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XXVI Ciclo

Studies on fatty liver disease, fetuin and cardiovascular disease

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ABSTRACT

Background 1) In Intrahepatic cholestasis of pregnancy (ICP) genetic alterations in the ATP-binding cassette subfamily B member 4 (ABCB4) and ATP-binding cassette subfamily B member 11 (ABCB11) have been associated to the onset of ICP in predisposed women and UDCA has been proposed as therapeutic target. However no many data are available on the correlation between genetic alterations and phenotypic manifestation of ICP.

2) Recently several lines of evidence indicate the importance of both quantitative and qualitative (saturated *vs* unsaturated) changes in dietary fatty acids (FAs) as relevant players in association with bile acids (BAs) in the pathogenesis of NAFLD. In addition a role of bile acids as regulators of hepatic and extrahepatic lipid metabolism has been clearly shown. Therefore beyond the acknowledged impact on cholesterol secretion and degradation it is now clear that bile acids may regulates the expression of different kind of genes involved in glucose and lipid metabolism and interact with the dietary composition of Fatty Acids. In addition they may represent interesting candidates for the treatment of several chronic liver and metabolic diseases like Non-alcoholic steatohepatitis (NASH) and diabetes.

3) Non-alcoholic fatty liver disease (NAFLD) is deemed to be an independent risk factor for cardiovascular disease (CVD) which is the leading cause of death in these individuals. Fetuin-A inhibits arterial calcification, induces insulin resistance and is increased in NAFLD. Although the pathogenic mechanisms linking NAFLD with CVD are incompletely understood, the role played by fetuin-A has gained increasing interest.

Aims The aims of my research projects are:

- first shear light on some of the controversy and uncertainty still accompanying knowledge on the regulatory networks linking bile acids and metabolism of cholesterol and triglycerides. This has been done also by using new bile acid molecules that might be potential targets for pharmacological prevention and/or treatment of NAFLD and possibly of atherosclerosis.
- second evaluate if NAFLD, CVD and fetuin-A are different in patients with CVD of 4 different anatomical sites: carotid arteries, lower extremities, abdominal/ thoracic aorta and coronary arteries (CAD+).

Methods For the study of cholestasis in pregnancy: DNA of 33 unrelated Italian women with obstetric cholestasis were screened by automated sequencing for mutations in the entire coding sequence of ABCB4 and ABCB11 genes.

For the study of bile acids effects on lipid metabolism: an *in vitro* model of steatosis, induced in HepG2 cells by different FAs (palmitic and oleic), was used also to test the effect of a novel bile acid (6-ECDCA) on lipid storage, cellular toxicity and gene expression.

Finally the third topic was studied by evaluating the relationship between NAFLD (ultrasound), CVD (standard techniques) and anthropometric, metabolic indices and fetuin-A concentration (ELISA).

Results Genotyping of ICP patient's revealed 11 mutations, 5 of whom were novel variants and a correlation between genotype and phenotype was recorded.

The evaluation of the effect of 6-ECDCA in our steatosis model showed an increase in hepatocyte steatosis after incubation with palmitic acid, a decrease in the toxicity induced by palmitic acid, an inhibition of the induction of Acetyl-CoA carboxylase 2 (ACACB) promoted by palmitic acid and an induction of fatty acid oxidation through CPT1A expression.

The prevalence of NAFLD in arteriopatic patients was 53.80%, a percentage higher than that of the general population. Patients with peripheral arterial disease had the greatest number of factors of metabolic syndrome and the lowest HDL cholesterol levels. Fetuin-A was highest in the CAD+ group and, in each group, fetuin-A was more elevated in NAFLD than in non-NAFLD.

ABCB4 AND *ABCB11* MUTATIONS IN INTRAHEPATIC CHOLESTASIS OF PREGNANCY IN AN ITALIAN POPULATION

INTRODUCTION

Bile secretion and bile salt transport systems

A major function of the liver is the transport of bile salts from blood to bile, the key mechanism for the secretion of cholesterol, insoluble in water, from the body (*Kubitz R et al. 2012*). In bile cholesterol is solubilized into mixed micelles containing large amounts of bile acids (BAs) and phospholipids (*Kubitz R et al. 2012*). BAs are normally stored in the gallbladder and cycle between the intestine and the liver via the enterohepatic circulation (EHC). Hepatic conjugation of bile acids with glycine or taurine increase their polarity and therefore their solubility. These modifications make BAs less transportable in a passive way, thus requiring the use of specific transport systems like membrane transporters for cellular uptake and secretion, which occur at opposite poles of the cells. The EHC represents an important circuit between liver and intestine, and is needed for normal physiologic function (*Zwicker BL et al. 2013*) (Fig. 1).

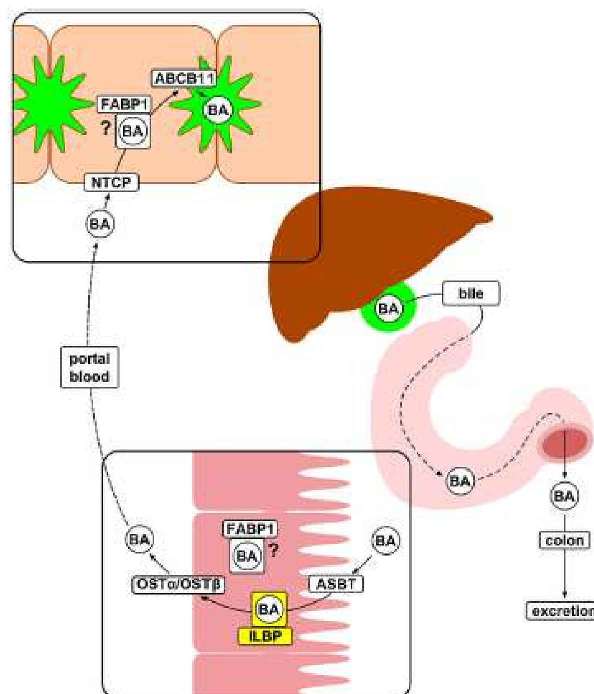


Figure 1. Transport of bile acids within the enterohepatic circulation and excretion out of the body. Bile acids (BAs) are synthesized by the liver and released into the lumen of the small intestine via bile. The majority of the conjugated BAs are recovered in the distal portion of the small intestine via an active process involving the apical sodium dependent bile acid transporter (ASBT). Unabsorbed BAs travel to the colon where they are further modified by gut bacteria. BAs that are not reabsorbed by the colon are excreted out of the body. BAs are excreted out of ileal enterocytes via the heterodimeric organic solute transporter alpha-beta (OSTα/OSTβ), and carried back to the liver via portal blood. The sodium-taurocholate cotransporting polypeptide (NTCP) located on the basolateral (sinusoidal) membrane of liver hepatocytes reabsorbs the BAs from portal blood. Fatty acid binding protein 1 (FABP1) may be involved in the transport of BAs to the apical (canalicular) membrane where the product of *ABCB11* gene, named BSEP (Bile Salt Export Pump), or sPgp (sister of P-glycoprotein), facilitates the secretion of newly synthesized and recovered BAs to bile. *Image from: Zwicker BL, Agellon LB. "Transport and biological activities of bile acids." Int J Biochem Cell Biol. 2013 Jul;45(7):1389-98.*

As shown in figures 1 and 2 effective transport systems have evolved to ensure bile acid excretion, these include the bile salt export pump (BSEP, gene symbol *ABCB11*) at the canalicular (apical) membrane of hepatocytes as well as the sodium-taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptides (OATPs) which mediate uptake of bile salts from blood into hepatocytes. Secretion of bile acids from hepatocytes back into blood may be achieved by the organic solute transporter alpha and beta (OSTα/OSTβ) in the small intestine and in the liver by the multidrug resistance associated protein 4 (MRP4) which functions as a bile acid efflux pump at the sinusoidal membrane of human hepatocytes. At the canalicular membrane, the multidrug resistance protein 3 (MDR3, gene symbol *ABCB4*) "flops" phosphatidylcholine into bile. MDR3 forms a functional unit together with the heterodimeric cholesterol transporter ABCG5/8 and BSEP, because their substrate (phosphatidylcholine, cholesterol, bile acids) together constitute mixed micelles (*Kubitz R et al. 2012*) (Fig. 2).

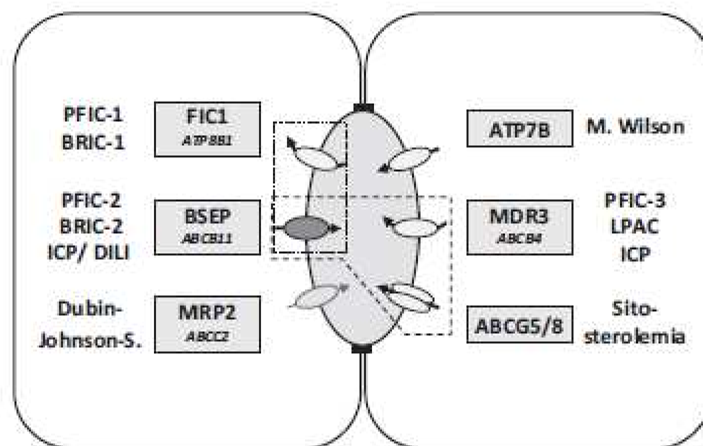


Figure 2. Canalicular transporter proteins and associated liver diseases. *Image from: Kubitz R et al. "The bile salt export pump (BSEP) in health and disease." Clin Res Hepatol Gastroenterol. 2012 Dec;36(6):536-53.*

The bile salt export pump BSEP (*ABCB11*) belongs to the MDR/TAP subfamily of ATP binding cassette transporter (ABC transporter) together with P-glycoprotein (P-gp/or MDR1, gene: *ABCB1*) and MDR3 (*ABCB4*) (*Kubitz R et al. 2012*). Every ABC transporter consists of two transmembrane domains (TMDs) coupled with two nucleotide binding domains (NBDs); the two TMDs dimerize to form the substrate translocation pathway, while the NBDs control the conformation of the TMDs through ATP-induced dimerization and hydrolysis-induced separation (*Procko E et al. 2009*).

BSEP is a high affinity liver-specific transporter responsible for the bile salt-dependent bile flow transporting mainly monovalent bile acids (*Kubitz R et al. 2012*). These proteins transport biliary constituent against steep concentration gradients across the canalicular membrane coupling the energy of ATP binding and hydrolysis to substrate transport (*Procko E et al. 2009*).

Due to the crucial localization of BSEP and others ABC transporters, and the multiple effects of bile acids, alteration of these transporters may directly or indirectly affect processes such as liver regeneration, glucose homeostasis, energy expenditure, atherosclerosis or carcinogenesis (*Kubitz R et al. 2012*). Elevated concentration of hydrophobic bile acids may in addition be toxic to the liver due to their detergent properties and may activate proapoptotic pathways. All these effects may explain why these molecules play a central role in many liver diseases (*Kubitz R et al. 2012*).

Cholestatic disorders

Cholestatic liver diseases are caused by a range of hepatobiliary insults and involve complex interactions among environmental and genetic factors (*Hirschfield GM et al. 2013*) (Fig. 3). Little is known about the pathogenic mechanisms of specific cholestatic diseases, which has limited the ability to manage patients with these disorders. Recently, genome-wide studies (GWAS) have provided insight into the pathogenesis of several cholestatic liver disorders like gallstones disease, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Moreover genetic variants that alter the expression and/or function of bile acid transporters that affect the composition of bile have been associated with cholestasis (Hirschfield GM et al. 2013).

Several ABC transporters such as BSEP, MDR3 and ATP8B1 are genetically mutated in some familial cholestatic syndromes, which can be defined as acquired forms of cholestasis. Two variants of these genes, encoding transporters that affect the secretion of bile acids and phospholipds, have been associated with cholestasis, namely *ABCB11*, which encodes the bile salt export pump (BSEP), and *ABCB4*, which encodes hepatocanalicular phosphatidylcholine flippase (MDR3) (*Hirschfield GM et al. 2013*).

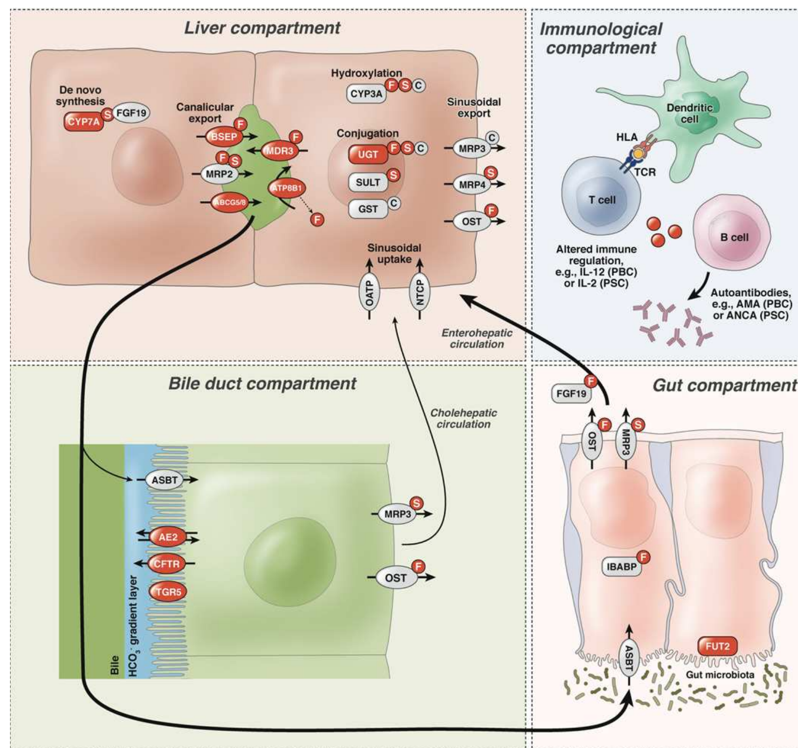


Figure 3. Modeling of genetic influence in cholestatic liver diseases. The liver compartment comprises genes involved in hereditary cholestatic syndromes and gallstone disease (highlighted in red). The same loci and loci in the biliary compartment may also serve as modifiers in immunemediated injury, as exemplified by the immunologic compartment. Susceptibility to cholestatic liver disease has also recently been shown to be associated with genetic factors influencing microbial community composition, as illustrated by the gut compartment that also involves the enterohepatic circulation of bile acids. *Image from: Hirschfield GM, et al. "The genetics of complex cholestatic disorders." Gastroenterology. 2013 Jun;144(7):1357-74.*

Intrahepatic cholestasis of pregnancy (ICP)

Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a liver disease which can occur in the third trimester of pregnancy. It is associated with raised serum bile acids, that resolves

completely after delivery, and increased rates of adverse fetal outcomes, (*Glantz A et al. 2004*). Pruritus is the main clinical feature of this condition. Maternal prognosis is usually benign, with a subsequent normalization of serum liver tests. However the increase of serum bile acids and, probably, of their toxic metabolites, have been suggested to be responsible for foetal complications, mainly foetal distress, intrauterine death and preterm delivery (*Glantz A et al. 2004*).

The aetiology of ICP is not completely understood, but there is evidence that environmental, hormonal and genetic factors play a role in its development. Familial clustering, the presence of ethnic and geographic variations and, more recently, the observation that mutations in genes coding for hepatobiliary transport proteins are involved in the aetiology of multiple form of cholestatic syndromes including PFIC (progressive familial intrahepatic cholestasis that is associated, in its three different variants, with altered *ATP8B1*, *ABCB11* or *ABCB4*) and BRIC (benign recurrent intrahepatic cholestasis that is associated, in its two variants, with altered *ATP8B1* and *ABCB11*) suggest a genetic basis in ICP (*Pauli-Magnus C et al. 2004; Wasmuth HE et al. 2007; Dixon PH et al. 2009; Painter JN et al. 2005*).

Evidence for the genetic predisposition to ICP have come from studies of a cholestatic disorder such as the Progressive Familial Intrahepatic Cholestasis (PFIC) and Benign Recurrent Intrahepatic Cholestasis (BRIC) syndromes, characterized by a defect in bile secretion caused by homozygous mutations in ATP-binding-cassette proteins. Case reports have described ICP in mothers of affected children that were heterozygous for mutations of *ABCB4* and *ABCB11* genes (*Pauli-Magnus C et al. 2004; Wasmuth HE et al. 2007; Dixon PH et al. 2009*).

A few ‘‘common’’ BSEP mutations (including p.E297G, p.D482G and p.N591S) have been detected in ICP-patients in heterozygous form and account for about 1.4% (7/491) of ICP-patients (*Dixon PH et al. 2009*). Apart from rare mutations, the common BSEP polymorphism p.V444A (c.1331T > C, rs2287622, valine to alanine at position 444) has been linked to ICP. The C-allele is more frequent in ICP-patients as compared to healthy pregnant women (*Pauli-Magnus C et al. 2004; Dixon PH et al. 2009; Kubitz R et al. 2012*).

The phospholipidflopppase MDR3 (gene symbol: *ABCB4*) is expressed in the canalicular membrane of hepatocytes and mediates the biliary excretion of phosphatidylcholine, which is required for the formation of mixed micelles in bile. Several mutations of *ABCB4* have been identified, which cause cholestatic liver diseases of varying severity including progressive familial intrahepatic cholestasis type 3 (PFIC-3), intrahepatic cholestasis of pregnancy (ICP) and the low phospholipid associated cholelithiasis syndrome (LPAC) (*Kubitz R et al. 2011*).

AIMS OF THE STUDY

Many studies have investigated the role of ABCB4 mutations in patients with ICP but the contribution of ABCB11 mutations is still not completely understood; moreover, most studies have analysed ABCB11 and ABCB4 mutations in specific population such as Swedish, Finnish, French, German and British European women affected by ICP. In addition studies in Italian patients are still few and limited to the study of the contribution of ABCB4 mutations in the development of ICP (*Anzivino C et al. 2013*). Therefore, the aim of this study is to clarify the genetic contribution of ABCB4 and ABCB11 in the development of ICP in a cohort of Italian patients, and to investigate the possible relationship between genotype and phenotype.

MATERIALS AND METHODS

Patients and controls

Women with ICP were prospectively enrolled at the Mother-Infant Department, University of Modena and Reggio Emilia, University Hospital Policlinico-Modena. In the years 2006–2009, 33 unrelated women with ICP were available to participate in the present study.

The diagnosis of cholestasis of pregnancy was based on the following criteria: (I) a history of generalized pruritus, with or without jaundice, developed in the second or third trimester of pregnancy, in the absence of any dermatologic or other systemic medical conditions causing pruritus, (II) an increase in laboratory indices of cholestasis: serum bile acids equal or exceeding 16 μmol/L and/or serum alanine and aspartate aminotransferase >31 U/L, (III) spontaneous resolution of clinical symptoms and normalization of laboratory indices after delivery, (IV) exclusion of other aetiology of liver disease (mainly viral infections and metabolic diseases) (*Anzivino C et al. 2013*). Biochemicals parameters were recorded for each ICP patients with particular regard to: serum transaminase (AST, ALT), bile acids, bilirubin and GGT (Table 1).

100 control subjects, who experienced physiological pregnancies, were enrolled and screened for the new genetic variants. They were interviewed about age, parity, number of eventual abortions and their possible cause, and geographical origin. After being informed about the aims and design of the study, all the patients and controls gave their written informed consent to participate into the study.

Venous blood samples were drowed and genetic variants were screened for the genes *ABCB4* and *ABCB11* by the mean of a direct sequencing technique. The study protocol was performed according to the ethical guidelines of the Declaration of Helsinki in its latest version and has been approved by the local Ethical Committee.

Clinical details of the 33 patients enrolled in the study

Parameters	Normal range	Patients[a]
Mean age (years)		33.5 ± 3.9
Parity		
1		9 (27)
2 or more		24 (72)
Mean ALT (U/L)	(2-31 U/L)	244.5 ± 189.9
Mean AST (U/L)	(2-31 U/L)	127.3 ± 86
Median total bilirubin (mg/dL)	(0.2-1.2 mg/dL)	0.59 (0.36-0.81)
Mean total serum bile acids (μmol/L)	(0-16 μmol/L)	64.5 ± 52.1
Mean GGT (U/L)	(7-35 U/L)	18.9 ± 10.3
Onset of pruritus		
2nd trimester		13 (39)
3rd trimester		20 (61)
Operative delivery (induction of labour and/or caesarean section)[b]		22 (66)

Table 1. Clinical details of the 33 patients enrolled in the study.
AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-gluatmil transpeptidase.
[a] Data indicate, whenever appropriate, mean value ± SD or absolute number of patients (%). For bilirubon levels, the median value and percentiles (25-75) are shown.
[b] Operative deliveries due to complications related to ICP (foetal distress and/or intolerable pruritus and/or persistent elevation of AST and ALT). *From Anzivino C et al "ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population." Dig Liver Dis. 2013 Mar;45(3):226-32.*

DNA extraction, amplification and sequence analysis of *ABCB4* and *ABCB11* exons

Ten ml of venous blood were collected in each patient. Genomic DNA was extracted from 200 µl of peripheral blood using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The DNA concentration was quantified by spectrophotometry (Beckman DU 650).

Polymerase chain reaction (PCR) was performed in order to amplify the 28 exons of *ABCB4* and *ABCB11* genes by intronic primers designed to span all exons and at least 100 bp of flanking intronic sequence at the 5' and 3' end of each exon, the primers sequences were kindly granted by Pauli-Magnus et al. (*Pauli Magnus C et al. 2004*). Genomic and cDNA sequences were derived from known sequences (*ABCB4*: GenBank accession number AC005068.2 for noncoding exons -3 to 1 and coding exon 2 and 3, AC006154 for exons 4-12, AC0005045 for exons 13-28 and NM_000443 for cDNA; *ABCB11*: GenBank accession number AC008177 for promoter and exon 1-21, AC069165 for exon 22-28 and NM_003742 for cDNA). 100ng of DNA were amplified by the Expand High Fidelity PCR system kit (Roche) in a final volume of 50 µl containing: 5 µl of 10X PCR buffer (20mm Tris-HCl pH 7.5, 100 mM KCl, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol), 0.4 µM of specific primer (MWG-Biotech), 200 µM of dNTPs mix, 1 U of High Fidelity Taq DNA polymerase, and sterile water to reach the final volume of 50 µl. The reaction was processed using a Thermocycler GeneAmp PCR System 2007 (Applied Biosystems) through the following steps: initial denaturation at 94°C for 2 minutes, followed by 34 cycles of denaturation at 94°C for 45 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 45 seconds, concluding with a final extension at 72°C for 7 minutes.

The PCR products were separated by electrophoresis on 2% agarose gel and then purified with the High Pure PCR Product Purification Kit (Roche), according to the manufacturer's instructions.

Two sequencing reactions were performed for each exon in order to amplify both strands each in a final volume of 20 µl containing: 3 µl of DNA purified, 1 µl of specific primers (MWG-Biotech), 2 µl of Big-Dye terminator cycle sequencing kit (Applied Biosystems) and sterile water to reach a final volume of 20 µl. The reaction was processed using a Thermocycler GeneAmp PCR System 2007 (Applied Biosystems) through an initial denaturation at 96° C for 10 seconds and 30 cycles of denaturation at 96° C for 10 seconds, annealing 50° C for 5 seconds, extension at 60° C f or 4 minutes. The products of reaction were purified by precipitation with NaAcetate 3 M and ethanol. The pellet was dissolved in 13 µl of formamide and was then loaded into the ABI PRISM ® 3130 Genetic Analyzer. The comparison of the sequence of these bases with the control sequences identifies nucleotide variations, both in homozygosity and in heterozygosity. The sequences were analysed with Chromas Lite software.

