Panel F: AGG cluster (c-statistic 0.756, $p < 0.001$), esRAGE (c-statistic 0.706, $p < 0.01$)

Figure 3. *Panel A.* Median esRAGE levels in non carriers and carriers of the "protective cluster" of polymorphisms, including LOX-1 IVS4-14A plus ADIPO 45GG and 256 GT/GG plus IL-10 10-1082G, according to the presence or absence of metabolic syndrome (MS).

Panels B-C. Univariate correlation between plasma sCD40L levels (Panel B) or oxidized LDL (Panel C) and esRAGE in patients with or without non-alcoholic fatty liver disease (NAFLD).

Empty symbols represent patients without NAFLD. Full symbols represent patients with NAFLD.

Variable	No NAFLD			NAFLD			
	FCHL (n=23)	MS (n=15)	FCHL + MS (n=12)	FCHL (n=24)	MS (n=24)	FCHL + MS (n=12)	P value
Age, yr	58 (55-59)	55 (52-58)	56 (53-58)	54 (49-57)†	55 (53-57)	56 (54-58)	0.001
Gender (M, %)	13 (56.5)	9 (60)	4 (40)	14 (58.3)	14 (58.3)	7 (77.8)	0.730
BMI, Kg/m^2	25.6 (24.4-26.1)	27.1 (26.8-27.4)*	27.1 (26.8-27.5)*	25.5 (24.7-26.3)	27.9 (27.3-28.7)*	28.3 (28.0-28.7)*	0.0001
WC, cm	88 (80-99)	101 (89-102)	90 (89-102)	94 (85-99)	99 (87-102)	103 (96-105)	0.005
SAP, mmHg	130 (130-135)	140 (135-140)*	140 (135-145)*	135 (130-140)	145 (140-149)*	140 (135-145)	0.0001
DAP, mmHg	80 (75-85)	85 (85-90)*	90 (85-91)*	80 (75-84)	87 (85-90)*	90 (85-90)*	0.0001
LDL, mmol/L	4.52(4.42-4.7)	2.89 (2.82-2.97)*	4.13 (4.08-4.19)*	4.5 (4.39-4.65)	3.07 (3-3.15)*†	4.24 (4.16-4.32)*	0.0001
HDL, mmol/L	1.29 (1.19-1.34)	0.98 (0.9-1.01)*	1.06 (1.03-1,09)*	1.24 (1.19-1.32)	1.03 (0.98-1.06)*	1.06 (0.98-1.09)*	0.0001
TG, mmol/L	1.56 (1.42-1.72)	2.07 (1.89-2.16)*	2.2 (2.12-2.28)*	1.58 (1.45-1.73)	2.21 (2.15-2.33)*†	1.85 (2.22-2.39)*	0.0001
APO A, g/L	1.00 (0.93-1.02)	0.88 (0.85-0.90)*	0.90 (0.86-0.91)*	0.99 (0.92-1.04)	0.81 (0.79-0.85)*†	0.83 (0.81-0.85)*	0.0001
APO B, g/L	1.50 (1.45-1.59)	1.05 (1.02-1.08)*	1.48 (1.46-1.56)	1.51 (1.45-1.57)	1.00 (0.99-1.05)*	1.58 (1.53-1.60)	0.0001
Lp(a), µmol/L	0.93 (0.79-1.04)	0.86 (0.68-0.93)	0.89 (0.79-1.04)	0.95 (0.76-1.09)	0.73 (0.68-1.00)	0.89 (0.71-1.00)	0.180
FPG, mmol/L	4.89 (4.78-5.17)	6.44 (6.39-6.5)*	6.39 (6.33-6.44)*	5.17 (4.83-5.5)	6.5 (6.39-6.67)*	6.56 (6.44-6.67)*	0.0001
Insulin pmol/L	39.6 (32.6-43.7)	96.5 (93.7-98.6)*	92..4 (90.9-95.2)*	39.6 (29.2-47.2)	113.2(108.3-126.4)*†	128.5 (124.3-131.9)*†	0.0001
HOMA-IR	0.7 (0.6-0.8)	1.9 (1.8-1.9)*	1.8 (1.8-1.9)*	0.7 (0.6-0.9)	2.2 (21-2.5) *†	2.3 (2.2-2.7) *†	0.0001
ALT, U/L	26 (23-29)	29 (24-31)	30 (29-32)	31 (29-35)†	36 (33-38)†	38 (36-40)*†	0.0001
γGT, U/L	30 (27-34)	38 (33-46)	47 (43-50)*	36 (27-54)†	66 (63-70)*†	74 (69-76)*†	0.0001
FLI	41 (30-55)	43 (30-45)	44 (42-46)	55 (37-62)	69 (67-73)*†	81 (79-90)*†	0.0001
NAFLD Score	-0.16 (-0.32-0.02)	0.46 (0.44-0.53)*	0.44 (0.36-0.46)*	0.28 (0.15-0.47)†	0.68 (0.62-0.73)*†	0.67 (0.61-0.71)*	0.0001
CRP, nmol/L	11.43 (8.57-14.29)	10.48 (9.52-13.33)	11.43 (10.48-12.38)	14.29 (10.48-18.10)†	13.33 (12.38-13.33)	13.33 (11.43-13.33)	0.009