Prediction of functional consequence of non-synonimous variants

Potential consequences of non-synonymous variants in *ABCB4* and *ABCB11* genes were predicted by the mean of the computational method PolyPhen-2 (Polymorphism Phenotyping; http://genetics.bwh.harvard.edu/pph2), which estimates the likelihood of a particular non-synonymous coding SNP to cause a functional impact on the protein, based on an alignment of physiochemical and evolutionary properties of related proteins. The PolyPhen-2 algorithm is reported as PSIC (position-specific independent count) score, where scores below 1.5 design a "benign substitution"; scores between 1.5 and 2 and scores above 2 define a "possibly damaging" and "probably damaging" substitution, respectively.

RESULTS

The DNA sequence analysis was performed on the 27 coding exons of the *ABCB4* and *ABCB11* genes and their respective exon-intron boundaries in 33 pregnant women with ICP. DNA of the control subjects was screened only for new mutations and to test frequency of the single nucleotide polymorphisms (SNPs) found. Sequence analysis of *ABCB4* and *ABCB11* genes revealed the presence of mutations in 10 out of 33 patients (33%); no mutations were found in any of the 100 control subject screened.

Analysis of *ABCB4* gene

After sequence analysis, five variants were identified in the coding region of *ABCB4*: a substitution affecting a splice site (p.1738LfsX744) and a frameshift mutation (p.1587DfsX603) that were never found in other studies and were absent in the control subjects, so were novel mutations.

Three non-synonymous changes (p.L73V, p.T175A and p.N510S) were previously described in literature (Pauli-Magnus C et al. 2004; Tomaiuolo R et al. 2009). All the variants were found in a single chromosome; their location and PSIC scores in the study population are shown in table 2.

ABCB4 mutations				
Patients no.	Nucleotide change and effect on protein	Location	PSIC scores by PolyPhen-2 analysis	Refs
1	c.217 C>G (p.L73V)	Exon 4	0.489	[4]
2	c.523 A>G (p.T175A)	Exon 6	0.774	[4]
3	c.1529 A>G (p.N510S)	Exon 13	**2.075**	[11]
4	**c.1758_1759 ins G (p.1587DfsX603)**	Exon 15	***X***	[3]
5	**c.2211(+1) G>T (p.1738LfsX744)**	5' Intron 17	***X***	[3]
ABCB11 mutations				
6	c.403 G>A (p.E135K)	Exon 6	0.502	[12]
7	**c.852 T>A (p.V284D)**	Exon 9	**2.175**	[3]
8	c.1445 A>G (p.D482G)	Exon 14	1.364	[12-14]
9	**c.1674 G>C (p.Q558H)**	Exon 15	1.383	[3]
10	c.2093 G>A (p.R698H)	Exon 18	0.821	[4,15]
11	**c.2191 C>T (p.P731S)**	Exon 19	0.851	[3]

Table 2. *ABCB4* and *ABCB11* mutations identified in 11 patients and PSIC scores by PolyPhen-2 algorithm. New mutations are shown in bold. X indicates mutations that predict a premature protein truncation. PSIC scores < 1.5 define benigne substitutions; PSIC scores > 2.0 (bold) define probably damaging substitutions. *From Anzivino C et al "ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population." Dig Liver Dis. 2013 Mar;45(3):226-32.*

The first novel mutation is a heterozygous insertion of guanine between the nucleotides 1758-1759 that cause a frameshift mutation with the insertion of 17 new amino acids leading to the formation of an early stop codon (TAG) at the position 604 of the mRNA (p.1587DfsX603) (Fig. 4).

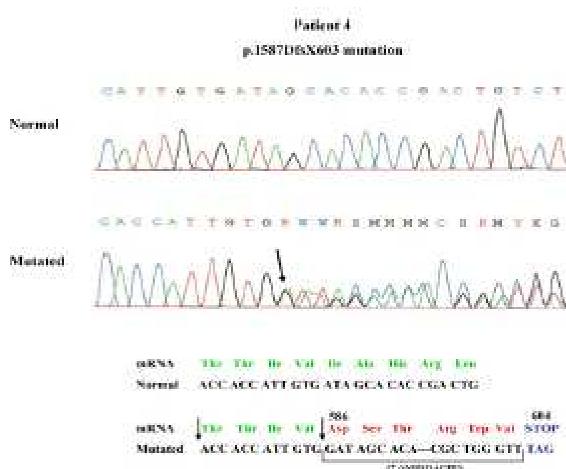


Figure 4. Electropherogram of the frameshift mutation with indication of its possible consequence on the mRNA sequence. Normal and mutated sequences are reported. A heterozygous insertion of a guanine (G) between the nucleotides 1758-1759 causes a shift of the reading frame with the insertion of 17 new amino acids (red) and the formation of an early stop codon at the position 604 of the mRNA (p.1578DfsX603) (blue). *Image from: Anzivino C et al "ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population." Dig Liver Dis. 2013 Mar;45(3):226-32.*

The second new variant (c.2211(+1) G>T) was located in the 5' of intron 17 and occurred in the splicing donor site where a guanine was substituted with a thymine (G>T). This mutation probably lead to an alteration of the correct splicing activating a cryptic splicing donor site and including 4 bases of intron 17 in the mRNA sequence that cause a frameshift with an insertion of 7 new amino acids and the formation of an early stop codon (TGA) (p.1738LfsX744) (Fig. 5).

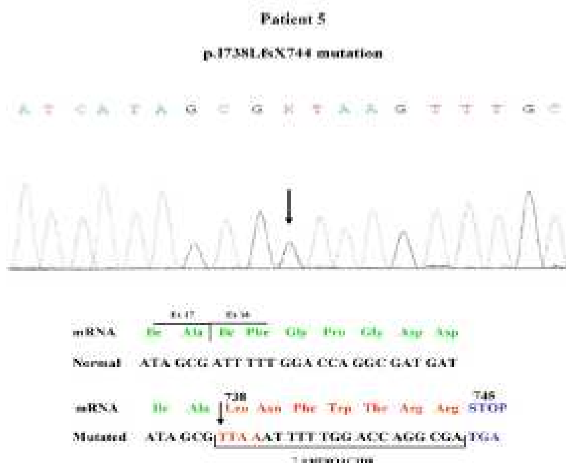


Figure 5. Electropherogram of the splicing mutation with indication of its possible consequence on the mRNA sequence. Normal and mutated sequences are reported. A heterozygous substitution of a guanine (G) with a thymine (T) alters the correct exon splicing activating a cryptic splice donor site with the inclusion of 4 bases of intron 17 in the mRNA sequence, consequently determining the shift of the coding frame with the formation of an early stop codon (TAG) (blue) located 7 amino acids (red) downstream (p.1738LfsX744). *Image from: Anzivino C et al "ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population." Dig Liver Dis. 2013 Mar;45(3):226-32.*

Sequence analysis of exon 13 showed an heterozygous substitution of adenine with a guanine (c.1529 A>G) in position 1529 of cDNA, resulting in an amino acid change at the codon 510 where an asparagines was substituted with a serine (p.N510S) (Fig. 6). The PSIC score reports that the p.N510S mutation as probably damaging for the protein function.

Clinical data of ICP patients carrying *ABCB4* mutations are shown in table 3.

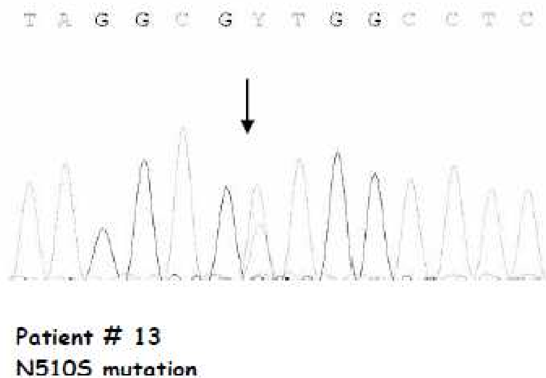


Figure 6. Electropherogram of the heterozygous base substitution in exon 13 of *ABCB4* gene.

Parameters	Patient 1 L73V	Patient 2 T175A	Patient 3 N510S	Patient 4 1587DfsX603	Patient 5 1738LfsX744
Onset of pruritus	3rd trimester	3rd trimester	3rd trimester	3rd trimester	2nd trimester
Parity	3	2	2	2	1
Previous ICP	Yes	No	Yes	Yes	Yes
Peak of AST (U/L)	82	79	133	204	43
Peak of ALT (U/L)	123	156	238	382	76
Peak of bilirubin (mg/dL)	0.14	0.81	Nd	2.8	2.07
Peak of GGT (U/L)	6	25	Nd	67	54
Total bile acids (µmol/L)	28.7	41	128	Nd	114.5
Delivery	Caesarean section ($37w^{+5}$)[a]	Caesarean section (36w)[a]	Caesarean section (39w)[a]	Caesarean section (32w)[a]	Caesarean section ($33w^{+4}$)[a]
Cholelithiasis	No	No	No	Yes	No
UDCA therapy	Yes	No	No	No	Yes

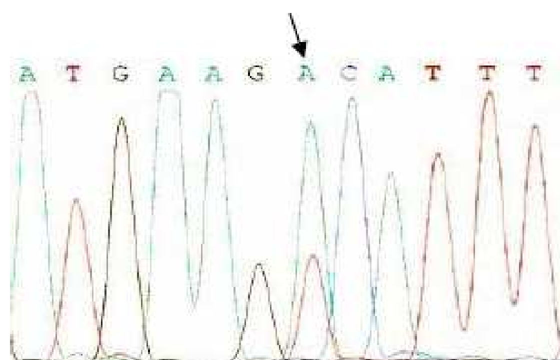
Table 3. Clinical details of patients with mutations in *ABCB4* gene.
AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-gluatmil transpeptidase; Nd: not determined.
[a] Caesarean section due to pregnancy related to ICP (foetal distress and/or intolerable pruritus and/or persistent elevation of AST and ALT).
UDCA: ursodeoxycholic acid. *From Anzivino C et al "ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population." Dig Liver Dis. 2013 Mar;45(3):226-32.*

Analysis of *ABCB11* gene

Among all the variants found in *ABCB11* gene, six were localized in the coding sequence, three (p.V284D, p.Q558H and p.P731S) were new mutations never found in other studies and were also absent in our control group.

Variants p.E135K, p.D482G and p.R698H were reported in previous studies (*Pauli-Magnus C et al.2004; Lang Tet al. 2006; Byrne JA et al. 2009; Strautnieks SS et al. 1998; Strautnieks SS et al. 2008*). All new variants were found in a single chromosome; their location and PSIC scores are shown in table 1.

The first novel variant was found in exon 9 where, a heterozygous substitution of a thymine with an adenine in position 852 of cDNA (c.852T>A), lead to an amino acid change at position 284 of BSEP protein, where a valine is replaced by aspartic acid (p.V284D) (Fig. 7). This patient experienced a severe clinical feature of ICP and that was associated with an elevated PSIC score (Table 1).



Patient # 1
V284D mutation

Figure 7. Electropherogram of the heterozygous base substitution in exon 9 of *ABCB11* gene.

The second mutation (c.1674 G>C) was a substitution in exon 15 of a guanine with a cytosine at position 1674 of cDNA, this lead, in the protein, in a substitution of a glutamine with a histidine at position 558 (p.Q558H) (Fig. 8).

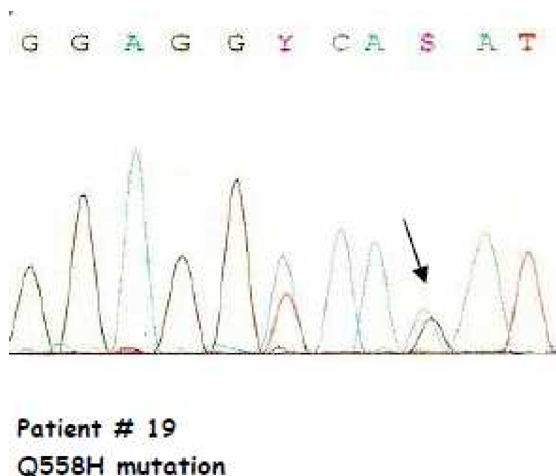


Figure 8. Electropherogram of the heterozygous base substitution in exon 15 of *ABCB11* gene.

The last new variant was identified in exon 19 and involves the exchange of a proline with a serine at position 731 (p.P731S) of the protein, this was caused by a substitution of a cytosine with a thymine at position 2191 of the cDNA (c.2191 C>T) (Fig. 9). PolyPhen-2 algorithm assigned it a PSIC score of 0.85 (Table 1), which defines a benign substitution, consistent with the mild clinical feature of the patient.

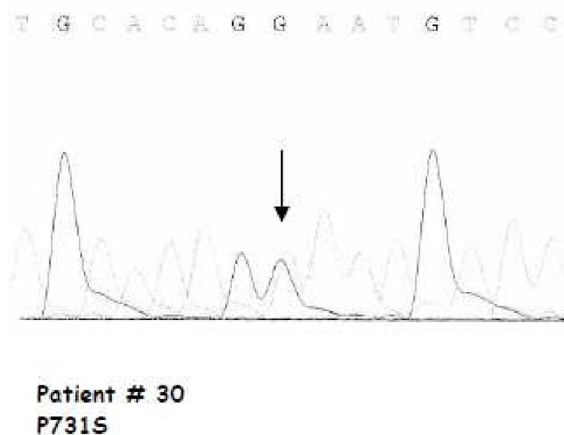


Figure 9. Electropherogram of the heterozygous base substitution in exon 19 of *ABCB11* gene.

The two mutations previously described in literature, p.E135K on exon 6 and p.D482G on exon 14, are associated with two similar phenotype (*Anzivino C et al. 2013*), and the PSIC score classifies them as benign variants (Table 1).

Finally, the variant p.R698H is referred as a polymorphism (*Pauli-Magnus C et al. 2004; Lang T et al. 2006*), however it was not detected in any of the 100 control subjects screened in the study. The PSIC score classifies it as benign (Table 1). Clinical details of ICP patients with *ABCB11* mutations are summarized in table 4.

Parameters	Patient 6 E135K	Patient 7 V248D	Patient 8 D482G	Patient 9 Q558H	Patient 10 R698H	Patient 11 P731S
Onset of pruritus	3rd trimester	2nd trimester	3rd trimester	2nd trimester	2nd trimester	3rd trimester
Parity	2	1	2	2	2	2
Previous ICP	Yes	Yes	Yes	No	Yes	Yes
Peak of AST (U/L)	24	92	29	125	244	105
Peak of ALT (U/L)	26	215	37	315	514	198
Peak of bilirubin (mg/dL)	0.53	0.49	Nd	0.36	0.3	0.46
Peak of GGT (U/L)	7	25	Nd	23	14	16
Total bile acids (µmol/L)	93.6	112.4	28	Nd	20.4	23.4
Delivery	Induction of labour ($36w^{+4}$)[a]	Caesarean section (38w)[a]	Induction of labour ($38w^{+4}$)[a]	Caesarean section (36w)[a]	Caesarean section (38w)[a]	Induction of labour (38w)
Cholelithiasis	No	No	No	No	No	No
UDCA therapy	Yes	Yes	No	Yes	Yes	Yes

Table 4. Clinical details of patients with mutations in *ABCB11* gene.
AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-gluatmil transpeptidase; Nd: not determined.
[a] Caesarean section due to pregnancy related to ICP (foetal distress and/or intolerable pruritus and/or persistent elevation of AST and ALT).
UDCA: ursodeoxycholic acid. *From Anzivino C et al "ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population." Dig Liver Dis. 2013 Mar;45(3):226-32.*

DISCUSSION

Cholestasis of pregnancy is a multifactorial disease. A possible pathogenetic role was initially attributed to the sequence variations in *ABCB4* gene (*Pauli-Magnus C et al 2004*), then the attention was extended to other genes directly involved in bile formation, such as the *ABCB11* gene (*Pauli-Magnus C et al. 2004; Wasmuth HE et al. 2007; Dixon PH et al. 2009*). Also ethnic and geographic variability seems to have a role in the aetiology of ICP but, until now, most of the studies have been performed in Swedish, French and English populations (*Pauli-Magnus C et al. 2004; Wasmuth HE et al. 2007; Dixon PH et al. 2009; Bacq Y et al. 2009*). Previous studies in Italian women with ICP (*Floreani A et al. 2008; Floreani A et al. 2006; Tavian D et al. 2009*) considered only genetic variability of the MDR3 gene, whose genetic variations have been consistently reported as causative of ICP (*Pauli-Magnus C et al. 2004; Wasmuth HE et al. 2007; Jaquemin E et al. 2001; Mullenbach R et al. 2003*).

The present study provides further evidence for a pathogenetic role of MDR3 genetic variation in a subset of Italian patients with ICP and identifies new MDR3 and BSEP mutations possibly responsible for ICP and its clinical expression. Clearly the causal relationship needs to be confirmed by functional studies.

Sequence analysis of both genes revealed the presence of 11 possibly disease-causing mutations in 11 out of 33 (33%) ICP patients; the same variants were screened and resulted absent in the control population.

Nine variant sites were single nucleotide substitutions, one was a frameshift and one was a splice-site mutation. All the variants identified in both genes were detected in the heterozygous state confirming previous published data (*Pauli-Magnus C et al. 2004, Dixon PH et al. 2009, Floreani A et al. 2008; Tavian D et al. 2009*).

As regards to *ABCB4* gene, 5 mutations were found, two of them were not simple amino acid changes but represent a frameshift and a splicing mutations (*Anzivino C et al. 2013*). The frameshift p.1587DfsX603

results in the introduction of an early stop codon and predicts a truncated protein of 603 amino acids instead of 1286. This mutation is localized in the cytoplasmatic region of MDR3 that represents the ATP binding site. This mutation might cause the production of a dysfunctional protein or alters its insertion into the membrane; this is line with the severe phenotype of the patient (table 3 – patient 4). Moreover the screening of other members of this patient's family identified a dominant mode of inheritance of this mutation with female restricted expression.

The second new variant on *ABCB4* is a splicing mutation (p.1738LfsX744) which probably creates a truncated protein of 744 amino acids. Considering the severity of patient phenotype it could be speculate that the defect on this allele might cause a dysfunction in MDR3 protein.

The missense mutation p.N510S is associated with the worst phenotype that could be justified by its localization; in fact the amino acid substitution affect the first nucleotide binding domain suggesting that it could determine a marked impairment in protein function.

On the other hand all the mutations found in *ABCB11* were single amino acid changes; and, among them, three were novel mutations (*Anzivino C et al. 2013*). According to PSIC scores, given by PolyPhen-2 algorithm, variant p.P731S, classified as a benign mutation, is associated with a mild cholestatic phenotype, whereas the p.Q558H and p.V284D were classified as probably damaging mutation and are associated with more severe clinical manifestations of cholestasis.

The p.V284D mutation determines a substitution of a valine (a neutral polar amino acid) with an aspartic acid (an acid amino acid); the different nature of amino acid and its localization in the intracellular loop between transmembrane domain 4 and 5 might lead to an altered protein conformation and/or function. Other studies identified other substitutions in the same position, thus demonstrating the importance of the conservation of this valine in position 284 (*Pauli-Magnus C et al. 2004; Lang T et al. 2006*).

The p.Q558H missense mutation is localized in the first nucleotide binding domain (NBD1) of the ABC transporter and the substitution involves amino acids with different chemical properties, thus a structural change of the protein is possible.

In conclusion this study identified 11 causative variants possibly responsible for ICP in our populations (11/33); of these five were novel mutations in five unrelated patients, and a genotype/phenotype correlation was present in most variants. Among these mutations five are localized on *ABCB4* and six on *ABCB11* and the most severe phenotypes are associated with the *ABCB4* mutations, confirming a pathogenic involvement of MDR3 variants in this disease.

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EFFECT OF 6-ECDCA, A NEWLY SYNTHESIZED BILE ACID ON STEATOSIS EXTENT, GENE EXPRESSION AND LIPOGENESIS IN HepG2 CELL COLTURES

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and Non-alcoholic steatohepatitis (NASH)

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease that spans from simple steatosis to non-alcoholic steatohepatitis (NASH), NASH-cirrhosis and hepatocellular carcinoma (*Farrell GC et al. 2006*). Estimated prevalence of NAFLD by ultrasonography ranges from 6.3% to 33% with an average of 20% in the general population, but rises in groups of patients who have associated risk factors like obesity, type 2 diabetes mellitus, dyslipidemia and metabolic syndrome (*Chalasani N et al. 2012; Loria P et al. 2010; Nascimbeni F et al. 2013*). Primary metabolic NAFLD is now considered to be the hepatic manifestation of the metabolic syndrome, a cluster of interrelated clinical features in which insulin resistance (IR) associates with elevated fasting glycemia, dyslipidemia, hypertension and visceral obesity (*Kotronen A et al. 2008*).

The molecular basis of NAFLD development and of its progression to fibrosis have not yet been fully characterized. Recent data suggest that steatosis (the accumulation of inhert triglycerides) may represent a mechanism of protection against free fatty acids (FFAs) induced toxicity (*Yamaguchi K et al. 2007; Li Z et al. 2009*). NASH and fibrosis would result from the failure of this protection (*Arrese M. 2009*).

We hypothesized that the ability of liver cells to accumulate fatty acids into tryglicerides (therefore to induce steatosis) might be critically influenced by the hepatic lipid composition. In agreement our research group has previously shown in hepatocyte cell cultures that palmitic acid (PA – a saturated fatty acid) and oleic acid (OA – a monounsaturated fatty acid) have different effect on steatosis extent and hepatocellular damage (*Ricchi M et al. 2009*). In particular the results obtained by the study of Ricchi M et al. clearly showed that incubation of HepG2 cells with OA was associated with a greater increase of triglycerides accumulation as compared to PA (Fig. 1). However the extent of apoptosis, as evaluated by DAPI staining and caspase-3/7 activity assay, was greater (at equimolar doses) with PA incubation than with OA (Fig. 2). In addition when cells were incubated with a mixture of the two fatty acids they were partially protected from apoptosis (*Ricchi M et al. 2009*).

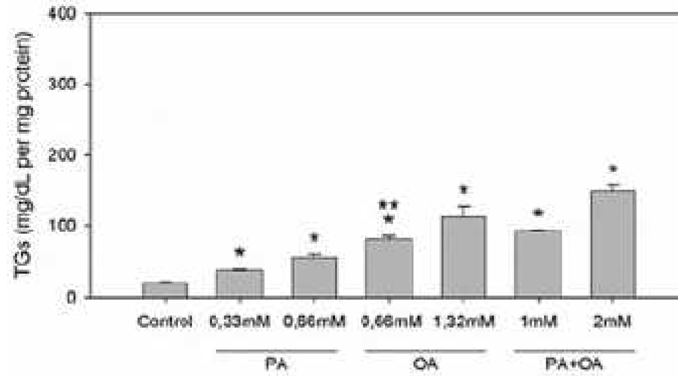

Figure 1: Effect of different fatty acids on triglycerides accumulation. HepG2 cell cultures were incubated with palmitic acid (PA) (0.33 and 0.66 mM), oleic acid (OA) (0.66 and 1.32 mM) or mixtures of the two fatty acids (1 mM or 2 mM) for 24 h. Triglyceride (TG) accumulation was evaluated as the concentration of TGs in cell lysates after NaOH lysis. Columns represent mean values ± standard error of three different experiments conducted in triplicate. *P < 0.05 vs control; **P < 0.05 vs PA 0.66 mM. *From Ricchi M, et al. J Gastroenterol Hepatol. 2009.*

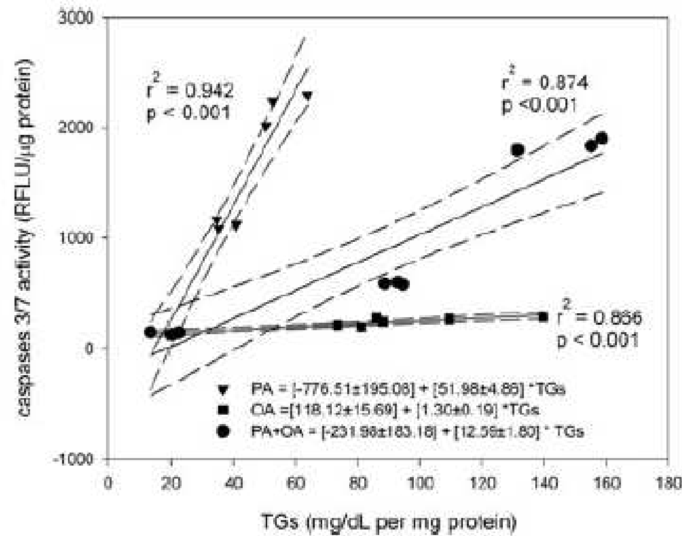

Figure 2: Relationship between extent of triglyceride (TG) accumulation and caspases 3/7 activity. Regression lines represent the relationship between the two parameters after incubation of HepG2 cells respectively with palmitic acid (PA ▼), PA + oleic acid (OA; ●) and OA (■). Single points are the mean of three experiments that have in parallel evaluated TGs content and caspases activity. The slope values of PA vs PA + OA (t = 6.805; P < 0.0001) and OA vs PA + OA (t = -5.353; P < 0.0001) regression lines are significantly different. *From Ricchi M, et al. J Gastroenterol Hepatol. 2009.*

Moreover incubation with OA induced the activation and/or the stimulation of lipogenic genes (*PPAR-γ* and *SREBP-1*) while incubation with PA was associated with greater expression of *PPAR-α* involved in enhanced β-oxidation and oxidative stress (*Ricchi M et al. 2009*).

These data suggest that cellular damage in NASH might be regulated by lipid partitioning. Saturated fatty acids, as PA, which are not able to be incorporated into tryglicerides in lipid droplets are more damaging than monounsaturated fatty acids as OA (Fig. 3).

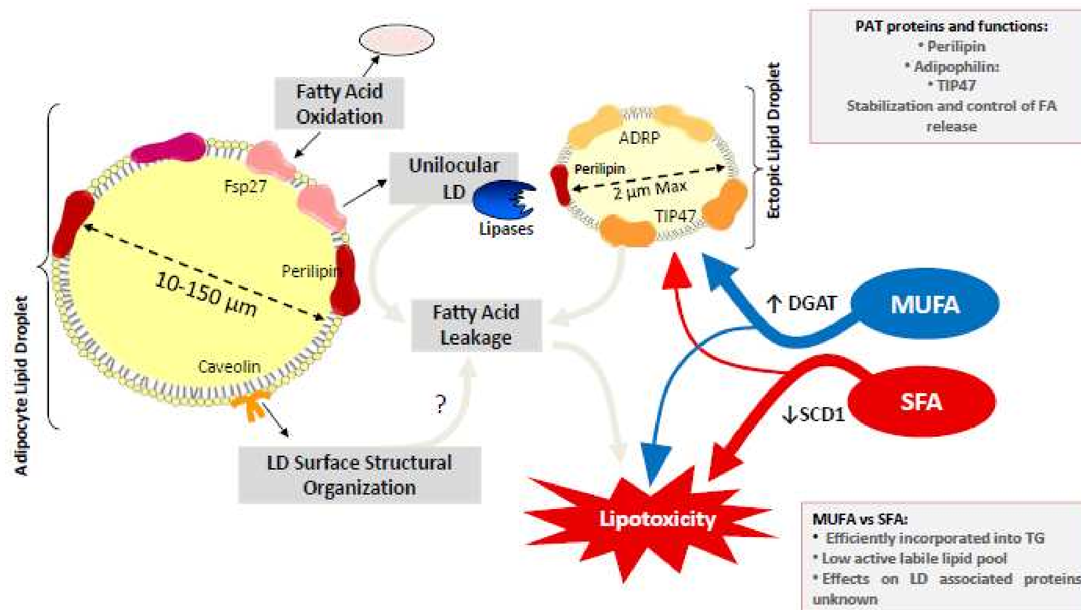

Figure 3: Schematic representation of the molecular mechanism underlying the greater damaging effect of saturated fatty acids (SFAs) as compared to monounsaturated fatty acids (MUFAs). MUFAs are readily incorporated into TGs in lipid droplets and without any increase in free fatty acids while SFAs, wich are not incorporated into TGs, might induce lipotoxicity and liver damage due to the increased proportion of free fatty acids. *Modified from: Le Lay S, Dugail I. Prog Lipid Res. 2009;48:191-5.*

FXR mediated BAs regulation of gene expression

The bile acid receptor (BAR), also known as farnesoid X receptor (*FXR*) or *NR1H4* (nuclear receptor subfamily 1, group H, member 4) is a nuclear receptor that is encoded by the *NR1H4* gene in humans and expressed at high levels in the liver and intestine. Chenodeoxycholic acid (CDCA) and other Bas are natural ligands for *FXR*. Similar to other nuclear receptors, when activated, *FXR* translocates to the cell nucleus, forms an heterodimer with *RXR* and binds to its response elements on DNA up- or down-regulating the expression of its target genes. *FXR* indirectly modulate the synthesis of cholesterol by inhibiting cholesterol 7alpha-hydroxylase (*CYP7A1* - the rate-limiting enzyme in bile acid synthesis from cholesterol) through the induction of the small heterodimer partner (*SHP*) which, in turn, binds and represses the promoter of *CYP7A1*. In this way a negative feedback pathway is established in which synthesis of bile acids is inhibited when cellular levels are high (*Matsubara T et al. 2012; Seok S et al. 2013*). Moreover *FXR* regulates, directly or through *SHP* induction, a wide variety of target genes critically involved in the control of BAs, lipid and glucose homeostasis, and in the regulation of immune responses (Table 1) (*Adorini L et al. 2012*).

Gene function	Upregulated	Downregulated
Regulation of bile acid synthesis, transport and metabolism	*BAAT* *BACS* *BSEP* *CYP3A4* *FGF15/19* *IBABP* *MDR2* *MDR3* *MRP2* *OATP8* *OST-α, β* *SHP* *SULT2A1* *UGT2B4*	*ASBT* *CYP7A1* *CYP8B1* *LRH-1* *NTCP* *OAT2* *UTG2B7*
Regulation of lipid metabolism	*ApoC-I* *ApoC-II* *ApoC-IV* *ApoE* *C3* *FAS* *Insig-2* *PLTP* *PDK4* *PPAR-α* *SRB-1* *Syndecan-1* *VLDLR*	*ANGPTL3* *ApoA-I* *ApoC-III* *HL* *HNF4A* *MTP* *Paraoxonase 1* *SREPB-1c*
Regulation of glucose metabolism	*AKR1B7* *GLUT4* *GSK3* *PEPCK*	*FBP-1* *G6Pase* *PEPCK*
Inhibition of inflammatory responses		*NF-κB*
Regulation of coagulation	*Fibrinogen α, β, γ* *Kininogen*	
Vascular remodeling	*DDAH-1* *eNOS* *ICAM-1* *VCAM-1*	*Endothelin-1*
Antibacterial activity	*CAMP* *CAR12* *IL-18* *iNOS*	

Table 1: Genes transcriptionally regulated by FXR or FXR-SHP. FXR regulates, directly of via small heterodimer partner (SHP), a wide variety of target genes by binding to FXR response elementseither as a monomer or as a heterodimer with RXR. *From Adorini L et al. Drug Discov Today. 2012.*

Liver triglycerides (TGs) production is regulated by the fatty acid synthesis rate, which results from the combination of the transcriptional activity of *PPARα* (*NR1C1* - which stimulates fatty acid β-oxidation) and *SREBP-1c* (which controls fatty acid synthesis). SREBPs, and in particular *SREBP*-1, are basic helix-loop-helix leucine zipper transcription factors, that control the expression of genes involved in lipogenesis, such as acetyl-CoA carboxylase (*ACACB*), fatty acid synthase (*FASN*), acetyl-CoA synthetase (*AceCS*), glycerol-3-phosphate acyltransferase and stearoyl-CoA desaturase-1 (*SCD* - an enzyme involved in the synthesis of monounsaturated fatty acids MUFAs).

Therefore BAs, through binding to *FXR* and regulating its downstream pathway (*SHP*, *NR0B2*, *PPARα*, *ApoC-II*), may modulate TGs levels. Watanabe M and colleagues, in two different transgenic mouse models (*KK-A^y* and *ob/ob*), found that BAs (in particular cholic acid-CA administration) lower serum TGs level by targeting *SREBP1c* gene expression. They furthermore demonstrated that is the activation of *FXR* itself that, through *SHP* induction, reduces *SREBP1c* expression and this is in perfect analogy with the mechanism proposed to explain the reduction of *CYP7A1* expression by BAs, which also involves *SHP* as a mediator (*Watanabe M et al. 2004*).

Bile acids as “metabolic” regulators

Recent papers have highlighted a key role of bile acids (BAs) as regulators of metabolism. Through their ability to bind and activate tissue specific nuclear receptors, particularly *FXR*, BAs can regulate the expression of different genes involved in their own metabolism but also on lipid and glucose metabolism and therefore in the control of energetic metabolic state (*Lefebvre P et al. 2009*).

BAs have long been known to affect triglyceride homeostasis. In humans, bile acid-binding resins induce the production of VLDL-TGs, whereas treatment of cholesterol gallstones with chenodeoxycholic acid (CDCA) has been shown to reduce hypertriglyceridemia. The reciprocal relationship between bile acid biosynthesis and TGs production could be explained in two ways: at the transcriptional level, bile acids, which are the endogenous ligands of the farnesoid X receptor (*FXR*, *NR1H4*), activate the transcription of several genes that could modulate TGs levels, such as the atypical nuclear receptor short heterodimer partner (*SHP*, *NR0B2*), *PPARα*, and *ApoC-II*. Alternatively, at the metabolite level, a reduction in BAs biosynthesis could increase hepatic cholesterol and oxysterol levels, that in turn will influence the function of the lipogenic *SREBP-1c* by attenuating its processing and activation. This could lead to decreased TGs production (*Watanabe M et al. 2004*) (Fig. 4).

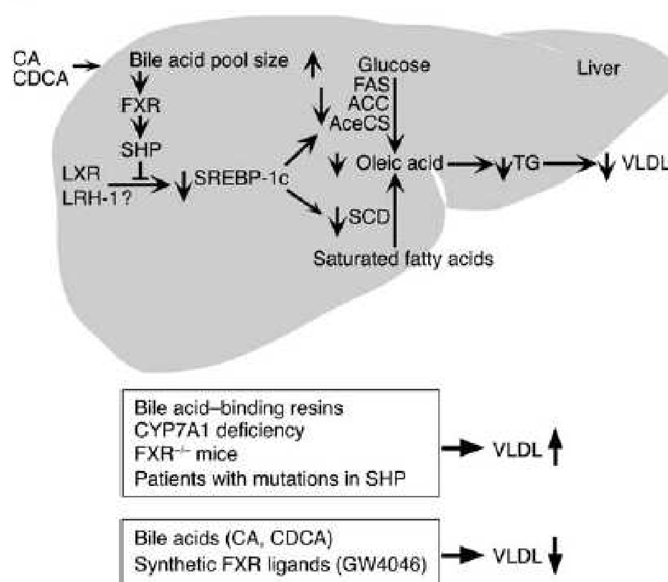

Figure 4: A FXR-SHP-SREBP-1c regulatory cascade. Schematic representation of the proposed role of SHP in mediating the effects of FXR agonists on SREBP-1c expression and lipogenesis. *From Watanabe M. et al. J Clin Invest. 2004.*

BAs have been also shown to be agonists for the G protein-coupled receptor *TGR5* (Takeda G protein-coupled receptor 5). Treatment of cells expressing this GPCR with BAs induces the production of intracellular cAMP, activation of a MAP kinase signaling pathway, and internalization of the receptor. The receptor is implicated in the suppression of macrophage functions and regulation of energy homeostasis by BAs. Another effect of this receptor is to activate deiodinases which convert the prohormone thyroxine (T4) to the active hormone triiodothyronine (T3). T3 in turn activates the thyroid hormone receptor which increases metabolic rate (*Watanabe M et al. 2006*).

BAs signaling through *FXR* and *TGR5* modulate several metabolic pathways, regulating not only BAs synthesis and enterohepatic recirculation, but also triglyceride, cholesterol, glucose and energy homeostasis.

In addition, BAs display well-documented anti-inflammatory and antifibrotic properties. Therefore, these agents represent interesting candidates for the treatment of several chronic liver and metabolic diseases, including NASH and diabetes (*Adorini L et al. 2012*).

Several preliminary studies have suggested the possible use of BAS for the treatment of metabolic diseases such as steatosis (*Okan A et al. 2002*) and diabetes (*Zhang Y et al. 2006*).

6α-ethyl-chenodeoxycholic acid (6-ECDCA, also known as Obeticholic Acid or INT 747) (Fig. 5 and 6) is a 6α-ethyl derivative of chenodeoxycholic acid (CDCA) and a first-in-class potent FXR agonist (*Adorini L et al. 2012*).

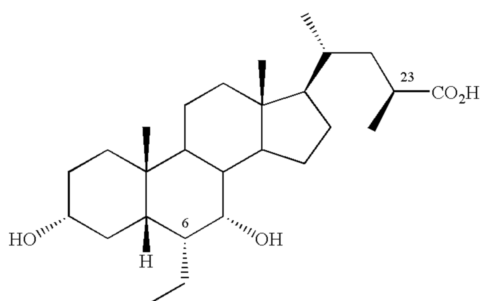

Figure 5: Chemical structure of 6-Ethylchenodeoxycholic acid (6-ECDCA). *From GuideChem.com*

This bile acid has been shown to have anticholeretic activity *in vitro* (*Li YT et al. 2007*), to inhibit vascular smooth muscle cells inflammation and migration, to promote adipocyte differentiation and regulate adipose tissue metabolism *in vivo* (*Rizzo G et al. 2006; Fiorucci S et al. 2005*).

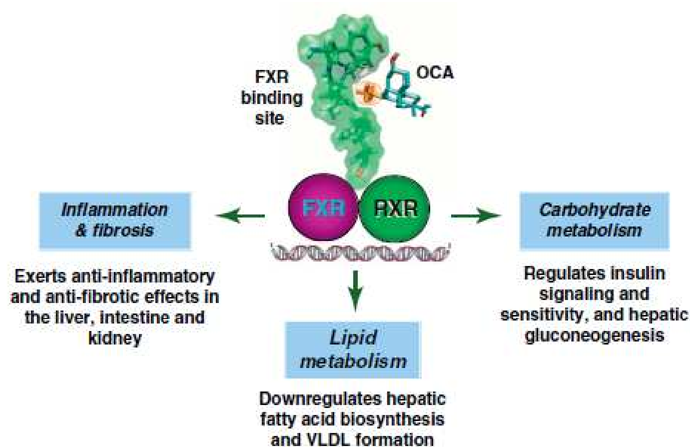

Figure 6: FXR activation by newly synthesized BAs, 6-ECDCA (OCA), might potentially beneficial effects in NASH. First class FXR agonist mediated regulation of glucose and lipid metabolism and inhibition of inflammatory responses, leading to decreased fibrosis have been shown in animal models of NAFLD/NASH and in a pilot clinical study in diabetic patients with NAFLD. *From Adorini L et al. Drug Discov Today. 2012.*

In Zucker obese rats 6-ethylchenodeoxycholic acid (6-ECDCA), has been shown to reverse insulin resistance, dyslipidemia and to protect from liver steatosis. In addition 6-ECDCA treatment decreased the serum level of glucose, free fatty acid and HDL, and the concentration of triglycerides, free fatty acid, and glycogen in the liver via *FXR* activation, thus suggesting that 6-ECDCA might be a potential agent for the treatment of NASH or NAFLD (*Cipriani S et al. 2010*).

Mudaliar S and colleagues reported recently also the efficacy and safety of 6α-ethyl-chenodeoxycholic acid in patients with type 2 diabetes and NAFLD. They found that, in this phase 2 trial, administration of 25 or 50 mg OCA for 6 weeks was well tolerated, increased insulin sensitivity, and reduced markers of liver

inflammation and fibrosis in patients with type 2 diabetes mellitus and nonalcoholic fatty liver disease (*Mudaliar S et al. 2013*). These data suggest that BAs analogs with high *FXR* agonism might be potential agent for the treatment of NAFLD.

AIMS OF THE STUDY

Based on the results obtained in the previous study by Ricchi M et al. on the different effect of palmitic (PA) and/or oleic acid (OA) on lipid accumulation and apoptosis in hepatocyte cell cultures, in the same *in vitro* model of hepatic steatosis induced in HepG2 cells, this study aimed:

1) to determine the effect of the novel, *FXR* agonist, synthetic BA (6-Ethylchenodeoxycholic acid, 6-ECDCA) on the extent of lipid storage induced by PA, OA or a mixture of the two fatty acids;
2) to evaluate if 6-ECDCA can prevent the toxicity induced by PA;
3) to explore the changes in HepG2 in lipid storage and cell toxicity related genes associated with 6-ECDCA incubation with different fatty acids.

This section presents the preliminary data of our experiments.

MATERIALS AND METHODS

Hepatocyte cell cultures, fatty acids and bile acid solutions

HepG2 cells (derived from a well differentiated human hepatoblastoma cell line that retain many characteristics of normal differentiated quiescent hepatocytes, and are p53 wild type), were purchased from Istituto Zooprofilattico Sperimentale (Brescia, Italy). Long-chain Fatty Acids (FAs), palmitic (16:0) and oleic (18:1) were provided as sodium salts (Sigma-Aldrich, Milan, Italy). Palmitic acid and Oleic acid were dissolved in H_2O (stock solution 100 mM) (*Ricchi M et al. 2009*).
Stock solutions were kept at -20℃ before the exper iments. Solutions and reagents used for cell cultures were from GIBCO Life Technologies Ltd (Grand Island, NY, USA).
The bile acid 6-Ethylchenodeoxycholic acid (6-ECDCA), a potent *FXR* agonist with an half maximal effective concentration (EC_{50}) of 99 nM, is a synthetic derivative of Chenodeoxycholic acid (CDCA) with hydrophobic properties. Lyophilized 6-ECDCA was kindly provided by Intercept Pharmaceuticals (La Jolla, CA, USA) and then it was dissolved in EtOH 100% (stock solution 100 mM) and kept at -20℃ until use.

Induction of steatosis and evaluation of 6-ECDCA effects on lipid storage and cellular toxicity

HepG2 cell cultures were incubated for 24 hours with D-MEM medium containing 1% bovine serum albumin (BSA), 1% penicillin-streptomycin solution (P/S) and supplemented with FAs (palmitic acid (PA) and oleic acid (OA) alone or in association) at the following final concentrations: a) PA 0,66 mM; b) OA 0,66 mM and c) a mixtures of the two free fatty acids (PA and OA) at the final concentrations of 0,66 mM (OA 0,44 mM and PA 0,22 mM, ratio 2:1) with or without the addition of 6-Ethylchenodeoxycholic acid (6-ECDCA) at two different final concentrations of 10 and 50 μM; control cells cultures were grown in D-MEM medium added

with the vehicle in which fatty acids and 6-Ethylchenodeoxycholic acid (6-ECDCA) were dissolved (Fig. 7). (*Fiorucci S et al. 2005*).

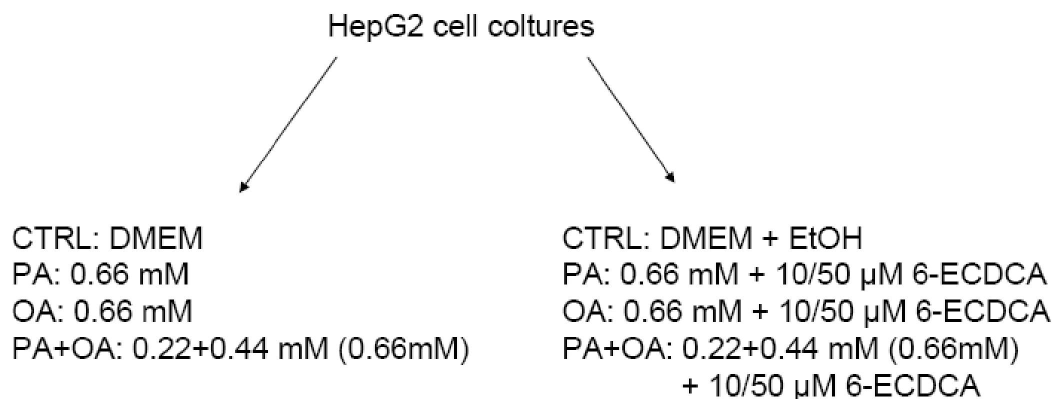

Figure 7: Schematic representation of the two groups of cell coltures analysed in the study: on the left side HepG2 cells treated only with the two fatty acids (PA or OA or a combination of the two) and control cells; on the right side HepG2 cells treated with the two fatty acids (PA or OA or a combination of the two) and with the two different concentrations of 6-ECDCA (10 and 50 µM) and control cells.

Evaluation of intracellular lipid content

Total intracellular lipid content was evaluated by Nile Red staining (Adipored, Cambrex); briefly, cells were grown in 96 black-plates and treated with FAs with or without 6-ECDCA. At the end of incubation cells were washed twice with phosphate buffered saline (PBS) and incubated with Adipored for 10 min. Fluorescence was evaluated with a microplate reader set to fluorometry parameters (excitation at 485 nm – emission at 535 nm) (Victor3-Perkin-Elmer, Monza, Italy) (*Ricchi M et al. 2006*).

Results of intracellular lipid conent content (RFLU) were normalized by protein content (µg/ml). Mean protein concentration of cell monolayers was determined according to a modified version of the Lowry method using serum albumin as a standard (Micro BCA Protein Assay Kit, Thermo Scientific-Pierce, Rockford, IL, USA). Fluorescence was evaluated with a microplate reader set to fluorometry parameters (562 nm) (Victor3-Perkin-Elmer, Monza, Italy).

Analysis of Caspase 3/7 induction

Caspases activity was evaluated by Apo-ONE Homogeneus Caspase-3/7 Assay (Promega, Italy) to assess the level of apoptosis induced in HepG2 cell coltures by the different treatments. Briefly, cells were grown in 96-well black plates. At the end of the 24 hours incubation period, a mixture containing lysis/permeabilization buffer and the caspases-3/7 substrate rhodamine 110,bis-(N-CBZ-l-aspartyl-l-glutamyl-l-valyl-l-aspartic acid amide; Z-DEVD-R110; Promega, Milan, Italy), which exists as a profluorescent substrate prior to the assay, was added to each well. After 4 hours the amount of fluorescent product, which is proportional to the amount of caspase-3/7 cleavage, was measured with a microplate reader set to fluorometry parameters (excitation at 485 nm – emission at 535 nm) (Victor3-Perkin-Elmer, Monza, Italy). Results of Caspase-3/7 activity (RFLU) were normalized by protein content (µg/ml). Mean protein concentration of cell monolayers was determined according to a modified version of the Lowry method using serum albumin as a standard (Micro BCA Protein

Assay Kit, Thermo Scientific-Pierce, Rockford, IL, USA). Fluorescence was evaluated with a microplate reader set to fluorometry parameters (562 nm) (Victor3-Perkin-Elmer, Monza, Italy) (*Ricchi M et al. 2006*).