Table 1. Clinical, anthropometrical and biochemical characteristics of study patients.
The last column on the right refers to the p p-value of the differences across the study groups (by ANOVA with Scheffè post-hoc test or Kruskal Wallis with Bonferroni relative post-hoc tests, to adjust for multiple comparisons)
† p<0.05 of post-hoc test *vs* the corresponding group without NAFLD
*p<0.05 of post-hoc test *vs* FCHL group

Variable	No NAFLD			NAFLD		
	FCHL (n=23)	MS (n=15)	FCHL + MS (n=12)	FCHL (n=24)	MS (n=24)	FCHL + MS (n=12)
Age	0.105 *0.127*	0.123 *0.111*	0.114 *0.112*	0.111 *0.117*	0.121 *0.111*	0.103 *0.119*
Gender (M, %)	0.175 *0.078*	0.109 *0.122*	0.126 *0.099*	0.145 *0.088*	0.104 *0.123*	0.116 *0.108*
BMI	-0.177 *0.077*	-0.202 *0.058*	-0.209 *0.058*	-0.251 *0.052*	**-0.409** ***0.002***	**-0.309** ***0.039***
WC	-0.196 *0.064*	-0.239 *0.053*	-0.218 *0.057*	**-0.268** ***0.049***	**-0.445** ***0.0009***	**-0.388** ***0.002***
SAP	-0.107 *0.119*	-0.146 *0.088*	-0.139 *0.092*	-0.116 *0.108*	-0.193 *0.067*	-0.171 *0.080*
DAP	-0.116 *0.108*	-0.144 *0.089*	-0.133 *0.094*	-0.111 *0.117*	-0.201 *0.058*	-0.184 *0.073*
LDL	**-0.278** ***0.046***	-0.094 *0.148*	-0.193 *0.062*	**-0.291** ***0.041***	-0.196 *0.060*	**-0.279** ***0.046***
HDL	0.219 *0.057*	0.199 *0.059*	0.198 *0.059*	**0.271** ***0.049***	**0.292** ***0.041***	**0.290** ***0.041***
TG	-0.234 *0.051*	-0.193 *0.067*	-0.199 *0.058*	**-0.274** ***0.049***	**-0.290** ***0.041***	**-0.277** ***0.048***
APO A	0.250 *0.52*	0.086 *0.148*	0.090 *0.139*	-0.249 *0.52*	**-0.272** ***0.049***	-0.248 *0.053*
APO B	**-0.292** ***0.041***	-0.206 *0.054*	**-0.279** ***0.047***	**-0.392** ***0.029***	-0.201 *0.058*	**-0.292** ***0.042***
Lp(a)	-0.097 *0.144*	-0.101 *0.136*	-0.105 *0.126*	-0.193 *0.066*	-0.177 *0.074*	-0.201 *0.059*
FPG	**-0.279** ***0.047***	-0.204 *0.055*	-0.199 *0.059*	**-0.270** ***0.048***	**-0.399** ***0.003***	**-0.383** ***0.007***
Insulin	0.171 *0.077*	-0.206 *0.054*	-0.200 *0.058*	0.239 *0.053*	**-0.427** ***0.001***	**-0.302** ***0.038***
HOMA-IR	-0.202 *0.057*	-0.211 *0.052*	-0.206 *0.054*	**-0.271** ***0.049***	**-0.456** ***0.0008***	**-0.402** ***0.004***
ALT	-0.085 *0.207*	-0.094 *0.148*	-0.088 *0.175*	**-0.305** ***0.039***	**-0.311** ***0.037***	**-0.308** ***0.040***
γGT	-0.163 *0.080*	-0.189 *0.068*	-0.176 *0.074*	**-0.313** ***0.038***	**-0.391** ***0.019***	**-0.388** ***0.021***
FLI	-0.094 *0.161*	-0.108	-0.101	**-0.294** ***0.041***	**-0.299**	**-0.288**