Molecular pathway

In HepG2 cell line total RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (BioRad, Milan, Italy) and quantified with the Experion Automated Electrophoresis System (BioRad, Milan, Italy) following the manufacturer's instructions. About 1 µg of total RNA was reverse-transcribed with the iScript™ cDNA Synthesis Kit (BioRad, Milan, Italy) on C1000™ Thermal Cycler (BioRad, Milan, Italy). iTaq Real Time polymerase chain reactions (RT-PCR) were performed on cDNA samples using the iQ™ Multiplex Powermix (BioRad, Milan, Italy) according to CFX96 Real-Time PCR Detection System (BioRad, Milan, Italy).
The iTaq multiplex strategies for each gene have been developed as Assay-on-Demand by Applied Biosystems for target genes (*FXR*, *SHP*, *CYP7A1*, *SREBP1C*, *FASN*, *DGAT1*, *SCD*, *ACACB* and *CPT1A*). Gene expression profiling was achieved using the comparative cycle threshold (CT) method of relative quantification (the calibrator samples were non-treated cells, with *β2-microglobulin* and *GAPDH* RNA used as endogenous controls). Data are expressed as log2 of the relative quantity (RQ) defined also as 'fold induction versus the controls'.
Lipogenesis was evaluated by estimating the expression level of *FXR* target genes (*SHP* and *CYP7A1)* and of lipid metabolism genes *SREBP1C*, *FAS*, *SCD* and *DGAT1*.
Fatty acid oxidation was determined by analyzing the expression level of *ACACB* and *CPT1A* genes.

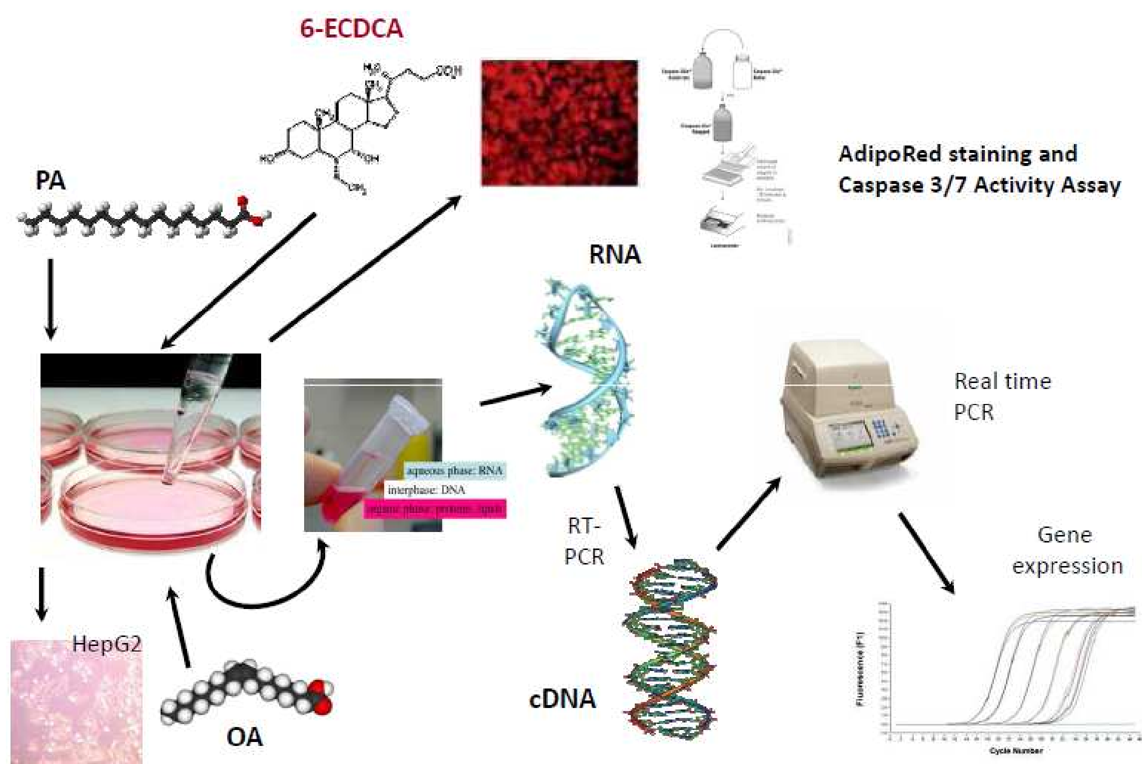

Figure 8: Schematic representation of the methods followed in the study. HepG2 cell coltures were treated with fatty acids (palmitic, oleic or their combination) to induce steatosis and incubated or not with the bile acid 6-ECDCA to evaluate its effects on lipid accumulation, apoptosis and gene expression. The intracellular lipid content was evaluated by staining with AdipoRed, apoptosis was evaluated by Caspase 3/7 Activity Assay and finally, after RNA extraction and reverse transcription to cDNA, gene expression was analyzed using Real time PCR.

Statistical analysis

Results of intracellular lipid content and apoptosis analysis were expressed as mean ± standard deviation (SD) of a minimum of three experiments conducted in triplicate. The significance of differences was assessed by Student's *t*-test or Mann-Whitney test for independent data. Significance was accepted at the $P < 0.05$ level. Statistical analysis was performed with the aid of GraphPad Prism statistical software (version 5.0 for Windows, GraphPad Software Inc., La Jolla, CA, USA). Instead the graphs representing the analysis of gene expression are related to individual preliminary experiments, therefore, was not carried out a statistical analysis.

RESULTS

Effect of different fatty acids combined to 6-ECDCA on intracellular lipid content in HepG2 cells

After 24 hours incubation, triglyceride accumulation was evident in all cells exposed to fatty acids with or without 6-ECDCA in comparison to control cells, as indicated by AdipoRed staining (Fig. 9). In agreement with previus results (*Ricchi M et al. 2009*) the degree of triglyceride accumulation was significantly higher in

HepG2 cells treated with OA than with PA. The combination of the two fatty acids was associated with a steatosis extent similar to that induced by OA (not significant) (Fig. 9).

The co-incubation of HepG2 cells with fatty acids and 6-ECDCA was associated with a slight (but not significant) increase in triglyceride accumulation in cells incubated with PA but not in those incubated with OA or with a mixture of the two fatty acids.

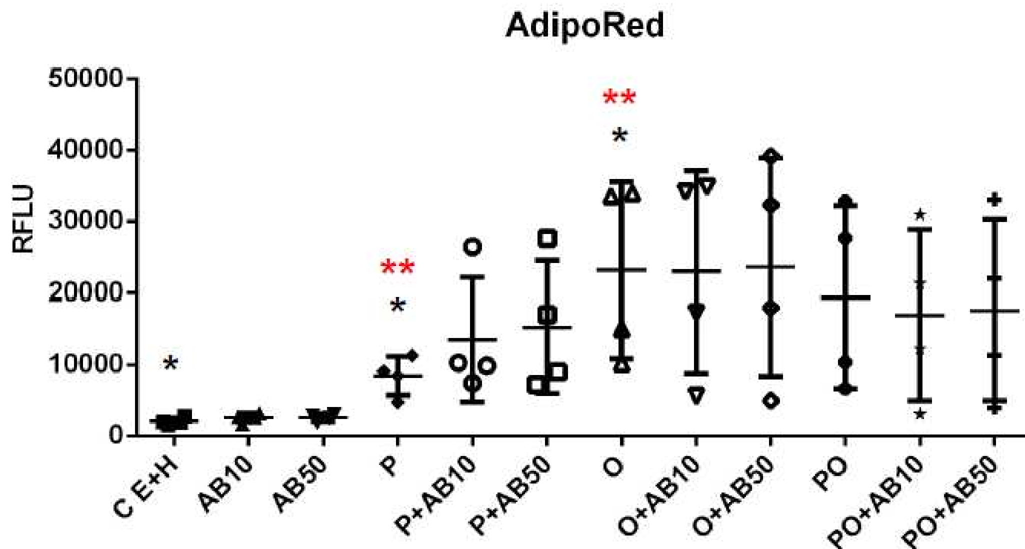

Figure 9: Effect of different fatty acids alone or in combination with 6-ECDCA on triglyceride accumulation. HepG2 cells were incubated with palmitic acid (**P**; 0.66 mM), oleic acid (**O**; 0.66 mM) or a combination of the two fatty acids (**PO**; **P** 0.22 mM + **O** 0.44 mM) alone or co-incubated with 6-ECDCA (**AB** 10 or 50 µM). Single experiments (conducted in triplicate) are represented by single points, mean values and standard deviation (SD). * *P* < 0.05 *vs* control (Student's *t*-test); ** *P* < 0.05 *vs* palmitic acid (**P**; 0.66 mM) (Mann-Whitney test).
P= palmitic acid (0.66 mM); **O**= oleic acid (0.66 mM); **PO**= (palmitic acid 0.22 mM + oleic acid 0.44 mM, total 0.66 mM, 1:2 ratio); **AB**= bile acid, 6-ECDCA (10 or 50 µM).

Effect of 6-ECDCA on apoptosis induced by palmitic and oleic acid

As previosly reported in the study by Ricchi M et al. palmitic acid at the concentration of 0.66 mM increased significantly (P < 0.05) the percentage of apoptotic cells in HepG2 cell cultures compared to control cells and to HepG2 cells treated with OA or a combination of the two fatty acids at the same concentration (0.66 mM). In contrast, oleic acid, used at the same concentrations of 0.66 mM did not induce any increase in the number of apoptotic cells as compared to untreated controls. Co-incubation with the two FFAs at the concentrations of 0.66 mM did not modify the rate of apoptosis as compared with control cells. (*Ricchi M et al. 2009*).

The treatment of HepG2 cells with 6-ECDCA (that alone display a rate of apoptosis similar to those of untreated cells) reduced the toxicity induced by palmitic acid in a dose dependent fashion but it did not substantially modified the effect on apoptosis induced by oleic acid or by the combination of the two fatty acids (not significant) (Fig. 10).

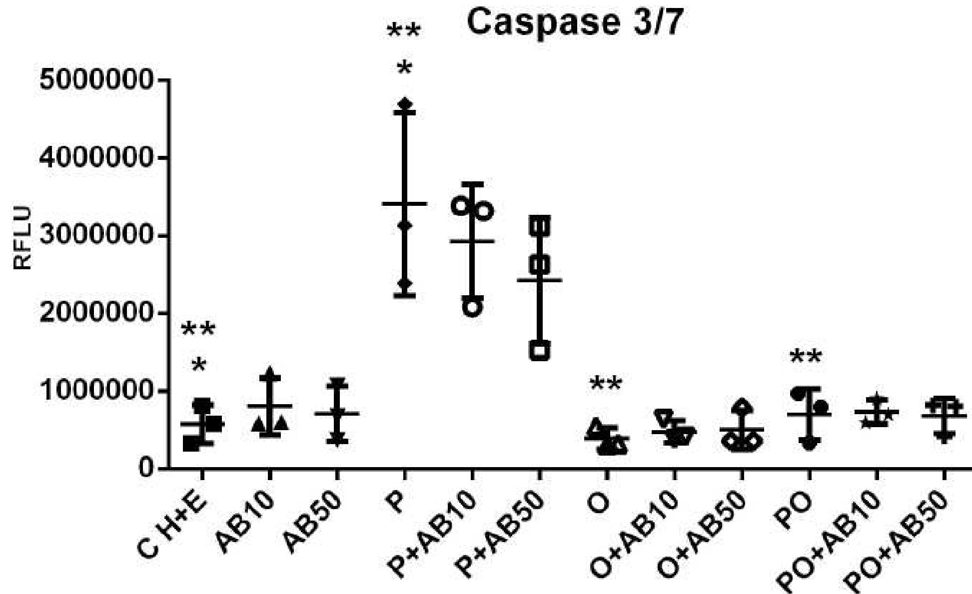

Figure 10: Effect of different fatty acids alone or in combination with 6-ECDCA on Caspase-3/7 Activity. HepG2 cells were incubated with palmitic acid (**P**; 0.66 mM), oleic acid (**O**; 0.66 mM) or a combination of the two fatty acids (**PO**; **P** 0.22 mM + **O** 0.44 mM) alone or co-incubated with 6-ECDCA (**AB** 10 or 50 µM). Single experiments (conducted in triplicate) are represented by single points, mean values and standard deviation (SD). * $P < 0.05$ *vs* control (Student's *t*-test); ** $P < 0.05$ *vs* palmitic acid (**P**; 0.66 mM) (Student's *t*-test).
P= palmitic acid (0.66 mM); **O**= oleic acid (0.66 mM); **PO**= (palmitic acid 0.22 mM + oleic acid 0.44 mM, total 0.66 mM, 1:2 ratio); **AB**= bile acid 6-ECDCA (10 or 50 µM).

Effect of 6-ECDCA on *FXR* and on its downstream signals

.
As shown in Figure 11, 6-ECDCA had little effects on *FXR* expression either alone or in combination with the two fatty acids. We observed however an increase in *SHP* expression and a decrease in *CYP7A1* expression both for 6-ECDCA alone or in combination with PA and/or OA. These data suggest that 6-ECDCA, at 10 or 50 µM, through the activation of *FXR-SHP* intracellular pathway, down-regulate *CYP7A1* expression, and therefore BAs synthesis, in steatotic HepG2 cells. These data are in agreement with the specific *FXR* agonist action of 6-ECDCA and confirms that the BA acts properly in our hepatocyte cell culture.

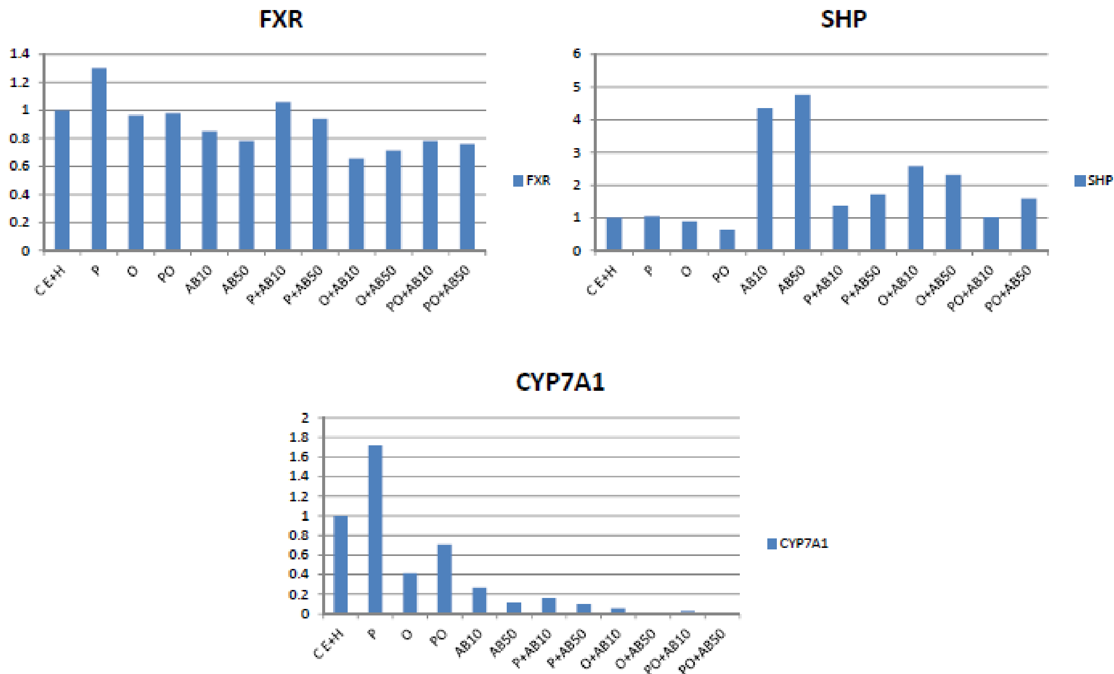

Figure 11: Effect of 6-ECDCA on *FXR (Farnesoid X receptor)*, *SHP (small heterodimer partner)* and *CYP7A1 (Cholesterol 7 alpha-hydroxylase also known as cholesterol 7-alpha- monooxygenase or cytochrome P450 7A1)* expression. HepG2 cells were incubated with palmitic acid (**P**; 0.66 mM), oleic acid (**O**; 0.66 mM) or a combination of the two fatty acids (**PO**; **P** 0.22 mM + **O** 0.44 mM) alone or co-incubated with 6-ECDCA (**AB** 10 or 50 μM). Results of a single experiment (conducted in triplicate) represented by mean values. (Statistical analisys not performed).
P= palmitic acid (0.66 mM); **O**= oleic acid (0.66 mM); **PO**= (palmitic acid 0.22 mM + oleic acid 0.44 mM, total 0.66 mM, 1:2 ratio); **AB**= bile acid 6-ECDCA (10 or 50 μM).

Modulation of the expression of lipid metabolism genes by fatty acids and 6-ECDCA in HepG2 cells

The analysis of the expression of lipogenic *sterol regulatory element binding protein* (*SREBP1c-)*, *fatty acid synthase (FASN*, *Diglyceryde acyltranferase (DGAT1-)* and *stearoyl-CoA desaturase-1* (*SCD)* genes in steatotic HepG2 treated with 6-ECDCA revealed that the 6-ECDCA tends to reduce lipogenic genes *SREBP1c*, *FASN*, *DGAT* expression mainly after incubation with PA and with a mixture of the two fatty acids (Fig. 12).

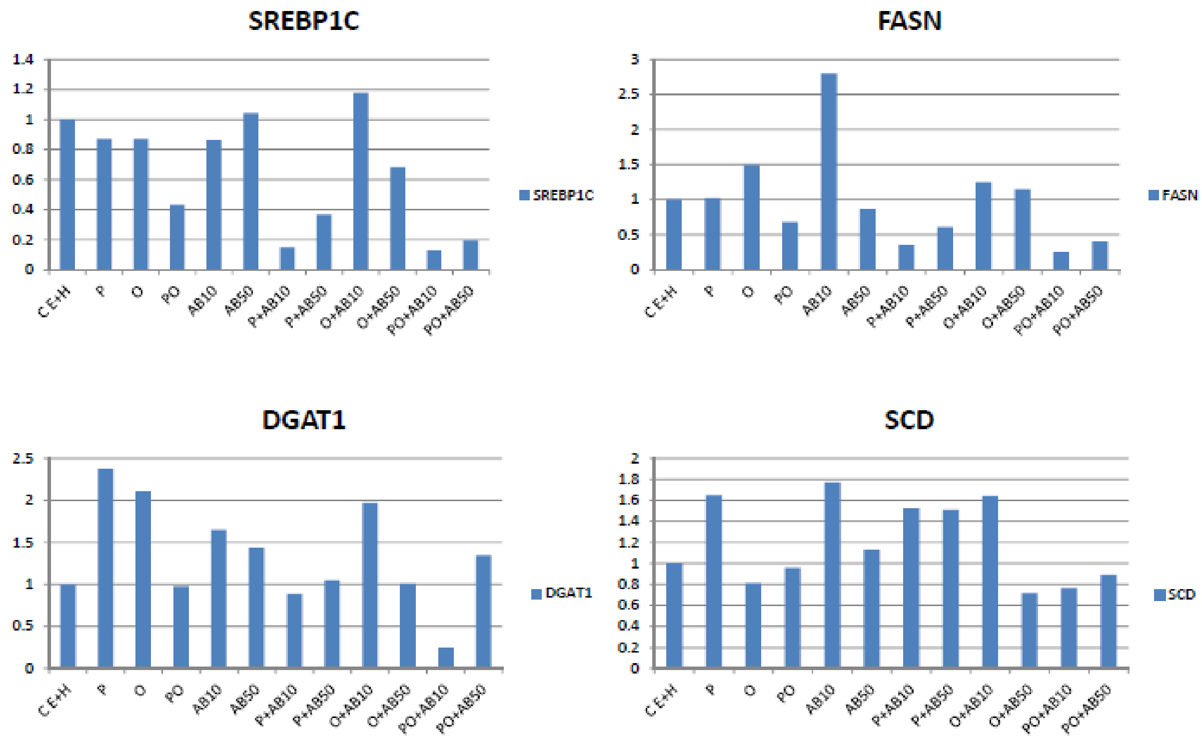

Figure 12: Effect of 6-ECDCA on *SREBP1c*, *FASN*, *DGAT1* and *SCD* expression. HepG2 cells were incubated with palmitic acid (**P**; 0.66 mM), oleic acid (**O**; 0.66 mM) or a combination of the two fatty acids (**PO**; **P** 0.22 mM + **O** 0.44 mM) alone or co-incubated with 6-ECDCA (**AB** 10 or 50 μM). Results of a single experiment (conducted in triplicate) represented by mean values. (Statistical analisys not performed).
P= palmitic acid (0.66 mM); **O**= oleic acid (0.66 mM); **PO**= (palmitic acid 0.22 mM + oleic acid 0.44 mM, total 0.66 mM, 1:2 ratio); **AB**= bile acid 6-ECDCA (10 or 50 μM).

To verify whether genes markers of fatty acid synthesis/oxidation might contribute to the effects of 6-ECDCA treatment in HepG2 steatotic cells, we measured the expression levels of Acetyl-CoA carboxylase 2 (*ACACB*) and Carnitine palmitoyltransferase 1A (*CPT1A*). As shown in Figure 13, PA alone stimulated the synthesis of fatty acids through the induction of *ACACB*. 6-ECDCA, co-incubated with PA reduced the expression of *ACACB* and increased the expression of *CPT1A* (the enzyme that controls fatty acids uptake by mithocondria).

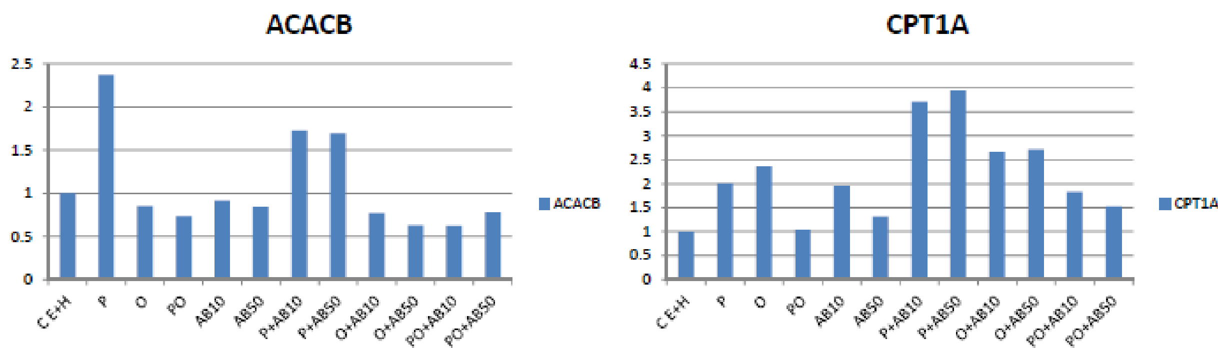

Figure 13: Effect of 6-ECDCA on *ACACB* and *CPT1A* expression. HepG2 cells were incubated with palmitic acid (**P**; 0.66 mM), oleic acid (**O**; 0.66 mM) or a combination of the two fatty acids (**PO**; **P** 0.22 mM + **O** 0.44 mM) alone or co-incubated with 6-ECDCA (**AB** 10 or 50 μM). Results of a single experiment (conducted in triplicate) represented by mean values. (Statistical analisys not performed).
P= palmitic acid (0.66 mM); **O**= oleic acid (0.66 mM); **PO**= (palmitic acid 0.22 mM + oleic acid 0.44 mM, total 0.66 mM, 1:2 ratio); **AB**= bile acid 6-ECDCA (10 or 50 μM).

DISCUSSION

Our experiments confirmed that, while the extent of steatosis was more severe in cells treated with OA than in those exposed to PA, opposite effects were found on cell apoptosis. This is in agreement with the previous study by Ricchi M et al. (*Ricchi M et al. 2009*).

Moreover in this study we evaluated the effects of a novel powerfull synthetic *FXR* agonist, 6-Ethylchenodeoxycholic acid (6-ECDCA), in the steatotic model of HepG2 cells treated with different fatty acids.

In our study, as showed by AdipoRed staining analysis, the co-incubation of palmitic acid with 6-ECDCA caused a slight, although not significant, increase in lipid accumulation. Evaluation of caspase-3/7 activity assay, on the other hand, demonstrated that 6-ECDCA caused a reduction in cellular toxicity induced by PA in HepG2 cells.

These data seem to support the hypothesis that steatosis might be a mechanism to prevent fatty acid toxicity and that 6-ECDCA, by increasing steatosis extent, prevents the damaging effect of palmitic acid (*Arrese M et al. 2009; Nolan CJ et al. 2009*). Alternative hypothesis to explain the reduced damaging effect of PA in co-incubation experiments with 6-ECDCA is that this BA might strongly inhibit the synthesis of more damaging BAs or might stimulate its mitochondrial oxidation thus preventing the damaging effect of free fatty acids.

Data on gene expression are very preliminary (one experiment), however it is clear, from the modulation of the expression induced on *SHP* and *CYP7A1*, that 6-ECDCA in this model, as it is expected, increases *SHP* and decreases *CYP7A1*, as compared to controls (*Watanabe M et al. 2004*). Addiction of 6-ECDCA to both fatty acids tend to increase *SHP* expression and to decrease *CYP7A1* as compared to the data observed with fatty acids alone suggesting that 6-ECDCA might affect the induced expression of these target genes by fatty acids. However there are currently no published data to support this hypothesis.

In these experiments 6-ECDCA has been shown to induce an inhibition of lipogenic genes particularly in the co-incubation with PA and that only when added to PA this new BA was found to decrease *ACACB* and increase *CPT1A*. This would indicate that this new BA may reduce steatosis by inhibiting lipogenesis and by decreasing the synthesis of malonil CoA that in turn would activate *CPT1A* the rate limiting enzyme of

mithochondrial fatty acids uptake and of their oxidation. Both findings are in agreement with Watanabe data (*Watanabe M et al. 2004*) and with the absolute lower level of steatosis found after incubation of HepG2 cells with PA + 6-ECDCA, but contrasts with the fact that co-incubation of 6-ECDCA with PA tend to increase hepatic steatosis induced by PA alone. Further experiments are needed to confirm and clarify this findings, in particular HPLC characterization of fatty acids pattern and evaluation of the 'free' fraction.

Taken all together these results seem to suggest that 6-ECDCA could protect from the toxicity induced by PA probably by enhancing lipid accumulation or by stimulating oxidation of free fatty acids thus preventing their damaging effects.

In conclusion, 6-ECDCA is an efficacious *FXR* agonist under testing for various diseases associated with bile acid disfunction, such as liver fibrosis and inflammatory bowel disease (*Matsubara T et al. 2013; Adorini et al. 2012*). 6-ECDCA has been evaluated in phase II clinical trials in patients with type 2 diabetes mellitus and NAFLD and was found to be well tolerated and effective in increasing insulin sensitivity and reducing markers of inflammation and fibrosis (*Mudaliar S et al. 2013*). 6-ECDCA shows antifibrotic activity in three liver fibrosis models through activation of *FXR* (*Matsubara T et al. 2013; Adorini et al. 2012*).

Based on these data 6-ECDCA might be potentially useful in the treatment of NAFLD/NASH by stimulating the incorporation of palmitic acid in "inhert triglycerides", reducing hepatocyte apoptosis and possibly stimulating fatty acid oxidation through mitochondrial handling of fatty acids.

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ROLE OF FETUIN-A ON THE RELATIONSHIP BETWEEN NON-ALCOHOLIC FATTY LIVER DISEASE AND CORONARY ARTERY DISEASE

INTRODUCTION

Non-alcoholic Fatty Liver Disease (NAFLD)

Non-alcoholic Fatty Liver Disease (NAFLD) spans from simple steatosis through Non-alcoholic Steatohepatitis (NASH) with or without fibrosis/cirrhosis and primary liver cancer (*Loria P et al. 2010*). NAFLD is strongly associated with metabolic syndrome (MS) and insulin resistance (IR) (*Vanni E et al. 2010; Maurantonio M et al. 2011*). Fatty liver is histologically defined as an accumulation of fat, mainly in the form of triglycerides, in more than 5% of hepatocytes without inflammation or hepatocellular damage (*Smith BW et al. 2011*). Working on the hypothesis that NAFLD was a continuum from simple steatosis to NASH, in 1998 Day and colleagues proposed, as a pathogenetic process, a two “hit” model: the first “hit” was given by the deposition of triglycerides in the cytoplasm of hepatocytes, but the disease did not progress further until it occurred the second “hit” that promoted inflammation, cell death and fibrosis (*Day CP et al. 1998; Van Rooyen DM et al. 2011*). In spite of the theory of “two-hit” the progression from simple fatty liver to NASH appears to be rare in humans (unlike rodents); although simple steatosis and steatohepatitis are still considered as two histological subtypes of a unique spectrum of NAFLD, these entities are likely to differ not only from the histological point of view, but also from the pathophysiological one.
More recently the hypothesis of “distinct-hit” according to which a combinations of genetic and molecular events might cause the activation of distinct pathways which can lead lead to simple steatosis or NASH has been putted forward (*Yilmaz Y 2012*) (Fig. 1).

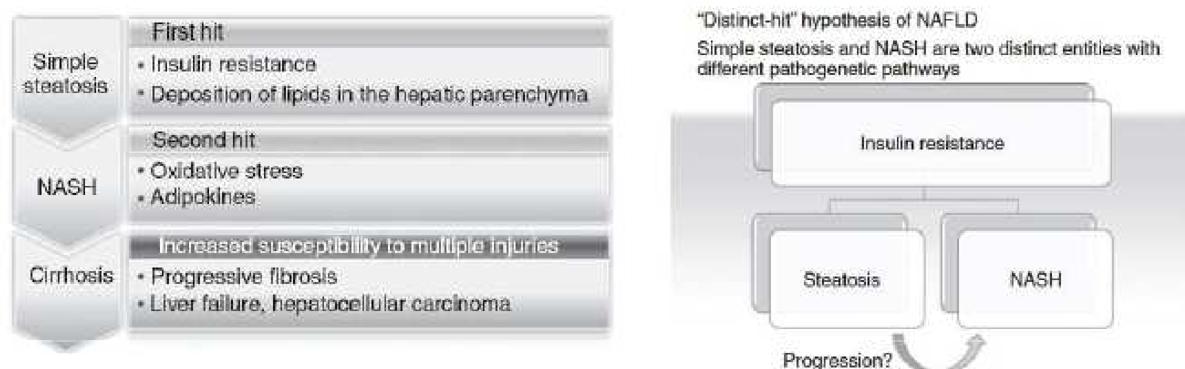

Figure 1: “Two-hit” versus “distinct-hit” pathogenesis of NAFLD. *From Yilmaz Y. Aliment Pharmacol Ther. 2012.*

Another pathogenetic theory is that of “one-hit” according to which IR is the only and necessary pathogenic step (*Lonardo A et al. 2010*). In this context steatosis may represent a protective mechanism. In individuals in whom this adaptive process fails NASH will develop (*Arrese M 2009*).

Atherosclerosis

Atherosclerosis is a systemic disease that involves the intima of the arteries of large and medium caliber; it is considered a chronic immuno-inflammatory, fibro-proliferative and progressive process dependent on the lipid pattern (*Sweeny JM et al. 2008*). Atherosclerosis is the result of an interaction between genetic and environmental factors that can modify the expression of some genes by promoting the development of the disease.

Atherosclerosis is a widespread disease of the arterial system that is caused by vessels injury. Risk factors for atherosclerosis include hypercholesterolemia, hypertension, obesity, metabolic syndrome and its sub-inflammatory state induced by IR. The role of nitric oxide (NO) in conjunction with reactive oxygen species (ROS) and the induction of inflammatory cytokines are also important. Atherosclerosis leads to ischemic events, including transient ischemic attacks (TIAs), thrombotic stroke and myocardial infarction. Therefore, atherosclerosis is not the result of only one risk factor, but a combination of various processes such as lipid homeostasis and the inflammatory response (*Mia-Jeanne VR et al. 2013*).

Atherosclerosis, the most common cause of coronary, cerebrovascular, and peripheral artery diseases, and occurs in epidemic proportions in western populations. There is evidence that elevated levels of oxidized LDL cholesterol (oxLDL-C) and other atherosclerotic risk factors, such as hypertension and cigarette smoking, as well as localized low shear stress, damage the artery's endothelial cells, which express leukocyte adhesion molecules. These surface structures capture monocytes that penetrate the intima. There, the monocytes mature into macrophages that take up oxLDL-C and thereby become foam cells. The latter produce inflammatory mediators that attract smooth muscle cells into the intima, where they proliferate and promote the accumulation of extracellular matrix. Together with the foam cells, the extracellular matrix contributes to the growth of atherosclerotic plaques (*Braunwald E. 2013; Wolf D et al. 2013*) (Fig. 2).

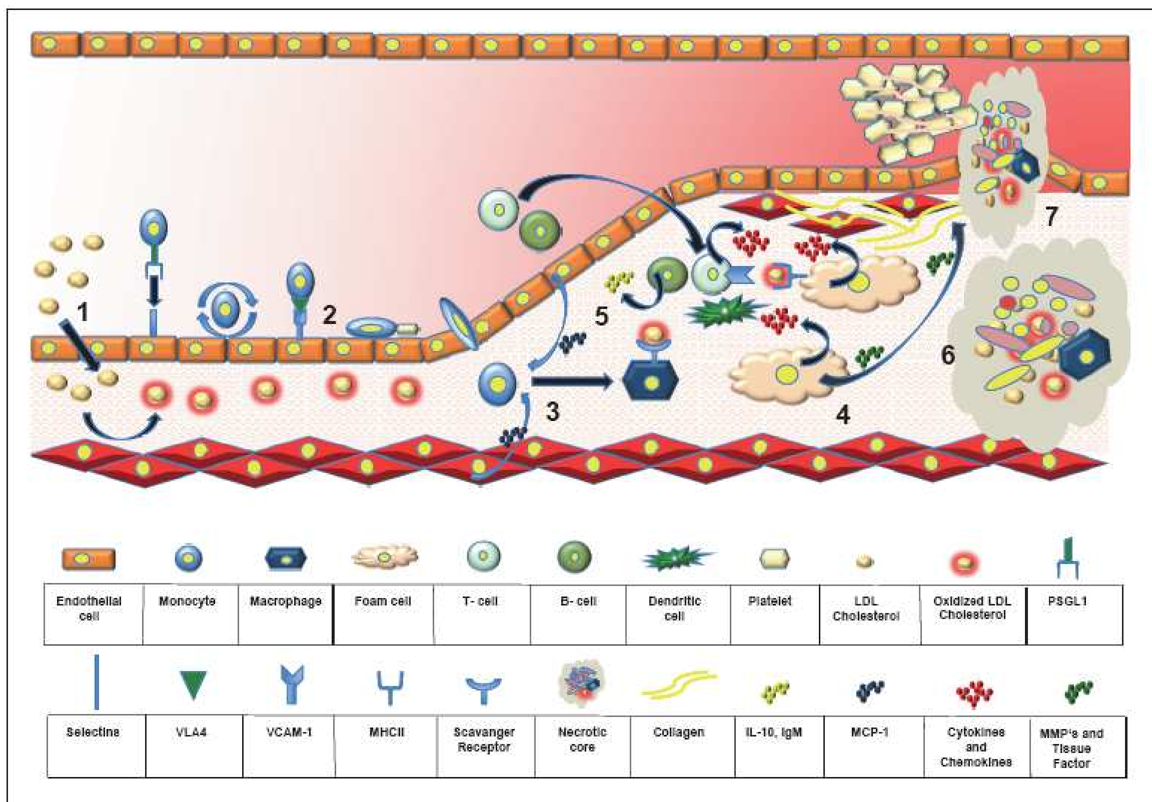

Figure 2: Atherosclerotic plaque formation, key events. (1) LDL cholesterol enters the arterial wall, is oxidized to oxLDL and activates endothelial cells (EC) expressing cell adhesion molecules. (2) Monocytes roll over the endothelium, adhere supported by platelets to the ECs and enter the intima. (3) Within the intima they transform to macrophages upon M-CSF stimulation. (4) By intaking oxLDL macrophages form foam cells which produce pro-inflammatory cytokines. (5) While the inflammation accelerates T-cells, B-cells and dendritic cells enter the plaque and regulate inflammation. (6) Cell detritus, lipids and extracellular matrix form a necrotic core. (7) Once the fibrous cap of collagen and smooth muscle cells is degraded by MMPs and ruptures, the necrotic core comes into contact to the blood steam, *From Wolf D. Hamostaseologie. 2013.*

NAFLD and risk of coronary heart disease

A large body of clinical and epidemiological evidence support that NAFLD is strongly associated with an increased prevalence and incidence of CHD (*Of our research group: Maurantonio M et al. 2011; Ballestri S et al. 2012; Ballestri S et al. 2014 In Press - of which this introduction is a brief synthesis - and other groups Musso G et al. 2011; Anstee QM et al. 2013; Targher G et al. 2013*).

NAFLD has been shown to be associated to both increased markers of subclinical atherosclerosis and increased prevalence of cardiovascular disease.

Many epidemiological data link NAFLD, both in adults and in adolescents, with several markers of subclinical atherosclerosis (i.e. endothelial dysfunction, arterial stiffness, increased carotid artery wall thickness, increased coronary calcium score) (*Maurantonio M et al. 2011; Ballestri S et al. 2012*).

In addition it has been reported that NAFLD is associated with ultrasonographic indices of circulatory endothelial dysfunction, independently of obesity and other classical CVD risk factors (*Villanova N et al. 2005; Salvi P et al. 2010; Pacifico L et al. 2010*). A meta-analytis showed that utrasonographically diagnosed NAFLD was strongly associated with increased carotid-artery intima-media thickness (cIMT) and an increased prevalence of carotid atherosclerotic plaques (*Sookoian S et al. 2008*).

Also coronary artery calcium (CAC) score, considered an early marker of atherosclerosis, is greater in NAFLD patients (*Abdulla J et al. 2011*) independently from the traditional risk factors for CVD (*Chen CH et al. 2010; Kim D et al. 2012*).

A reduction of coronary flow reserve (CFR), an index of impaired coronary microcirculation, was also reported in patients with NAFLD that was independen from other conventional risk factors and from the presence of metabolic syndrome (*Lautamäki R et al. 2006; Yilmaz Y et al. 2010; Nakamori S et al. 2012*).

Many cross sectional studies (Table 1) (*Ballestri S et al. 2014*), have shown a strong relationship between NAFLD with increased prevalence and severity of CHD in both nondiabetic and diabetic subjects.

Table 1 Main cross-sectional study examining the association of non-alcoholic fatty liver disease with the presence and severity of clinical coronary heart disease, ordered by year

Ref.	Study characteristics	NAFLD diagnosis	CHD diagnosis	Main findings
Lin *et al*[31], 2005	2088 male workers undergoing an annual health examination screening; NAFLD in 29.5%	US	Patient history, ECG	NAFLD associated with higher prevalence of CHD, independently of obesity and other traditional CVD risk factors. The odds for CHD increased progressively with ultrasonographic severity of NAFLD
Targher *et al*[32], 2007	2839 type 2 diabetic outpatients; NAFLD in 69.5%	US	Patient history, review of patient records, ECG, doppler ultrasound of carotid and lower limb arteries	NAFLD associated with higher prevalence of coronary, cerebrovascular and peripheral vascular disease than their counterparts without NAFLD, independently of traditional CVD risk factors, hemoglobin A1c, medication use and MetS features
Arslan *et al*[33], 2007	92 consecutive Turkish patients admitted with ACS; NAFLD in 70%	US	CAG (elective)	NAFLD was an independent predictor of CHD (> 50% stenosis of ≥ 1 major coronary artery) after adjustment for traditional CVD risk factors and MetS features
Mirbagheri *et al*[34], 2007	317 Iranian patients admitted for either ACS, angina or suspected CHD; NAFLD in 54%	US	CAG (elective)	NAFLD was an independent predictor of 'clinically relevant' CHD (> 30% stenosis of ≥ 1 major coronary artery) after adjustment for CVD risk factors and MetS features
Alper *et al*[35], 2008	80 Turkish patients with MS (stable or unstable angina, prognostic reasons); NAFLD in 54%	US	CAG (acute and elective)	NAFLD was the only independent predictor of severe CHD (> 70% stenosis of ≥ 1 major coronary artery) after adjustment for established CVD risk factors and MetS features
Akabame *et al*[36], 2008	298 consecutive Japanese patients with suspected CHD; NAFLD in 20%	CT	CT (elective)	NAFLD was independently associated with remodeling lesions or lipid core of coronary plaques but not with calcified coronary plaques or stenoses
Acikel *et al*[37], 2009	355 consecutive Turkish patients admitted for ACS or CHD suspicion; NAFLD in 60%	US	CAG (acute & elective)	NAFLD was an independent predictor of CHD (> 50% stenosis of ≥ 1 major coronary artery) after adjustment for conventional CVD risk factors
Assy *et al*[38], 2010	29 Israeli patients with low or intermediate risk of CHD and NAFLD and 32 healthy controls matched for age and sex	CT	CT (elective)	NAFLD was associated with greater prevalence of calcified and non-calcified coronary plaques, independently of the MetS and plasma C-reactive protein
Targher *et al*[39], 2010	250 type 1 diabetic patients; NAFLD in 44.4%	US	Patient history, chart review, ECG, doppler ultrasound of carotid and lower limb arteries	NAFLD was associated with higher prevalence of coronary, cerebrovascular and peripheral vascular disease than their counterparts without NAFLD, independently of traditional CVD risk factors, medication use, hemoglobin A1c, and albuminuria
Sun *et al*[40], 2011	542 hospitalized Chinese patients with high suspicion of CHD; NAFLD in 46%	CT	CAG (elective)	NAFLD was associated with greater severity of CHD, independently of traditional CVD risk factors
Wong *et al*[41] 2011	612 Chinese patients with suspicion of CHD; NAFLD in 58%	US	CAG (elective)	NAFLD was associated with CHD, independently of established CVD risk factors and MetS features
Domanski *et al*[42], 2012	377 patients with NAFLD (retrospective chart review); 219 of these patients had NASH	Biopsy	History of CVD (stroke, unstable angina, myocardial infarction, congestive heart failure, or need for coronary revascularization)	No increased prevalence of CVD in NASH patients compared with those with non-NASH fatty liver
Agaç *et al*[43], 2013	80 Turkish patients with ACS; NAFLD in 81%	US	CAG (acute)	NAFLD was independently associated with a greater severity of CHD (by Syntax score)
Boddi *et al*[44], 2013	95 consecutive non-diabetic Italian patients admitted for ACS; NAFLD in 87%	US	CAG (acute)	Presence and severity of NAFLD was independently associated with a three-fold higher risk of multi-vessel CHD
Inci *et al*[45], 2013	136 consecutive Turkish patients with CHD (stable angina or positive stress test results)	US	CAG (elective)	NAFLD was associated with greater severity of CHD, independently of traditional CVD risk factors

ACS: Acute coronary syndrome; NAFLD: Non-alcoholic fatty liver disease; CAG: Coronary angiography; CT: Computed tomography; CVD: Cardiovascular disease; ECG: Electrocardiogram; MetS: Metabolic syndrome; NASH: Non-alcoholic steatohepatitis; US: Ultrasonography.

Table 1: Studies reporting the relationship between NAFLD and increased prevalence and severity of CHD in both nondiabetic and diabetic subjects. *From: Ballestri S et al. WJG 2014, In Press*.

Finally many studies (summarized in Table 2) using either biochemical markers, such as elevated serum liver enzymes (*Schindhelm RK et al. 2007; Fraser A et al. 2007; Goessling W et al. 2008; Dunn W et al. 2008; Ong JP et al. 2008; Ruhl CE et al. 2009; Yun KE et al. 2009*) and fatty liver index (FLI)

(*Bedogni G et al. 2006*), or ultrasound imaging (*Jepsen P et al. 2003; Targher G et al. 2007; Hamaguchi M et al. 2007; Haring R et al. 2009; Lazo M et al. 2011; Stepanova M et al. 2012; Zhou YJJ et al. 2012; Treeprasertsuk S et al. 2012*) or histology (*Matteoni CA et al. 1999; Dam-Larsen S et al. 2004; Adams LA et al. 2005; Ekstedt M et al. 2006; Rafiq N et al. 2009; Soderberg C et al. 2010; Domanski JP et al. 2012*) for the diagnosis of NAFLD, have demonstrated that NAFLD, is significantly associated with an increased risk of fatal and/or non-fatal CHD and CVD events in both nondiabetic and type 2 diabetic subjects.