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		0.114	*0.126*		*0.04*	*0.046*
NAFLD Score	-0.118	-0.126	-0.121	**-0.299**	**-0.292**	**-0.303**
	0.103	*0.099*	*0.108*	***0.041***	***0.041***	***0.041***
CRP	-0.211	-0.208	-0.213	-0.241	-0.233	-0.241
	0.056	*0.057*	*0.058*	*0.053*	*0.053*	*0.054*
sCD40L	**-0.337**	**-0.351**	**-0.367**	-0.477	**-0.401**	**-0.463**
	0.038	***0.036***	***0.035***	***0.0003***	***0.003***	***0.0007***
ETP	**-0.382**	**-0.350**	**-0.373**	-0.479	**-0.490**	**-0.480**
	0.031	***0.036***	***0.034***	***0.0003***	***0.0002***	***0.0003***
IL-6	-0.068	-0.094	-0.102	-0.166	-0.234	-0.231
	0.293	*0.158*	*0.127*	*0.089*	*0.052*	*0.053*
TNF-α	-0.117	-0.126	-0.128	-0.177	-0.231	-0.229
	0.113	*0.099*	*0.103*	*0.076*	*0.052*	*0.054*
IL-10	**-0.291**	**-0.297**	**-0.301**	0.367	**0.391**	**0.382**
	0.042	***0.041***	***0.04***	***0.035***	***0.019***	***0.031***
Adiponectin	0.203	0.200	0.193	**0.393**	**0.400**	**0.418**
	0.061	*0.062*	*0.069*	***0.019***	***0.013***	***0.005***
Leptin	-0.096	-0.118	-0.103	-0.196	-0.203	-0.200
	0.219	*0.103*	*0.127*	*0.060*	*0.061*	*0.063*
Ox-LDL	-0.181	-0.168	-0.207	**-0.429**	**-0.299**	**-0.399**
	0.075	*0.084*	*0.060*	***0.001***	***0.04***	***0.008***