Table 2 Main prospective studies relating non-alcoholic fatty liver disease to increased risk of incident coronary heart disease or cardiovascular events, ordered by methodology used for the diagnosis of non-alcoholic fatty liver disease

Ref.	Study characteristics	Years of follow-up	NAFLD diagnosis	Study outcomes	Main findings
Fraser *et al*[47], 2007	Meta-analysis of 10 population-based cohort studies	7.3	Liver enzymes	Fatal and non-fatal CVD events	Elevated serum GGT level was associated with increased incidence of CVD events, independently of alcohol intake and traditional CVD risk factors
Schindhelm *et al*[48], 2007	Population-based cohort, *n* = 1439 subjects (Hoorn Study)	10.0	Liver enzymes	Fatal and non-fatal CHD events	Elevated serum ALT level was associated with CHD events, independently of the MetS and traditional CVD risk factors
Goessling *et al*[49], 2008	Community-based cohort, *n* = 2812 (Framingham Offspring Heart Study)	20.0	Liver enzymes	Fatal and non-fatal CVD events	Elevated serum ALT level was not associated with CVD events at multivariate analyses
Dunn *et al*[50], 2008	Population-based cohort, *n* = 7574 (NHANES-III)	8.7	Liver enzymes	All-cause and cause- specific mortality	Increased all-cause and CVD mortality rates in NAFLD but only in 45-54 year age group, independently of conventional CVD risk factors and C-reactive protein
Ong *et al*[51], 2008	Population-based cohort, *n* = 11285 subjects (NHANES-III)	8.7	Liver enzymes	All-cause and cause- specific mortality	Increased rates of all-cause, CVD and liver-related mortality in NAFLD. Liver disease was the third leading cause of death among persons with NAFLD after CVD and cancer-related mortality
Ruhl *et al*[52], 2009	Population-based cohort, *n* = 14950 (NHANES-III)	8.8	Liver enzymes	All-cause and cause- specific mortality	Elevated serum GGT level was associated with mortality from all causes, liver disease but not from CVD causes. Serum ALT level was associated only with liver disease mortality
Yun *et al*[53], 2009	Community-based cohort, *n* = 37085 (Health Promotion Center)	5.0	Liver enzymes	CVD or diabetes-related mortality	Elevated serum ALT level was independently associated with increased CVD or diabetes-related mortality
Calori *et al*[54], 2011	Community based-cohort, *n* = 2074 (Cremona study)	15.0	FLI index	All-cause and cause- specific mortality	FLI was independently associated with all-cause, hepatic, cancer and CVD mortality. When HOMA-insulin resistance was included in multivariate analyses, FLI retained its statistical association with hepatic-related mortality but not with all-cause, CVD and cancer-related mortality
Lerchbaum *et al*[55], 2012	Consecutive sample of patients, *n* = 3270 subjects routinely referred to coronary angiography	7.7	FLI index	All-cause and cause- specific mortality	High FLI was independently associated with increased all-cause, CVD, non-cardiovascular and cancer mortality
Jepsen *et al*[56], 2003	Population-based cohort, *n* = 1804 with hospital diagnosis of NAFLD (Danish national registry of patients)	16.0	US	All-cause and cause- specific mortality	Increased rates of all-cause, CVD and liver-related mortality in NAFLD, independently of sex, diabetes, and cirrhosis at baseline
Targher *et al*[57], 2007	Outpatient cohort, *n* = 2103 type 2 diabetic subjects (Valpolicella Heart Diabetes Study)	6.5	US	Fatal and non-fatal CVD	Increased rates of fatal and non-fatal CVD events in NAFLD, independently of age, sex, body mass index, smoking, diabetes duration, hemoglobin A1c, LDL-cholesterol, MetS features, medication use
Soler Rodriguez *et al*[58], 2007	Community-based cohort, *n* = 1637 healthy Japanese	5.0	US	Non-fatal CVD events	Increased rates of non-fatal CVD events in NAFLD, independently of age, sex, body mass index, alcohol intake, smoking, LDL-cholesterol, MetS features
Lazo *et al*[59], 2011	Population-based cohort, *n* = 11371 (NHANES-III)	14.5	US	All-cause and cause-specific mortality	NAFLD was not associated with increased all-cause and cause-specific (CVD, cancer and liver) mortality
Stepanova *et al*[60], 2012	Population-based cohort, *n* = 11613 (NHANES-III)	14.2	US	All-cause and cause-specific mortality	NAFLD was associated with increased prevalence of CVD, after adjusting for established CVD risk factors, but not with increased CVD mortality
Zhou *et al*[61], 2012	Community-based cohort study, *n* = 3543 adult men and women	4.0	US	All-cause and CVD mortality	Increased rates of all-cause and CVD mortality in NAFLD
Younossi *et al*[62], 2013	Population-based cohort, *n* = 1448 with NAFLD (NHANES-III)	14.2	US	All-cause and cause- specific mortality	NAFLD was independently associated with increased all-cause, CVD and liver-related mortality only among NAFLD patients with the MetS
Haring *et al*[63], 2009	Population-based cohort, *n* = 4160 German subjects (Study of Health in Pomerania)	7.2	US and liver enzymes	All-cause and CVD mortality	Elevated serum GGT level was independently associated with increased all-cause and CVD mortality in men
Kim *et al*[64], 2013	Population-based cohort, *n* = 1154 (NHANES-III)	14.5	US and advanced fibrosis score systems	All-cause and cause- specific mortality	NAFLD was not associated with increased all-cause mortality. However, NAFLD with advanced hepatic fibrosis (defined by NAFLD fibrosis score, APRI index or Fib-4) was independently associated with risk of all-cause mortality, of which the majority of deaths were due to CVD

Treeprasertsuk *et al*[65], 2012	Community-based cohort, *n* = 309 patients with NAFLD	11.5	US and CT	Fatal and non-fatal CHD	NAFLD patients had a higher 10-year CHD risk by FRS than the general population of the same age and sex. Almost identical number of FRS-predicted and actual new CHD events
Matteoni *et al*[66], 1999	Patient-based cohort, *n* = 132 NAFLD	18.0	Histology	All-cause and cause-specific mortality	Increasing liver-related mortality with the severity of NAFLD histology (according to four different histological subtypes). All-cause mortality and other causes of mortality were not significantly different across histological subtypes
Dam-Larsen *et al*[67], 2004	Patient-based cohort (Danish national registry of patients), *n* = 109 subjects with non-alcoholic SS	16.7	Histology	All-cause and cause-specific mortality	All-cause and cause-specific mortality did not significantly differ between patients with non-alcoholic SS and the general population
Adams *et al*[68], 2005	Community-based cohort, *n* = 420 patients with NAFLD	7.6	US/CT and histology	All-cause and cause-specific mortality	Increased rate of age- and sex-adjusted all-cause mortality in NAFLD than in the general population with CHD being the second cause of death
Ekstedt *et al*[69], 2006	Patient-based cohort, *n* = 129 consecutive patients with NAFLD and elevated serum liver enzymes (55% NASH)	13.7	Histology	All-cause and cause-specific mortality	Increased rates of CVD and liver-related mortality in patients with NASH, but not in those with SS, compared with in the reference population
Rafiq *et al*[70], 2009	Patient-based cohort, *n* = 173 patients with NAFLD (41.6% NASH)	13.0	Histology	All-cause and cause-specific mortality	CHD was the first cause of death in NAFLD cohort with no difference between NASH and non-NASH. Liver-related mortality, but not all-cause mortality, was higher in NASH *vs* non-NASH. No comparison was provided with the general population
Söderberg *et al*[71], 2010	Patient-based cohort, *n* = 118 patients with NAFLD and elevated serum liver enzymes (43% NASH)	24.0	Histology	All-cause and cause-specific mortality	Increased mortality rates of CVD, malignancy and liver disease in patients with NASH, but not in those with SS, compared with the matched general population

AST: Alanine aminotransferase; CHD; Coronary heart disease; CT: Computed tomography; US: Ultrasonography; FLI: Fatty liver index; FRS: Framingham risk score; GGT: Gamma-glutamyltransferase; HOMA: Homeostasis model assessment; MetS: Metabolic syndrome; NASH: Non-alcoholic steatohepatitis; SS: Simple steatosis; CVD: Cardiovascular.

Table 2: Main prospective studies relating NAFLD to increased risk of CHD or CV events ordered by methodology used for the diagnosis of NAFLD. *From: Ballestri S et al. WJG 2014, In Press.*

Although many data have shown that the association was independent of other metabolic risk factors, few studies have been performed in patients with biopsy proven NAFLD followed for long time period. Data are urgently needed in large cohorts of biopsy diagnosed NAFLD to provide a definite answer on the prognosis in NAFLD patients.

Pathogenetic link between CVD and NAFLD

Experimental data clearly show that the association of NAFLD with metabolic syndrome and diabetes may only in part explain the increased risk of CVD with NAFLD.

In IR, adipose and muscle cells oxidize lipids, resulting in increased released of FFAs, which can than be incorporated into TGs in the liver, leading to steatosis, representing the first step. In presence of steatosis, an activation of an inflammatory cascade associated with a complex interaction among hepatocytes, stellate, adipose and Kupffer cells results in progression to apoptosis/necrosis (NASH), fibrosis and consequently cirrhosis (*Perazzo H et al. 2014*).

However, the exact mechanisms for this complex relationship are not clear. Probably several highly interrelated factors among IR, oxidative stress and activation of a pro-inflammatory state contribute to the enhanced risk of CVD events in persons with NAFLD (*Perazzo H et al. 2014*) (Fig. 3).

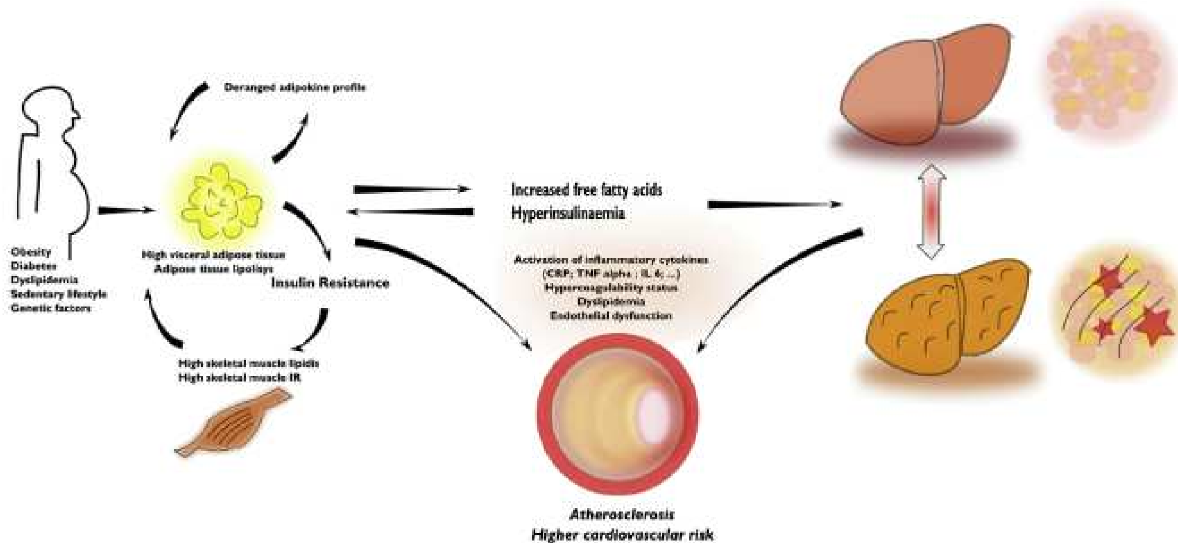

Figure 3: Possible potential mechanism of the association of NAFLD with CVD and their risk factors. The common risk factors that cause IR togheter with an altered lipid profile cause an increase in FFAs. This increase combined with the activation of the inflammatory cascade (with a complex interaction between hepatocytes, stellate, adipose and Kupffer cells) results in progression of apoptosis/necrosis (NASH), fibrosis and consequently cirrhosis. *From Perazzo H. Clin Liver Dis. 2014.*

Several factors that can explain the increased CVD risk in subjects with NAFLD; among these there are the increased lipolysis and VLDL secretion, the atherogenic lipoprotein profile, for example the increased in small dense LDL fractions and reduced in HDL fractions, the hyperglycemia due to hepatic overproduction of glucose, the increased release of inflammatory factors such as fibrinogen and C reactive protein (CRP) (*Gaggini M et al. 2013*).

Fat accumulation in the liver and oxidative stress induce the secretion of inflammatory markers such as, IL-6, TNF-α, Fetuin-A, CRP, and fibrinogen. These last metabolic abnormalities, common in subjects with NAFLD, may directly or indirectly promote atherosclerosis independently of other associated factor (*Gaggini M et al. 2013*) (Fig. 4) making NAFLD a key independent determinant of CVD. It has been hypothesized the existence of a liver vessel axis. Following this hypothesis the progression from fatty liver to NASH might be followed by the evolution from subclinical to clinical atherosclerosis (*Loria P et al. 2014*).

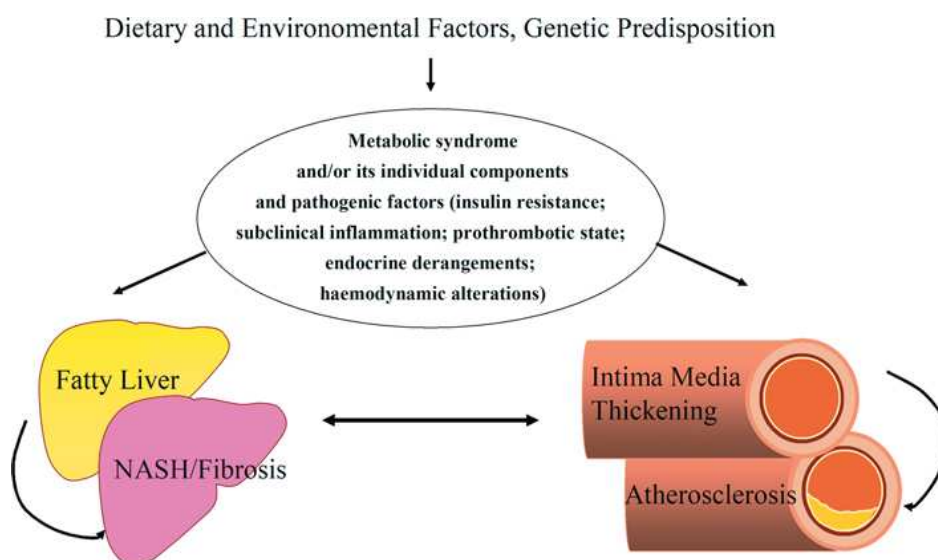

Figure 4: NAFLD and atherosclerosis share several biological precursors that suggest a similar pathogenesis and mutual influence of the two organs: in particular, the SM and its pathogenetic factors play a central role in both diseases. *From Loria P. Clin Sci (Lond.) 2008.*

Fetuin-A and biological roles in health and disease states

HISTORY - Fetuin-A is a multifunctional protein which is involved in delivery of fatty acids to cells and cholesterol efflux from cells, which may play a role in lipid transport during fetal life (*Kumbla L et al. 1991*). Not surprisingly, therefore, it was first isolated from fetal calf serum (*Mori K et al. 2011*).

Although the discovery of fetuin in bovine serum dates back to 1944 (*Mori K et al. 2011*), only in recent years several studies have focused on the role of this protein both in physiological and pathological conditions in humans.

DEFINITIONS, SYNONIMS AND GENETICS - Alpha-2-Heremans-Schmid glycoprotein (*AHSG*), also known as fetuin-A, is a protein that is encoded by the *AHSG* gene in humans (*Osawa M et al. 1997*). The Ahsg gene product belongs to a class of plasma binding proteins, in particular to the class of cysteine protease inhibitors: it is a serum glycoprotein of 55-59 kDa synthesized in the adult predominantly by hepatocytes (*Goustin AS et al. 2011*) (Fig. 5).

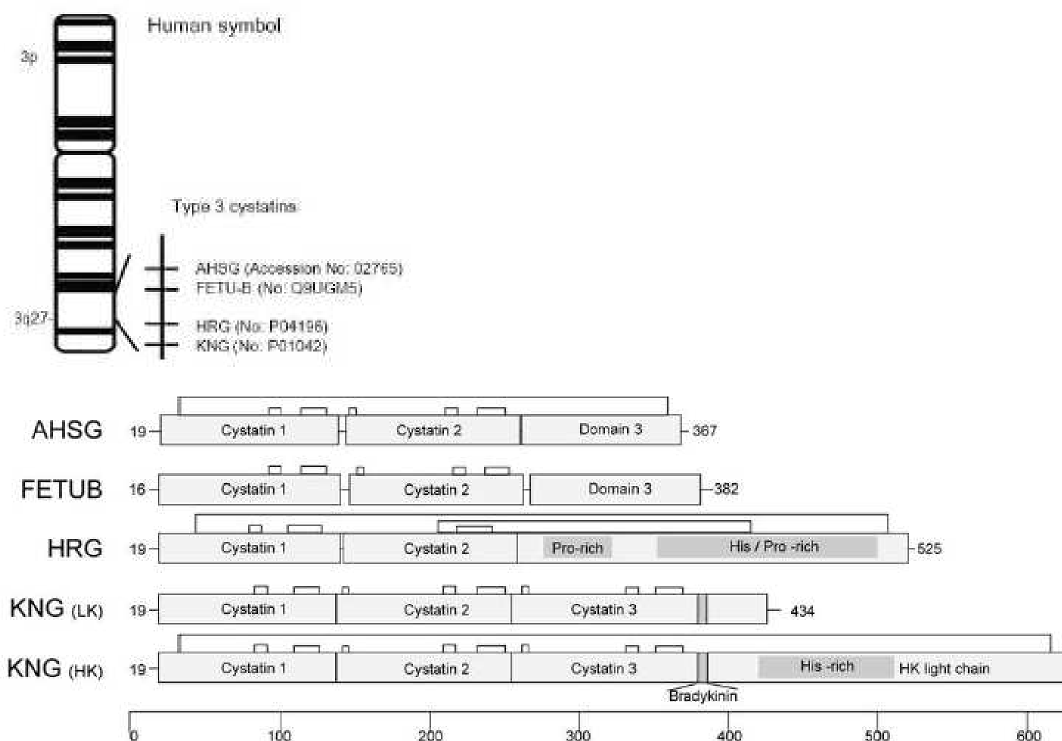

Figure 5: Organization of the type 3 cystatin family genes on human chromosome 3 and schematic structures of type 3 cystatins. *From Lee C. Front Biosci. 2009.*

A common variation (Thr230Met) in the *AHSG* gene is associated with a marked increase in beta2-adrenoceptor sensitivity in subcutaneous fat cells, which may be of importance in body weight regulation. A decreased expression of *AHSG* in men with Met/Met genotype influences human fat cell function at an early stage of the lipolytic cascade, preferably by specifically inducing β2-adrenoceptor function (*Lavebratt C et al. 2005*). Another work by Reineher and colleagues found polymorphisms of gene encoding fetuin-A associated with type-2 diabetes in children and insulin action in adipocytes (*Reinehr T et al. 2008; Jensen MK et al. 2013*).

CHEMICAL STRUCTURE - Fetuin-A has three carbohydrate units, which are present on a peptide chain linked with threonine and serine residues (*Singh M et al. 2012*) (Fig. 6).

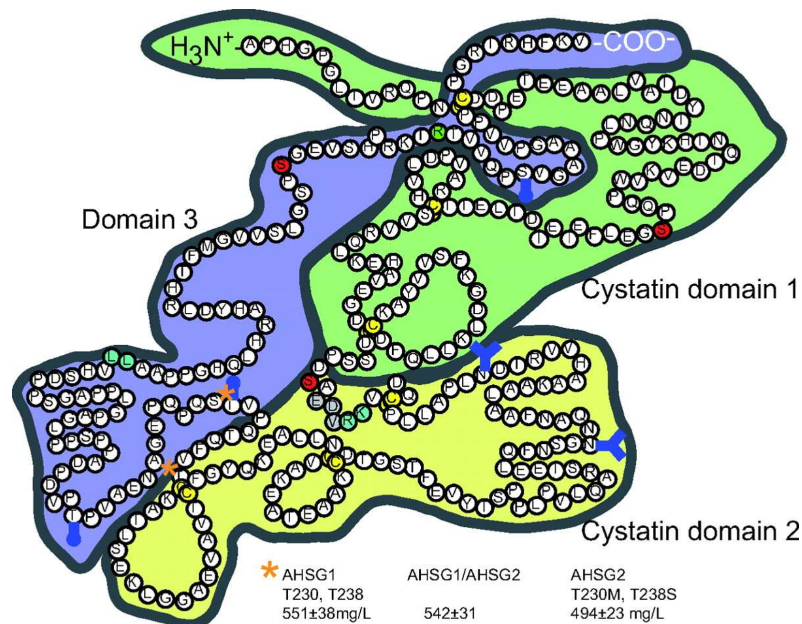

Figure 6: Cartoon of human fetuin-A/α2-HS glycoprotein showing cystatin-like domains 1 and 2 and a third unrelated domain in green, yellow, and blue shading, respectively. The cotranslational and posttranslational modifications disulfide bridges (C-C in yellow) Ser-phosphorylation sites (S in red), protease-sensitive sites (R-K, dibasic tryptic cleavage site; L-L, chymotryptic cleavage site; R-T, furin-sensitive cleavage site; all in green), allelic variants (T230/T238; M230/S238) depicted as orange asterisks, and Asn-linked complex N-glycosylation sites, and Ser/Thr O-glycosylation sites depicted as blue symbols, respectively. *From Jahnen-Dechent W. Circ Res. 2011.*

Structurally, the *AHSG* molecule consists of two tandemly arranged cystatin-like domains, which are post-translationally cleaved from a proprotein of a single mRNA and an unrelated domain (*Osawa M et al.1997*). Native alpha 2-HS glycoprotein undergoes a series of posttranslational modifications including proteolytic processing, multiple N-glycosylations and O-glycosylations, and sulfation of the carbohydrate side chains. Moreover, phosphorylation of alpha 2-HS that, like proteolytic processing, occurs only after transport to the late Golgi compartment, affects about the 20% of circulating fetuin-A (*Herrmann M et al. 2012*) (Fig. 7).

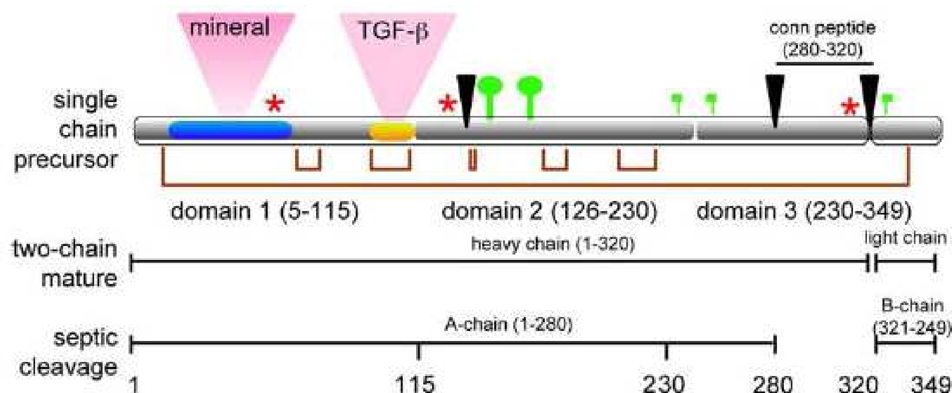

Figure 7: Post-translational modification of AHSG, a prototypic type 3 cystatin familiy member. AHSG consists of three domains, D1-D3, of ~115 amino acids each. Disulfide bridges indicated in brown rectangles are characteristic of the tandemly arranged cystatin domains D1 and D2. One interdomain disulfide links domain D3 to domain D1. Proteolytic cleavage sites are indicated by black wedges, serine phosphorylation sites are marked as red asterisks, N-glycosylation sites as large green and O-glycosylation sites as small green beacons. The so-called connecting peptide of AHSG is indicated. Binding regions for apatite and TGF-ß like growth factors are shown as pink triangles. Please note that carbohydrate binding proteins and phosphoamino acid-specific ligands may also bind and influence AHSG activity and stability. *From Lee C. Front Biosci. 2009.*

NORMAL SERUM CONCENTRATIONS - Serum fetuin-A concentration is a good indicator of liver cell function and it ranges from approximately 450-600 µg/ml in healthy individuals (*Kalabay L et al. 2007*).
Fetuin serum concentration drops after trauma, infection and malignancies through a down-regulation mechanism mediated by cytokines IL6 and IL1B; therefore, it is considered a negative acute phase protein (*Hennige AM et al. 2008*).

BIOLOGICAL ACTIVITY

Fetuin has been involved in a large variety of biological effects, which can be broadly classified into three chief categories: a) increased muscle and hepatic insulin resistance; b) increased adipose tissue insulin resistance and inflammation; and c) modulation of bone mineralization and ectopic calcification of soft tissues (*Mori K et al. 2011*) (Table 3).

Secreting cells	Modulators of secretion	Target cell	Cellular mechanism	Biological effect
Fetuin-A				
Hepatocyte	**Stimulators** ER stress ERK 1/2	Hepatocytes, Myocytes	↓ Tyrosine kinase and IRS-1 autophosphorylation → ↓ insulin receptor tyrosine kinase activity	↑ Muscle and hepatic insulin resistance
		Adipocytes	↓ Adiponectin secretion ↓ Interaction of circulating FFAs with TLR4 → ↑ FFA-induced proinflammatory adipokine secretion	↑ Adipose tissue insulin resistance and inflammation
		Bone and extracellular matrix (ECM)	Complexation with calcium and phosphate to form stable colloidal mineral–protein spheres (calciprotein particles, CPPs)	Modulation of bone mineralization and ectopic calcification of soft tissues

Table 3: Fetuin-A interaction among the liver, adipose tissue, and the bone. *Modified from Musso G. Trends Mol Med. 2013.*

a) Increased muscle and hepatic insulin resistance

As a member of the cystatin family of protease inhibitors, fetuin-A is a natural inhibitor of the insulin receptor. Indeed, the phosphorilated form of Fetuin binds to and inhibits the Insulin receptor (InsR) autophosphorilation in skeletal muscle and hepatocytes (*Srinivas PR et al. 1993; Jung CH et al. 2013*).
The interaction of insulin with the insulin receptor, leads to phosphorylation of the insulin receptor substrate proteins (IRS proteins) which are linked with two key pathways of cell signalling. First, the phosphatidyl inositol 3-kinase (PI3-K)-Akt/protein kinase B (PKB) pathway, which controls cellular glucose uptake and glycogen synthesis. Second, the Ras-mitogen activated protein kinase (MAPK) pathway that controls the cell transcription of various target genes (*Singh M et al. 2012 ; Song A et al. 2011*) (Fig. 8).