Table 2. Correlations between esRAGE and antropometric or biochemical variables in each group. In each cell, the first value represents the Kendall rank correlation Coefficient, the second value (in Italics) the P value
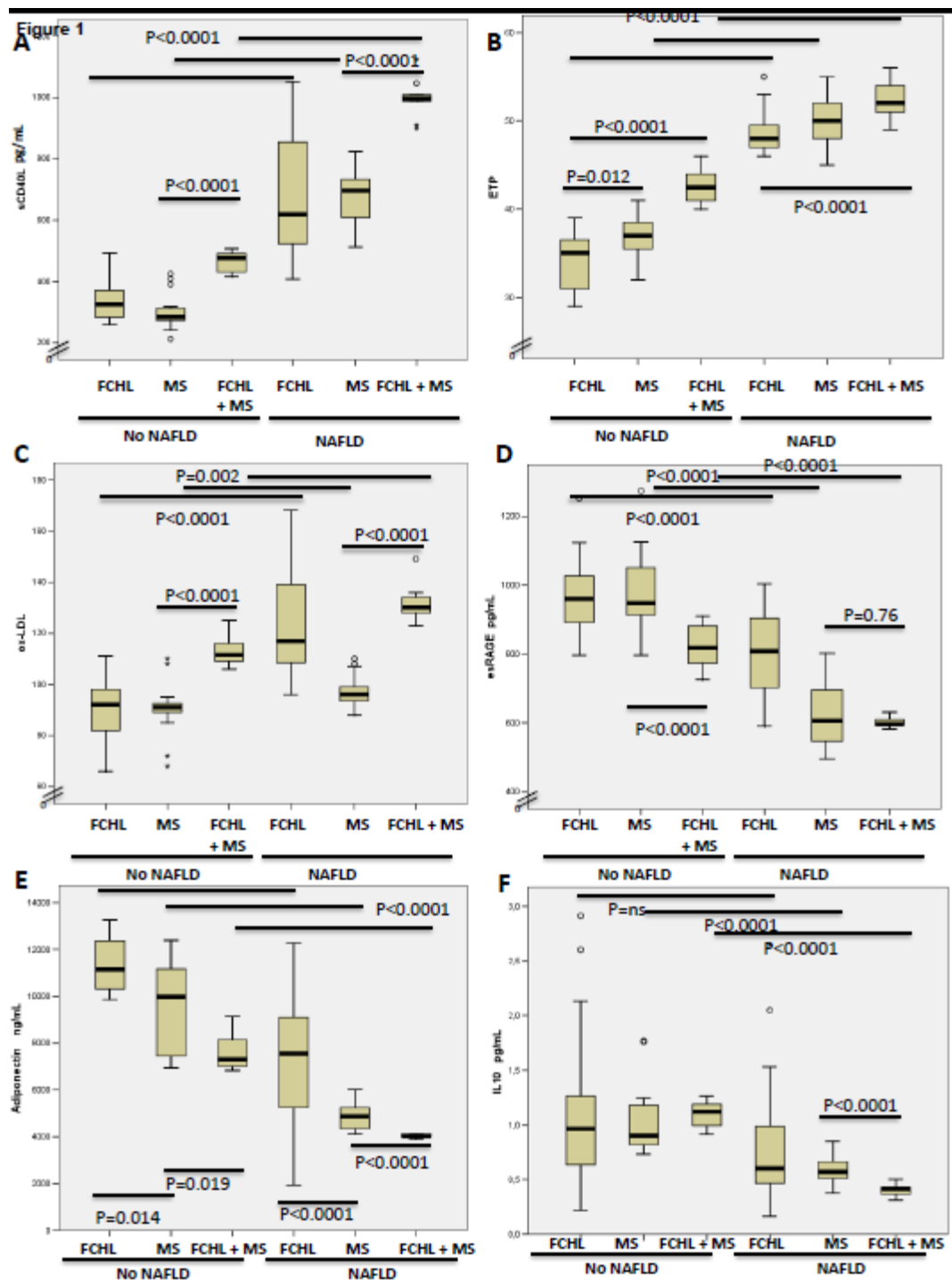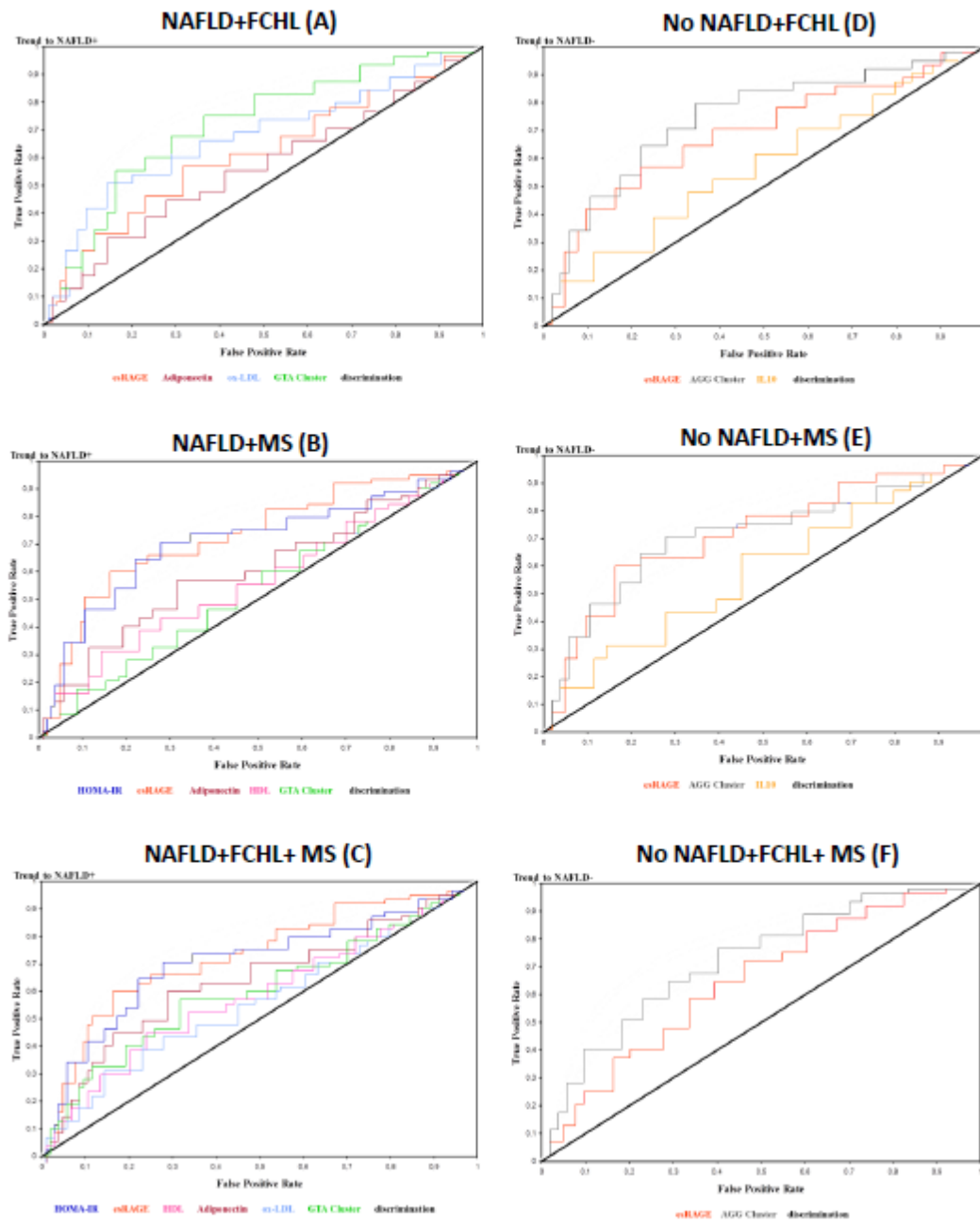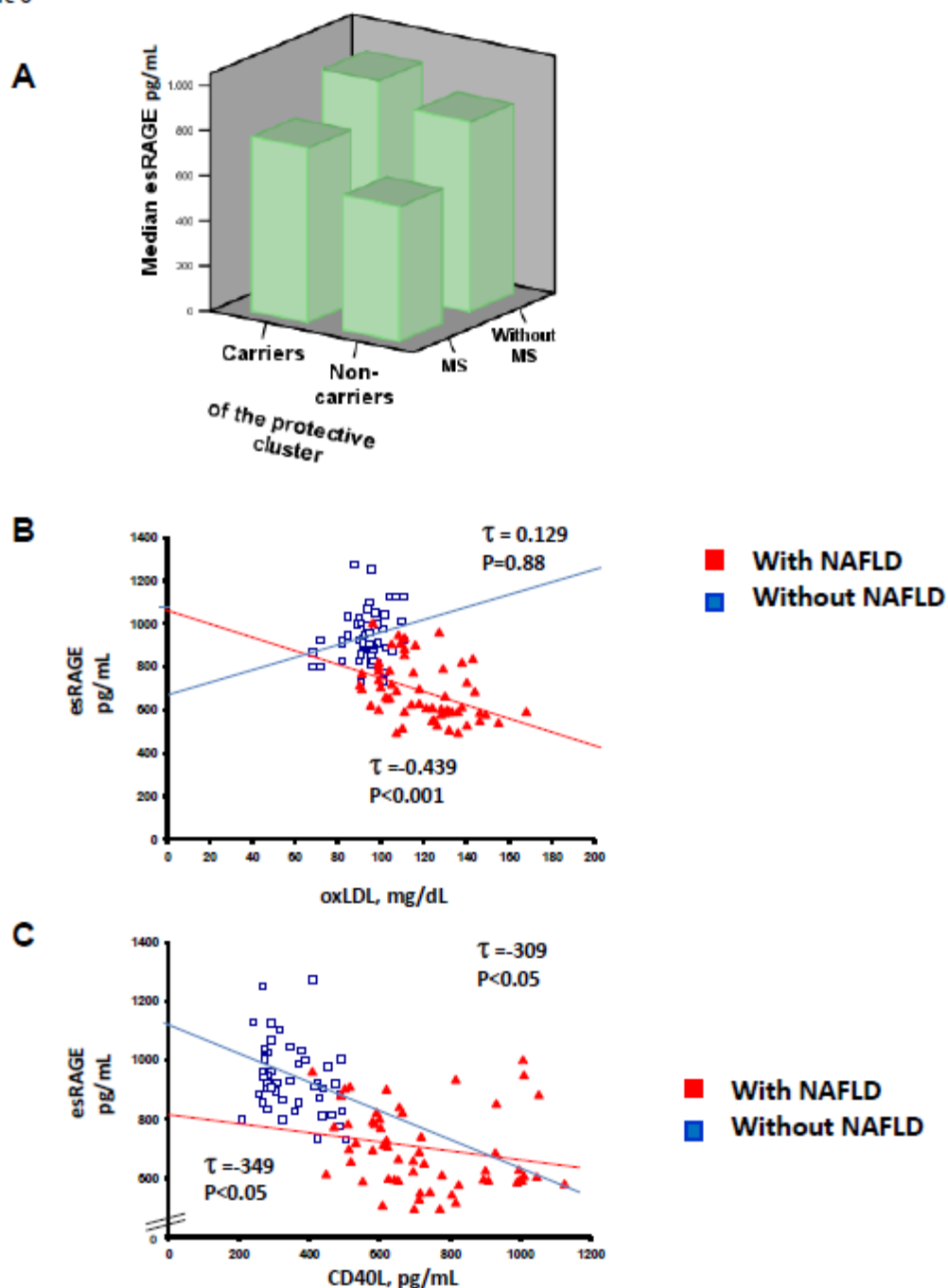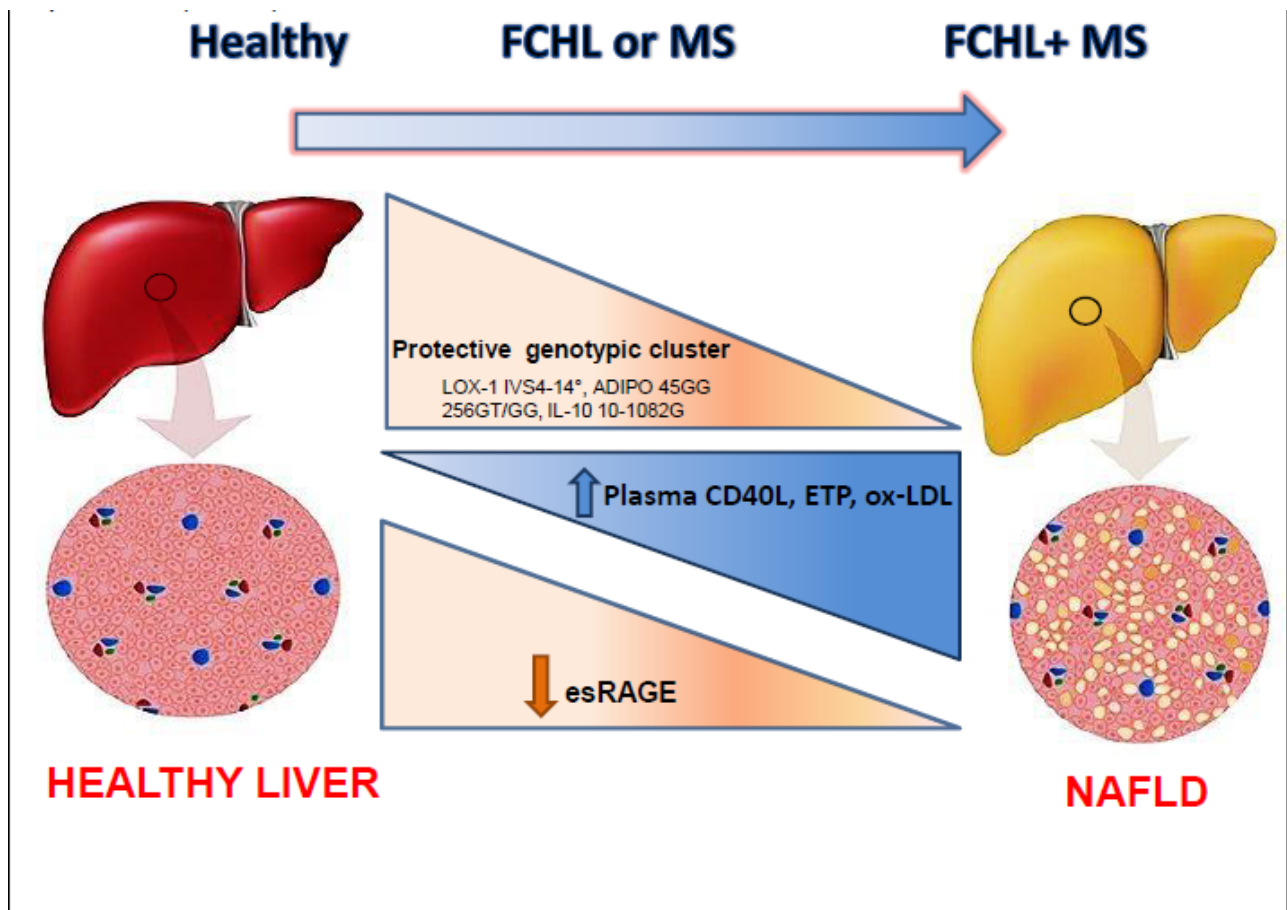
Figure 1

Figure 2

Figure 3

Graphical abstract