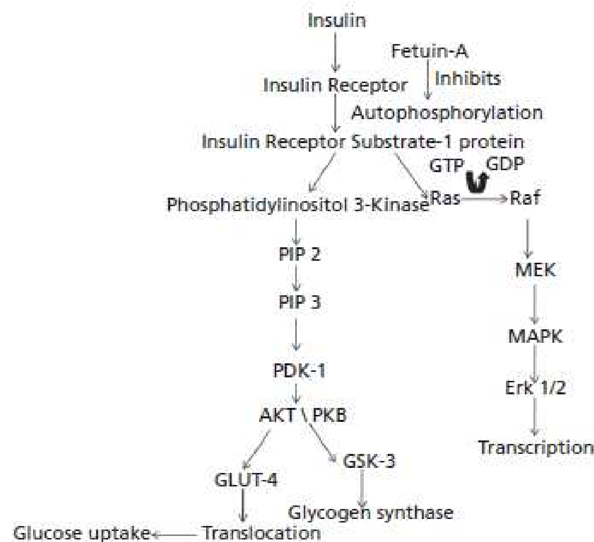

Figure 8: Insulin receptor in the presence of insulin phosphorylates the insulin receptor substrate proteins. These insulin receptor substrate (IRS) proteins are linked to the two main pathways: the phosphatidyl inositol 3-kinase (PI3-K)–AKT/protein kinase B (PKB) and the Ras–mitogen activated protein kinase (MAPK) pathway. Fetuin-A could inhibit insulin receptor tyrosine kinase activity by inhibiting the autophosphorylation of tyrosine kinase and IRS-1, which leads to insulin resistance and acts as a natural inhibitor of insulin receptor. Erk 1/2; extracellular signal-regulated kinase; GLUT4, glucose transporter-4; GSK-3, glycogen synthase kinase-3; MEK, tyrosine/threonine kinase; PDK-1, 3-phosphoinositide dependent kinase-1; PIP2, phosphatidyl inositol 4, 5 bisphosphate; PIP3, phosphatidyl inositol 1, 4, 5 triphosphate. *From Singh M. J Pharm Pharmacol. 2012.*

Fetuin-A is up-regulated in hepatic steatosis and the metabolic syndrome. These states are strongly associated with low-grade inflammation and hypoadiponectinemia. Hennige et al. found that fetuin-A suppresses adiponectin mRNA and circulating adiponectin serum levels and, on the other hand, it induces pro-inflammatory cytokine expression (like TNF, IL1B on THP1 monocytes and IL6 mRNA in adipocytes) (*Hennige AM et al. 2008*). Under this perspective, the fetuin is a pro-inflammatory molecule that stimulates the production of pro-inflammatory cytokines and modulates the synthesis of atheroprotective factors such as adipokine adiponectin (Fig. 9). Moreover Pal and colleagues suggest that fetuin-A could be an endogenous ligand for Toll-like receptor 4 (TLR4) through which lipids induce insulin resistance (*Pal D et al. 2012; Heinrichsdorff J et al. 2012*).

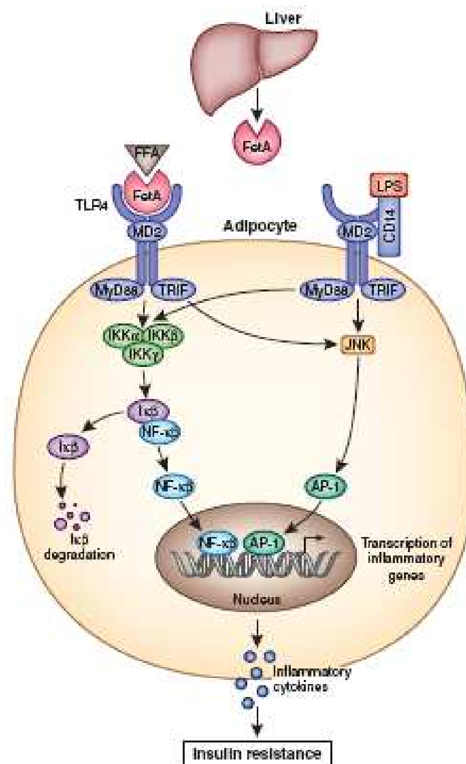

Figure 9: Fetuin-A function as an adaptor between FFAs and TLR4 signaling in lipid-induced inflammation. *From Heinrichsdorff J. Nature Medicine. 2012.*

Fetuin-A acts as a new cardiovascular risk factor and its level is associated with intima media thickness, and the severity of atherosclerosis is inversely correlated with the fetuin-A levels in serum (*Muendlein A et al. 2012; Szeberin Z et al. 2011*).

Recent studies have shown that fetuin-A serum concentration are correlated with levels of triglycerides, low-density lipoprotein (LDL) cholesterol, leptin and adiponectin accounting for the association of fetuin-A concentration with atherosclerosis via metabolic pathways including obesity, adipocyte dysfunction and insulin resistance (*Voros K et al. 2011*).

The group of Takata demonstrated that high glucose enhances *AHSG* transcription through activation of the ERK1/2 signaling pathway; therefore, increased *AHSG* expression in the liver may be a cause of glucose toxicity in the diabetic state (*Takata H et al. 2009*) (Fig. 8). The same group demonstrated also that increased serum fetuin-A levels are an independent marker of insulin resistance and atherogenic lipid profile in Japanese men (*Ishibashi A et al. 2010*).

b) Increased adipose tissue insulin resistance and inflammation

In obesity, liver fat increases and a correlation has been proposed to exist between increased concentration of fetuin-A and obesity (*Singh M et al. 2012*).

An elevated serum fetuin-A level is associated with the components of metabolic syndrome, such as high blood pressure, central obesity, high blood glucose and high triglycerides (*Xu Y et al. 2011*). Conversely, fetuin-A level decreases after weight loss (*Singh M et al. 2012*).

A recent *in vivo* study investigated the impact of weight loss on serum fetuin-A in simple steatosis and NASH. Fetuin-A serum concentrations were not increased in NAFLD patients (or subgroups) compared with

controls however while a significant decrease of inflammatory cytokines was found after surgery no significant changes of fetuin levels during weight loss were found except an increase shortly after surgery. NAFLD patients had an elevated mRNA and protein expression of fetuin-A that correlated with fetuin A concentration. No correlation of fetuin-A levels and signs of the metabolic syndrome, histological signs of NAFLD or adiponectin concentration were found (*Kahraman A et al. 2013*).

Other findings report that both non-esterified ('free') fatty acids and fetuin-A coexist at high levels in the serum of db/db mice, indicating an association between them. Palmitate stimulated NF-kappaB binding to the fetuin-A promoter resulting in increased reporter activity. These results suggest NF-kappaB to be the mediator of the palmitate effect. Palmitate-induced robust expression of fetuin-A indicates the occurrence of additional targets, and fetuin-A severely impaired adipocyte function leading to insulin resistance (*Dasgupta S et al. 2010*).

Toll-like receptor 4 (TLR4) has a key role in innate immunity by activating an inflammatory signaling pathway. Free fatty acids (FFAs) stimulate adipose tissue inflammation through the TLR4 pathway, resulting in insulin resistance. However, current evidence suggests that FFAs do not directly bind to TLR4, but an endogenous ligand for TLR4 remains to be identified. A recent work by Pal and others show that fetuin-A (FetA) could be this endogenous ligand and that it has a crucial role in regulating insulin sensitivity via Tlr4 signaling in mice. They found that FFA-induced proinflammatory cytokine expression in adipocytes occurred only in the presence of both FetA and Tlr4; removing both of them prevented FFA-induced insulin resistance. Taken together, these results suggest that FetA could be an endogenous ligand for TLR4 through which lipids induce insulin resistance. Accordingly, targeting Fetuin A may be a potential new candidate therapeutic option in managing insulin resistance and type 2 diabetes (*Pal D et al. 2012*) (Fig. 11).

c) Modulation of bone mineralization and ectopic calcification of soft tissues

Fetuin-A forms soluble complexes with calcium and phosphate and it is thus a carrier of otherwise insoluble calcium phosphate. Fetuin-A is a mineral carrier protein and a systemic inhibitor of pathological mineralization complementing local inhibitors that act in a cell-restricted or tissue-restricted fashion (*Brylka L and Jahnen-Dechent W 2013*) (Fig. 10).

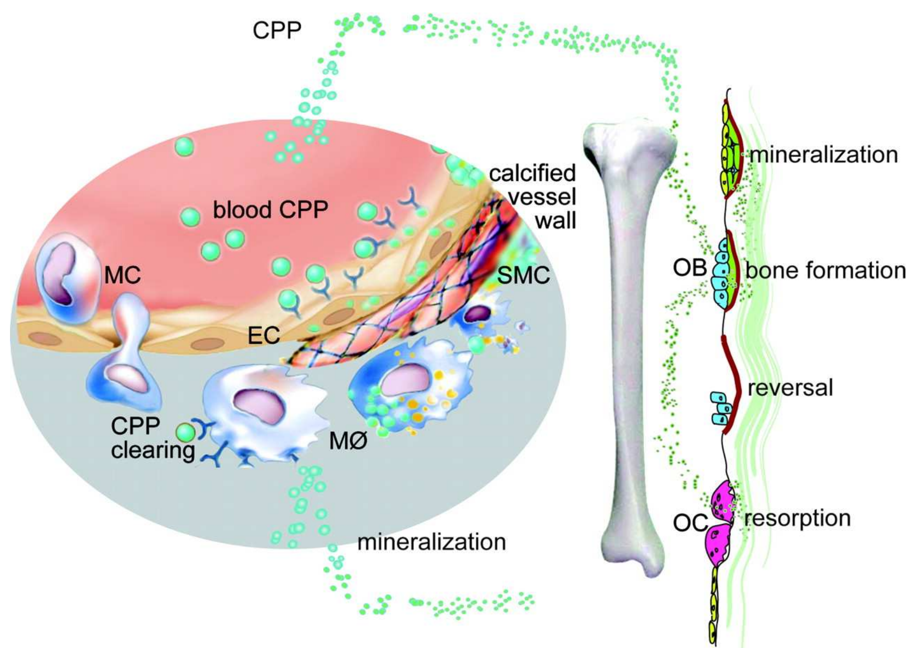

Figure 10: Hypothetical pathway of calciprotein particle (CPP) circulation and clearing by endothelial cells and tissues resident phagocytes. Fetuin-A stabilizes CPP in the circulation and mediates their efficient uptake. *From Jahnen-Dechent W. Circ Res. 2013.*

According to this view fetuin-A acts as a prototypic systemic inhibitor of mineralization as it complexes with calcium and phosphate to form stable colloidal mineral-protein spheres called calciprotein particles (CPPs). These are cleaved by tissue macrophages and hepatic Kupffer cells through scavenger receptors (*Musso G et al. 2013*). Collectively fetuin-A may be considered as mineral chaperones mediating the stabilization, safe transport, and clearance in the body of calcium and phosphate as colloidal complexes, therefore preventing ectopic calcification (*Heiss A et al. 2008*).

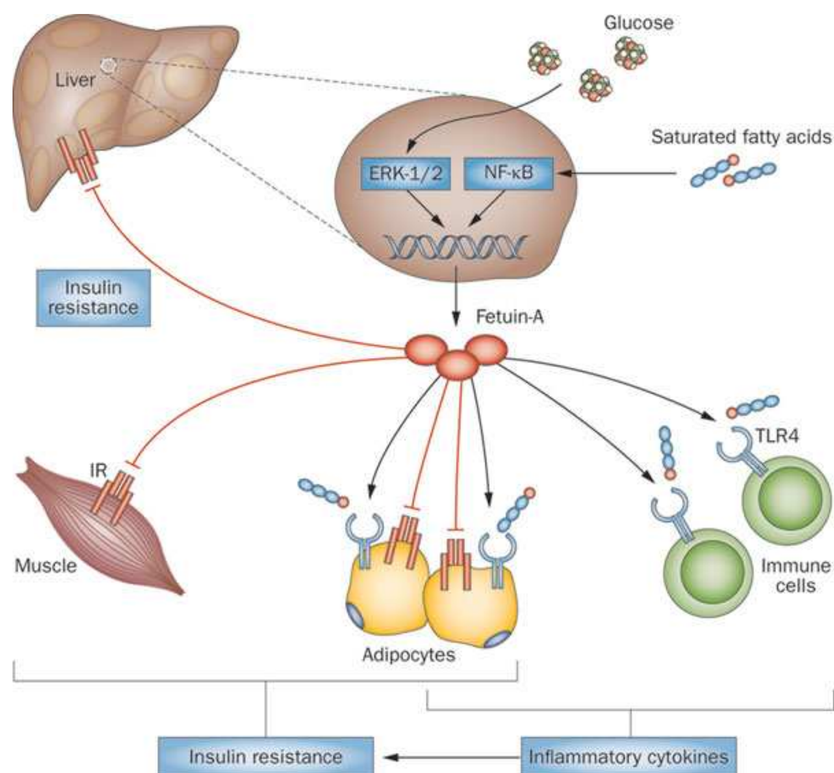

Figure 11: Hepatic production of the hepatokine fetuin-A is induced by increased glucose levels (via activation of ERK-1/2 signalling pathways) and by high saturated fatty acid levels (via activation of NF-κB). Fetuin-A is released into the circulation and inhibits insulin signalling by binding to the insulin receptor in insulin-responsive tissues, thereby inducing insulin resistance. Fetuin-A also serves as an adaptor protein for saturated fatty acids, allowing them to activate TLR4 and consequently induce inflammatory signalling and insulin resistance. *From Stefan N. Nat Rev Endocrinol. 2013.*

In cell cultures, Fetuin-A antagonizes the antiproliferative action of TGF-beta-1 binding directly to TGF-beta-1 and TGF-beta-2 and with greater affinity to BMP2, BMP4, and BMP6. Moreover, fetuin has also been shown to block binding of TGF-beta-1 to the TGF-beta receptor type 2. The serum and bone-resident fetuin binds to TGF-beta/BMP cytokines, and it blocks TGF-beta1 binding to cell surface receptors (*Szweras M et al. 2002*). Fetuin has been reported to act as a TGFβ antagonist by mimicking the TGFβ type II receptor. The results obtained by Verma-Gandhu M and colleagues indicate that the MCM obtained from patients with HCV-related liver fibrosis significantly stimulated collagen synthesis in HSCs. Collagen synthesis was also stimulated in HSCs using transforming growth factor beta (TGFβ), and this effect was neutralized using TGFβ antibody. Incubation of HSCs with fetuin (but not TGFβ antibody) significantly inhibited collagen synthesis in HSCs that were stimulated by HCV MCM samples (*Verma-Gandhu M et al. 2007*).

In conclusion fetuin A has been shown to be a multifunctional protein having an important role in main pathways involved in the pathogenesis metabolic diseases as insulin resistance, inflammation, fibrogenesis, lipid metabolism and calcium deposition in the artery wall. Data on the role of fetuin in NAFLD are still preliminary and data are needed to share light on its role in the pathogenesis of NAFLD and on the relationship with associated metabolic alterations.

AIMS OF THE STUDY

The main objectives of this project were:

1) to determine if NAFLD patients are at increased risk of developing CAD than non-NAFLD patients
2) to evaluate the relationship between plasma concentration of fetuin-A and anthropometric parameters in relation to CAD and/or NAFLD.

The results of the study were recently published: “*Relationship of Serum Fetuin-A Levels with Coronary Atherosclerotic Burden and NAFLD in Patients Undergoing Elective Coronary Angiography*” by Ballestri S, Meschiari E, Baldelli E, Musumeci FE, Romagnoli D, Trenti T, Zennaro RG, Lonardo A, Loria P and published on “*Metabolic Syndrome and Related Disorders*” 2013 (*Ballestri S, Meschiari E, Baldelli E, Musumeci FE, Romagnoli D, Trenti T, Zennaro RG, Lonardo A, Loria P. “Relationship of serum fetuin-a levels with coronary atherosclerotic burden and NAFLD in patients undergoing elective coronary angiography.” Metab Syndr Relat Disord. 2013 Aug;11(4):289-95*).

MATERIALS AND METHODS

Patients

The study included 70 patients consecutively submitted to elective coronary angiography for supected CAD between April 2010 and July 2010 at the Operating Unit (OU) of Cardiology at “Nuovo Ospedale Sant’Agostino Estense” (NOCSAE) in Baggiovara.

Patients were referred to OU of Internal Medicine and Metabolic Disorders to perform an abdominal ultrasound scanning to detect fatty liver within one week of coronary angiography.

Anamnestic, anthropometric, biochemical and ultrasonographic findings related to coronary disease patients were recorded by the OU of Internal Medicine and Metabolic Disorder. All patients selected (n=70) were subdivided in 2 groups according to the presence or absence of CAD:

- **Group 1**: 24 patients without coronary artery disease (CAD-), of which 9 with (NAFLD+/CAD-) and 15 without NAFLD (NAFLD-/CAD-);
- **Group 2**: 46 patients with coronary artery disease (CAD+), of which 20 with (NAFLD+/CAD+) and 26 without NAFLD (NAFLD-/CAD+) (Table 4).

Diagnosis	**Coronarography**		**Total**
	Positive (CAD+)	Negative (CAD-)	
NAFLD	20	9	29
Non-NAFLD	26	15	41
Total	46	24	70

Table 4: Study groups of all patients selected.

Informed written consent was obtained from all participating individuals before coronary angiography. All patients give their written consent to take part in the study.The protocl of this study was approved by the local Ethics Committee and performed in agreement with the Declaration of Helsinki.

NAFLD Diagnosis

In order to obtain a primary NAFLD diagnosis we used the following exclusion criteria for other known aetiologies of chronic liver disease: absent-to-low alcohol consumption (< 20 g/daily for women and < 30 g/daily for men; due to the low number of patients eligible for the study, 5 men with alcohol consumption < 50 g/daily were however enrolled), negativity to serological test for HCVAb and HBsAg, negativity to autoimmune disease (ANA, ASMA, LKM1 and AMA autoantibodies), proven absence of hepatotoxic drugs (anamnestic data), absence of vascular and inherited disease (alpha1-antitrypsin deficiency, hemochromatosis and Wilson disease).

Abdominal ultrasound scanning was performed to diagnose NAFLD after overnight fasting with a 3.5-5 MHz convex probe and a high-resolution B-mode scanner (Siemens Sonoline ANTARES™) in our Operating Unit of Internal Medicine and Metabolic Disorder.

Fatty liver was diagnosed based on increased liver echogenicity and the severity of steatosis was evaluated with the Ultrasonographic-Fatty Liver Indicator (US-FLI), a semi-quantitative ultrasonographic score recently proposed by our group as a diagnostic indicator (*Ballestri S et al. 2012*).

US-FLI ranges from 2 to 8. The condition necessary for the diagnosis of steatosis is the presence of liver/kidney echo contrast, graduated as mild/moderate (score 2) and severe (score 3). Additional criteria include the presence (score 1 for each one) or absence (score 0 for each one) of posterior attenuation of ultasound beam, vessels blurring, difficult visualization of gallbladder wall or diaphragm and areas of focal sparing. For the ultrasonographic diagnosis of steatosis, the minimum score required was a US-FLI ≥ 2 (*Ballestri S et al. 2012*).

Anamnestic data

All patients underwent an interview aimed at investigating their family and personal medical history including: previous diagnosis of type 2 diabetes, hypertension, altered lipid metabolism or dyslipidemia, current use of drugs associated with NAFLD or affecting lipid metabolism (like hypoglycemic, antihypertensive, lipid-lowering agents), previous diagnosis of thyroid diseases, familiar history of cardiovascular diseases or previous cardiovascular accidents, previous surgery, dietary habits, smoking (packages/year), alcohol consumption (g/daily), past and current use of medications or possible contacts with toxic agents associated with epatotoxicity.

Anthropometric parameters

In all patints weight (kg) and height (m) were measured and Body Mass Index (BMI) was calculated by the formula: [(kg)/height $(m)^2$]. BMI was used as a classification index of overweight and/or obesity according to the following table:

CLASSIFICATION	**BMI (kg/m^2)**
Underweight	<18.5
Normal weight	18.5 – 24.9
Overweight	25 – 29.9
Obesity class I	30 – 34.9
Obesity class II	35 – 39.9
Obesity class III	≥ 40

Table 5: Classification according to BMI.

Waist (cm), hip (cm) circumpherence and waist to hip ratio (W/H ratio) were also measured. Waist circumference is one of the parameters for the diagnosis of the MS. According to the ATPIII criteria (*National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). "Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation. 2002*) abnormal values are >88 cm in women and >102 cm in men. The W/H ratio is a further indicator of overweight complications and abnormal values are >0.85 in women and >1 in men.

Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) diameter were determined as described by Pontiroli and colleagues (*Pontiroli AE et al. 2002*) by using US scanning.

Finally we measured the patient's blood pressure at rest using, as a cut-off of high blood pressure, a measurement of 130/85 mm Hg.

Laboratory tests

Blood samples (20 cc) were collected after an overnight fasting. Laboratory evaluation included: blood cell count; coagulation parameters (PT, INR, aPTT ratio, fibrinogen); alanine and aspartate aminotransferases (ALT, AST); gamma-glutamyl transpeptidase (GGT); alkaline phosphatase (ALP); total and direct bilirubin; total and fractionated proteins; serum total cholesterol (CH); high-density lipoprotein (HDL) - CH; low-density lipoprotein (LDL) - CH; triglycerides (TG); fasting glucose; insulin; uric acid and urea; creatinine; electrolytes (Na^+, K^+); thyroid stimulating hormone (TSH); free thyroxine (FT4) and triiodothyronine (FT3); C-reactive protein (CRP).

In order to exclude other etiologies of chronic liver disease the following serological test were also performed: serum iron, transferrin, ferritin, transferring saturation; screening for major hepatotropic viruses (HBsAg and HCVAb), an evaluation of the presence of autoantibodies (ANA, ASMA, LKM1, AMA), serum concentration of copper, ceruloplasmin and alpha-1 antitrypsin.

Insulin Resistance (IR) was calculated according to the homeostasis model assessment (HOMA-IR) index as follows: HOMA index = [glycemia (mg/L) x serum insulin (µU/ml)] / 405 (*Matthews DR et al. 1985*).

LDL cholesterol was determined with Friedwald calculation: LDL-CH = total cholesterol CH - [HDL-CH + (TG/5)].

Fetuin-A analysis

Fetuin-A concentration was determined following the protocol of the commercial available kit "HUMAN FETUIN-A ELISA" (BioVendor, Czech Republic). All serum samples were processed in duplicate after a 1:10.000 dilution. Fetuin-A concentration in humans ranges from 450 to 600 µg/ml (*Singh M et al. 2012*).

Coronary Angiography (Coronarography)

All patients enrolled in the study underwent selective left and right coronary angiography performed in the hemodynamic laboratory of OU of Cardiology using a Brilliance Intensifier General Electric Innova (GE Healthcare, USA).

Based on literature data (*Leaman DM et al. 1981; Acikel M et al. 2009 and Assy N et al. 2010*), the presence of CAD was evaluated according to a 50% or greater diameter stenosis in epicardial coronary arteries or their major branches.

Gensini scoring system (GSS) was used to determine the severity of coronary atherosclerosis (*Gensini GG 1983*). The Gensini score was compute by quantifying coronary stenosis according to the degree of luminal narrowing and its anatomo-topographic importance (*Ballestri S et al. 2013*).

Comorbidities diagnosis

- T2DM diagnosis (according to American Diabetes Association): presence of serum fasting glucose levels ≥126 mg/dl or history of diabetes or current use of hypoglycemic drugs;
- Dyslipidemia diagnosis (according to NCEP ATP III): presence of hypercholesterolemia with total serum cholesterol values ≥200 mg/dl and hyper triglyceridemia with triglycerides serum levels ≥150 mg/dl;
- Hypertension diagnosis: presence of blood pressure >130/85 mmHg or previous history of hypertension or current use of antihypertensive drugs;
- Thyroid disease diagnosis: presence of anamnestic data of thyroid disease or current use of drugs to treat hyper/hypo-thyroidism or abnormal values of TSH (normal range 0,35-4,50 µIU/ml), FT4 (normal range 6,1-16,7 pg/ml) and FT3 (normal range 1,7-4,2 pg/ml);
- Metabolic Syndrome (MS) diagnosis according to American Heart Association (AHA) criteria (*Lonardo A et al. 2009*).

Statistical analysis

Statistical anlyses were performed using the statistical software package SPSS, version 18.0 for Windows (SPSS Inc., IL, USA).

Kolmogorov-Smirnov test was used to assess the normality of variables; results were expressed as mean ± SD (standard deviation) for continuous variables normally distributed; as median (25°-75° percentile) for those not normally distributed (diastolic blood pressure, ALT, AST, GGT, glucose, insulin, HOMA, creatinin and TSH); and as frequencies (percentages) for categorical variables.

Comparison between the means of continuous variables normally distributed were performed using the one-way analysis of variance (ANOVA) or Student's t-test, while the Kruskal-Wallis or Mann-Whitney test was used for non-normally distributed or ordinal variables. Fisher's exact test was used to compare nominal variables.

Stepwise multivariable logistic regression analysis were performed to identify single independent predictors of NAFLD and CAD (dependent variables). Exp (B) and 95% confidence intervals (CI) were reported with p values. In order to choose variables to be processed at the multivariate analysis, three steps were followed:

- first, based on generally available literature and on clinical judgment, a certain number of variables were selected;
- next a univariate model was generated;
- and finally, those variables that were identified as significant predictors for NAFLD or CAD at univariate analysis ($p<0.05$) were entered in the multivariate analysis.

The multivariate analysis to assess the independent predictors of NAFLD was performed twice to analyze the importance of two major correlates of NAFLD: BMI and MS separately. *Model 1* included age, gender and BMI as covariates and processed those variables found to be significant at univariate analysis. *Model 2* included age, gender and MS as covariates and processed those variables found to be significant at univariate analysis (*Ballestri S et al. 2013*).

A two-sided p value <0.05 was considered to be significant.

RESULTS

The study population consisted of 70 patients. We studied the same clinical, biochemical and ultrasonographic parameters by two groups of patients separately: patients with and without CAD were compared first (Table x) and next those with and without NAFLD (Table 6).

	CAD + (46)	CAD- (24)	P
Age (years)	67.6±11.4	68.9±13.6	0.686
Male gender, n (%)	35 (71.6)	13 (54.2)	0.102
BMI (Kg/m^2)	27.4±4.1	26.9±4.6	0.620
Waist Circ. (cm)	100.4±11.7	99.4±14.4	0.765
SBP (mmHg)	134.5±15.5	135.4±13.8	0.800
DBP (mmHg)	80.0 (75.0-85.0)	80.0 (76.2-88.7)	0.380
Smoke, n (%)	6 (26.1)	17 (37.0)	0.426
Hypertension, n (%)	36 (78.3)	15 (62.5)	0.171
Hyperlipidemia, n (%)	34 (73.9)	16 (66.7)	0.583
Diabetes, n (%)	11 (23.9)	5 (20.8)	1.000
MS, n (%)	22 (47.8)	12 (50.0)	1.000
NAFLD, n (%)	20 (43.5)	9 (37.5)	0.799
WBC (migl./mmc)	7.57±2.4	6.75±2.0	0.161
Platelets (migl./mmc)	193.0±51.0	181.7±48.4	0.377
AST (UI/l)	22.0 (18.4-26.0)	22.6 (20.2-31.6)	0.304
ALT (UI/l)	26.0 (19.0-36.8)	29.2 (18.2-42.7)	0.435
GGT (UI/l)	24.0 (18.0-29.5)	20.2 (15.5-28.8)	0.335
Total CH (mg/dl)	173.8±31.9	184.8±46.6	0.308
LDL-CH (mg/dl)	107.6±31.5	111.7±39.7	0.158
HDL-CH (mg/dl)	40.2±11.0	45.2±17.6	0.221
TG (mg/dl)	130.5±59.8	106.7±52.8	0.106
Glucose 0' (mg/ml)	98.0 (92.5-114.0)	96.0 (83.2-117.8)	0.371
Insulin 0' (µIU/ml)	5.3 (3.2-8.1)	4.8 (2.825-6.9)	0.409
HOMA	1.3 (0.8-2.0)	1.1 (0.70-1.7)	0.351
Uric acid (mg/dl)	6.2±1.1	5.9±1.8	0.416
GFR (mL/min/1.73m^2)	74.8±22.6	68.7±22.8	0.287
TSH (UI/ml)	1.4 (0.9-2.1)	1.3 (0.8-2.1)	0.738
Fetuin (µgr/ml)	374.0±123.9	445.8±146.5	**0.038**
Spleen area (cm^2)	39.6±8.0	44.0±7.4	0.197
SAT (mm)	14.4±5.3	15.2±8.0	0.626
VAT (mm)	61.7±23.9	46.0±27.0	**0.020**
VAT/SAT ratio	4.7±2.0	3.4±2.0	**0.020**
Drugs			
anti-hypertensive, n (%)	36 (78.3)	13 (54.2)	0.054
lipid-lowering, n (%)	31 (67.4)	10 (41.7)	**0.045**
hypoglycemic, n (%)	10 (21.7)	5 (20.8)	1.000

Table 6: Clinical, biochemical and US characteristics of patients according to presence or absence of CAD. Data are expressed as mean ± SD and median (25th-75th percentile) for continuous variables normally and not normally distributed. as frequencies (percentages) for categorical variables.
T-student's test for normally distributed variables. Mann-Whitney test for non-normally distributed variables. Fischer's Exact test for proportions.
ALT= Alanine aminotransferase; AST= Aspartate aminotransferase; BMI= Body Mass Index; CH= Cholesterol; DBP= Diastolic blood pressure; GGT= Gamma glutamil transferase; HDL= High Density Lipoprotein; HOMA= Homeostasis Model Assessment of Insulin Resistance; MS= Metabolic syndrome; SAT= Subcutaneous adipose tissue; SBP= Sistolic blood pressure; TG= Triglycerides; VAT=Visceral adipose tissue; WBC= White blood cells count.

	NAFLD (29)	Non-NAFLD (41)	P
Age (years)	64.5±10.5	70.6±12.7	**0.039**
Male gender, n (%)	20 (69.0)	28 (68.3)	1.000
BMI (Kg/m^2)	29.2±5.0	25.8±3.1	**0.003**
Waist Circ. (cm)	104.8±13.5	96.6±10.9	**0.007**
SBP (mmHg)	135.7±14.7	134.1±15.1	0.672
DBP (mmHg)	80.0 (80.0-90.0)	80.0 (72.5-85.0)	0.121
Smoke, n (%)	12 (41.4)	11 (27.5)	0.302
Hypertension, n (%)	22 (75.9)	29 (70.7)	0.786
Hyperlipidemia, n (%)	25 (86.2)	25 (61.0)	**0.031**
Diabetes, n (%)	11 (37.9)	5 (12.2)	**0.019**
MS, n (%)	19 (65.5)	15 (36.6)	**0.028**
CAD, n (%)	20 (69)	26 (63.4)	0.799
WBC (migl./mmc)	7.1±1.7	7.4±2.7	0.625
Platelets (migl./mmc)	199.1±54.5	182.1±46.1	0.163
AST (UI/l)	22.0 (19.0-25.7)	22.0 (19.0-28.6)	0.718
ALT (UI/l)	27.5 (19.4-41.8)	26.9 (17.3-39.9)	0.638
GGT (UI/l)	22.45 (19.0-27.7)	21.8 (14.6-31.1)	0.501
Total CH (mg/dl)	186.1±43.1	171.8±32.8	0.123
LDL-CH (mg/dl)	116.1±40.9	104.2±28.5	0.188
HDL-CH (mg/dl)	40.6±11.8	42.9±15.0	0.493
TG (mg/dl)	140.9±63.9	109.4±50.9	**0.026**
Glucose 0' (mg/ml)	101.0 (89.5-127.5)	96.0 (88.5-105.5)	0.171
Insulin 0' (µIU/ml)	5.7 (3.7-8.7)	4.3 (2.7-6.5)	0.134
HOMA	1.5 (1.0-2.3)	0.9 (0.6-1.6)	**0.042**
Uric acid (mg/dl)	5.9±1.3	6.2±1.4	0.342
GFR (mL/min/1.73m^2)	73.2±24.7	72.3±21.4	0.876
TSH (UI/ml)	1.4 (1.0-2.2)	1.4 (0.8-2.2)	0.416
Fetuin (µgr/ml)	429.5±134.3	376.5±133.5	0.115
Spleen area (cm^2)	46.4±6.1	38.1±7.4	**0.008**
SAT (mm)	16.9±6.8	13.1±5.5	**0.017**
VAT (mm)	63.1±27.8	51.7±23.8	0.084
VAT/SAT ratio	4.11±2.1	4.35±2.1	0.664
Drugs			
anti-hypertensive, n (%)	21 (72.4)	28 (68.3)	0.795
lipid-lowering, n (%)	23 (79.3)	18 (43.9)	**0.004**
hypoglycemic, n (%)	10 (34.5)	5 (12.2)	**0.038**

Table 7: Clinical, biochemical and US characteristics of NAFLD and non-NAFLD patients. Data are expressed as mean ± SD and median (25th-75th percentile) for continuous variables normally and not normally distributed. as frequencies (percentages) for categorical variables.
T-student's test for normally distributed variables. Mann-Whitney test for non-normally distributed variables. Fischer's Exact test for proportions.
ALT= Alanine aminotransferase; AST= Aspartate aminotransferase; BMI= Body Mass Index; CH= Cholesterol; DBP= Diastolic blood pressure; GGT= Gamma glutamil transferase; HDL= High Density Lipoprotein; HOMA= Homeostasis Model Assessment of Insulin Resistance; MS= Metabolic syndrome; SAT= Subcutaneous adipose tissue; SBP= Sistolic blood pressure; TG= Triglycerides; VAT=Visceral adipose tissue; WBC= White blood cells count.

Features of CAD+ vs CAD- patients

Patients with and without CAD were comparable for age and gender. A statistically significant difference was found between the two groups in fetuin-A serum levels, VAT thickness and VAT/SAT ratio. Fetuin-A levels were lower while VAT and VAT/SAT ratio were higher in patients with CAD. The prevalence of NAFLD, despite being greater for patients with CAD, did not reach a statistically significant difference by the presence (43.5%) or absence (37.5%) of CAD (p=0.799), probably as a results result of the limited number of patients recruited (Table 6).

Features of NAFLD+ vs NAFLD- patients

Patients with NAFLD were younger than those without NAFLD. BMI, waist circumference, TGs levels, fasting glucose, HOMA, spleen area and SAT thickness as well as the prevalence of metabolic derangements (hyperlipidemia, diabetes and MS) were significantly higher in NAFLD patients. Fetuin-A levels were higher in patients with NAFLD as compared to those without NAFLD but the difference was not statistically significant (p=0.115) (Table 7).

Independent predictors of CAD and NAFLD

Parameters that were found to be statistically significant for the descriptive analysis and those selected based on clinical judgement and literature data, were studied with multivariate stepwise logistic regression to identify independent predictors of CAD and NAFLD.

Using the positive outcome of coronary angiography (CAD+) as the dependent variable, it was constructed a model in which the fixed covariates are gender, age and NAFLD while the independent variable studied were fetuin-A levels. Data have shown that decreased fetuin-A levels independently predicted CAD in this study population (OR=0.995, p=0.049) (Table 8).

The table x shows the logistic regression for NAFLD: sex and age were included as fixed covariates; next were stepwise placed in the analysis BMI, MS and fetuin-A. At multivariate analysis we used two models: in the first the fixed covariates are gender, age and BMI, in the second we fixed sex, age, and MS. Fetuin-A (OR=1.005, p=0.036) togheter with BMI (OR=1.298, p=0.005) were independent positive predictors of NAFLD in *Model 1* (in which BMI was used as fixed covariate) (Table 9, panel A). However, fetuin-A was no longer significant in *Model 2* in which MS, used as fixed covariate, turned out to be the only independent predictor of NAFLD (OR=3.556, p=0.022) (Table 9, panel B)

	B	SE	OR	95% CI	P
Age	0.015	0.028	1.016	0.962-1.072	0.579
Female gender	-1.118	0.682	0.327	0.086-1.244	0.101
NAFLD	0.908	0.645	2.478	0.700-8.778	0.160
Fetuin-A	-0.005	0.002	**0.995**	0.991-1.000	**0.049**
Constant	1.507	2.146	4.512		0.483

Table 8: Independent predictors of CAD at multivariate analysis. Fixed covariates: gender, age and NAFLD.

	B	SE	OR	95% CI	P
Age	-0.037	0.027	0.963	0.914-1.016	0.168
Female gender	0.523	0.682	1.687	0.444-6.415	0.443
BMI	0.261	0.092	**1.298**	1.084-1.553	**0.005**
Fetuin-A	0.005	0.003	**1.005**	1.000-1.010	**0.036**
Constant	-7.045	3.764	0.001		0.061

Table 9, Panel A: Independent predictors of NAFLD at multivariate analysis. *Model 1*, fixed covariates: gender, age and BMI.

	B	SE	OR	95% CI	P
Age	-0.050	0.026	0.951	0.905-1.000	0.052
Female gender	0.368	0.651	1.445	0.403-5.175	0.572
SM	1.269	0.553	**3.556**	1.202-10.519	**0.022**
Constant	3.080	1.797	21.751		0.087

Table 9, Panel B: Independent predictors of NAFLD at multivariate analysis. *Model 2*, fixed covariates: gender, age and MS. Fetuin-A was not kept in this model.

After excluding diabetic patients (n=16) fetuin-A was no longer an independent predictor of CAD and NAFLD at multivariate analysis. VAT/SAT ratio independently predicted CAD (OR=4.210, p=0.031), while BMI (in *Model 1*, OR=1.224, p=0.011) and MS (in *Model 2*, OR=2.990, p=0.040) independently predicted NAFLD.

Correlation analysis of Fetuin-A, Gensini score and US-FLI

In the whole population study we did not found a significant correlation between fetuin-A and metabolic parameters. We found a negative correlation (Pearson's r: -0.341) between fetuin-A and WBC (p=0.005). Fetuin-A was negatively correlated with Gensini score (Spearman's ρ=-0.292) in patients with CAD, in line with the results of multivariate analysis, although the statistical significance was borderline (p=0.064). Correlation analysis by Spearman's ρ were performed between US-FLI and clinico-laboratory parameters in patients with NAFLD, and between Gensini score and clinical-laboratory parameters in those with CAD. US-FLI was correlated with antropometric indices and metabolic parameters (BMI: ρ=0.407, p=0.035; waist

circumference: ρ=0.416, p=0.031, insulin: ρ=0.494, p=0.010) but not with fetuin-A or the severity of Gensini score.

Gensini score was positively correlated with serum glucose levels (ρ =0.362; p=0.020) and negatively with SAT thickness (ρ =-0.339; p=0.040).

On the basis of literature data we compared fetuin-A levels according to diagnosis of diabetes or MS in the whole population study. Fetuin-A levels were higher in diabetics (408.4±40.4) than in non-diabetics (395.88±18.06) as well as in patients with MS (406.9±22.1) than in those without it (390.7±24.8), however in both cases the differences were not statistically significant (p=0,518 and p=0,292 respectively) (Table 10).

	MS+	**MS-**	**P**
Fetuin-a	406,87±22,08	390,72±24,82	0,292
	Diabetes+	**Diabetes-**	
Fetuin-a	408,36±40,43	395,88±18,06	0,518

Table 10: Correlation between Fetuin-A serum levels and MS or diabetes.

DISCUSSION

NAFLD is a clinico-pathologic syndrome characterized by ectopic fat accumulation associated with a chronic inflammatory state, in which IR, abnormal adipose tissue, oxidative stress and excessive FFAs are the main actors. These complex interactions results in a low-grade chronic inflammation, endothelial dysfunction and hypercoagulability status that results in potential progression of atherosclerosis and severe CVD outcomes (*Perazzo H et al. 2014*).

Major studies have reported that patients with NAFLD are at higher risk of atherosclerosis, CVD mortality and overall mortality compared with the general population, independently of known metabolic factors.

Accumulating evidence supports the link between NAFLD and CAD. Mirbarghery and colleagues were first in reporting an association between angiographically documented CAD and NAFLD, independently of other risk factors (*Mirbargheri SA et al. 2007*). Wong and colleagues confirmed this finding.

This study shows that NAFLD and coronary artery disease are not associated. However the fact that data show that among patients with positive coronary angiography (CAD+) 65.7% had NAFLD, while among those with NAFLD 43.3% had a negative coronary angiography (CAD-), suggest that the limited sample size might have produced a statistical falsely negative result.

The chief aim of this study was to evaluate if fetuin-A might modulate the cardiovascular risk in relation to the presence of NAFLD. Fetuin-A acts as an endogenous inhibitor of the tyrosine kinase (TK) insulin receptor in liver and muscle cells, therefore causing the development of IR in these tissues; moreover circulating fetuin-A serum levels are independently associated with MS and its components (*Dogru T et al. 2013*). Elevated fetuin-A levels also associate with NAFLD, however there are no data regarding the relationship between fetuin-A and increased CVD in NAFLD (*Dogru T et al. 2013*). In our study, the levels of fetuin-A did not correlate significantly with MS (p=0.292), although they were higher in patients with MS than in patients without. Again this could be a false negative value due to the limited size of the sample.

In this study multivariate analysis showed that fetuin-A predicts NAFLD independent of other metabolic factors, in agreement with recent data reported by Haukeland (*Haukeland JW et al. 2012*). Moreover a

recently published paper by Dogru demonstrated for the first time that circulating fetuin-A in NAFLD is independently associated with endothelial dysfunction and subclinical atherosclerosis in a study cohort that enrolled 115 NAFLD patients and 74 age-matched healthy controls (*Dogru T et al. 2013*). Following inclusion of the MS in the multivariate analysis model fetuin-A is no longer an independent predictor of NAFLD suggesting that the association of NAFLD and fetuin-A serum levels is largely mediated by the presence of MS.

Fetuin A is an attractive biological mediator of atherogenesis: given that **this glycoprotein prevents ectopic** calcifications, thus a reduction of fetuin-A concentrations might, in principle, promote calcifications of heart and vessels (*Mori K et al. 2012*). Despite these theoretical considerations, the role of fetuin-A as a cardiovascular risk factor remains controversial.

Multivariate analysis in this study showed **fetuin-A to be an independent predictor of CAD** in the whole population under evaluation (p=0.049). Interestingly, fetuin-A was negatively correlated with WBC in this study, suggesting that **decreased fetuin-A levels could be associated with atherogenesis** via increased subclinical inflammation. Such a view needs to be validated by assessing hs-CRP and the pro- (IL-6, IL-1B, TNF-alfa) and anti-inflammatory (adiponectin) cytokine profile in future studies. At partial variance with our data Dogru and colleagues found that fetuin-A positively correlated with TGs, HOMA-IR, ADMA (asymmetric dimethyl arginine), cIMT (carotid artery intima-media thickness); and negatively correlated with HDL-CH and adiponectin (*Dogru T et al. 2013*).

In this study patients with and without CAD, further to different fetuin-A serum concentrations, had different VAT thickness and VAT/SAT ratio. VAT has emerged as a key organ contributing to the development of CAD and increased visceral adiposity is significantly associated with the severity of CAD, even in subject without central obesity (*Lee YH et al. 2010*). No differences were found in the prevalence of classical CV risk factors (age, smoking, dyslipidemia, hypertension) in patients with/without CAD, probably due to the limited sample size. Moreover, although the prevalence of dyslipidemia was not different between CAD+ and CAD- groups, the percentage of patients taking lipid-lowering drugs was higher in CAD+ subgroup (p=0.045).

In conclusion, fetuin-A might represent a bridge between hepatic steatosis and CVD, although it is not yet clear completely clear how it is involved in determining cardiovascular risk. This glycoprotein increases in NAFLD, IR and MS, which are major risk factors for CVD, and decreases in patients with CVD. This could be due to the fact that one of the main actions of fetuin-A is to inhibit ectopic calcification, as shown in various studies both *in vitro* and *in vivo* (*Schäfer C et al. 2003; Merx MW et al. 2005*). In the recent work by Dogru and colleagues they found an association between fetuin-A and cIMT in subject with biopsy proven NAFLD, suggesting that fetuin-A might play a different and multifunctional role in the pathogenesis of atherosclerosis. It might be important even at the beginning of atherosclerotic disease and may have a protective role for the progression of atherosclerosis in the early stages of NAFLD (*Dogru T et al. 2013*). It could be speculate that a complex relationship between fetuin-A and atherosclerotic process should exist.

The major limit of this study is the limited study sample size, that might have impaired the statisctical power of the analysis. However, despite this, the study shows statistically significant data supporting a strong association between fetuin-A, NAFLD and CAD. Our findings are in agreement with recent published data of Dogru et al. that found an association between circulating fetuin-A levels in NAFLD and endothelial dysfunction and subclinical atherosclerosis (*Dogru T et al. 2013*).

In conclusion this study showed that increased fetuin-A levels is an independent predictor of NAFLD, and, on the other hand, decreased fetuin-A levels are associated with a lower risk of CAD diagnosed at elective coronarography.

NAFLD is almost universally deemed to be a cardiovascular risk factor (*Perazzo H et al. 2014*), but it remains unclear how we move from NAFLD, a state featuring elevated fetuin-A levels, through CAD which, based on these findings, displays decreased protein levels. Low levels of fetuin-A increase the risk of CAD and, therefore, this protein could be used to obtain a more adequate assessment of patients cardiovascular risk. However, further studies are needed to better understand the mechanism of action of fetuin-A in cardiovascular disease associated with NAFLD and what might be the factors that predict circulating levels of fetuin-A.

In addition to these results, our research group has recently investigated the prevalence of NAFLD and the serum levels of fetuin-A in 4 different anatomical sites: carotid arteries, lower extremities, abdominal/ thoracic aorta and coronary arteries to further clarify the role of fetuin in the relationship between NAFLD and CVD. Our results show that serum concentrations of fetuin-A were significantly different between the four groups with the highest values in the CAD+ group, furthermore in each group the fetuin-A was more elevated in patients with NAFLD than in non-NAFLD (data not shown).

Collectively these data seem to suggest that atherosclerotic disease in different vascular anatomical sites is associated with multiple risk factors and fetuin-A may be involved in atherosclerosis associated with NAFLD.

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