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Antioxidant diet and genotyping as tools for the prevention of liver disease

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Abstract. – It is well-known that 75% of risk factors of chronic liver disease (CLD) are related to nutrition. These circumstances potentially progress towards liver steatosis, fibrosis and hepatocellular carcinoma (HCC). It still represents an enormous problem for the economy of public health worldwide. Furthermore, validated prevention programs could be the solution.

Recent knowledge in understanding molecular determinants of energy liver metabolism and new genetic markers offers new insights into the pathogenesis of CLD and HCC. The main rationale of the present issue is to provide a summary of recent insights into the inherited variants regulating lipid metabolism (steatohepatitis) and acquired mutation for early diagnosis of HCC, specifically focusing on the significance of antioxidant agents and genotyping tests as a cost-effectiveness tool for the prevention of liver disease.

Several national healthy programs worldwide promote the daily use of antioxidant nutrients either for the prevention and/or as complementary and alternative medicines (CAM). This review could be advising for the planning of a large-scale clinical trial including a combination strategy of antioxidant agents and genotyping tests in patients with high risk of CLD.

Key Words:
NASH, NAFLD, Pharmacogenetics, Genotyping methods, Cost-effectiveness.

Introduction

The liver performs the regulation of diverse vital functions, like as metabolism, secretion, and storage. The metabolic activities are linked to its capability either to detoxifying endogenous substances of organisms (waste metabolites) and/or exogenous (xenobiotics).

The non-metabolic activity is primarily: supplying energy, synthesize crucial agents (carbohydrates, fats, proteins, enzymes and hormones), vitamins storage, secretion of enzymes via bile. Hepatic diseases have been recorded since the 1965s by many researchers and they still represent a problem for the economy of healthcare worldwide[1]. Hepatic disease includes: damaging of hepatocytes, tissues, and structure. Moreover, virus, parasites, neoplastic cells and autoimmune diseases (immune hepatitis, primary biliary cirrhosis), are the most causative biological factors of this damaging process. In addition, liver tissue damage, could be procured by several chemical agents, such as carbon tetrachloride (CCl_4), thioacetamide, dimethylnitrosamine (DMN), D-galactosamine/lipopolysaccharide (GalN/LPS, a significant number of drugs (high-doses of Paracetamol, Isoniazid, etc.), and incontestably, excessive assumption of alcohol[2].

Individuals with high-risk of hepatic disease are: (1) who have viral hepatitis (HBV or HCV); (2) who have hepatic steatosis may result in oxidative hepatocellular damaging, inflammation fat accumulation, and transformation of tissue in fibrosis, a process called, non-alcoholic steatohepatitis (NASH)[3]. These circumstances potentially progress towards liver cirrhosis and HCC[4].

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Newly identified genetic risk variants could provide a practical implement for the outcome and clinical management and of patients with CLD and/or HCC. The main rationale of this narrative review is to offer an overview of recent insights into the genetics of liver fat accumulation (steatohepatitis) and HCC, specifically focusing on the importance of antioxidant agents and inherited variants regulating lipid metabolism.

Currently, the pharmacological treatment of NASH is still lacking, and these new genetic insights should lead to the identification of new therapies.

Despite the huge advances in modern medicine, there are no helpful drugs that offer absolute safety to the organ. Moreover, often, therapy combining two or more drugs could induce severe side effects, especially, in the so-called frail patients[5].

To prevent unpredictable drug-botanicals interactions, it is imperative to know the individual metabolic profile by detecting Cytochrome P450 (CYP450) gene variants[6]. Thus, it is necessary to identify alternative pharmaceuticals for the individual management of hepatic diseases, with the aim of these agents being more effective and less toxic.

The authors won't to providing information and bibliographic support to researchers who are exploring treatments for hepatic safety. Also, they encourage the increasing of new investigations linking genomic data to antioxidant agents. This present review has, as its aim, the gathering of data based on works conducted in some botanicals that are administered recurrently as Complementary and Alternative Medicines (CAM) with hepatoprotective capacity[7].

Furthermore, recent progress in genotyping tests suitable as biomarkers of HCC and NALFD for diagnosis and prognostication are summarized.

Energy Metabolism

The liver is a hub organ, which governs the energy metabolism of the whole body with a multistep process strongly controlled. It serves as a core to metabolically connect to various tissues, including skeletal muscle and adipose tissue.

In the gastrointestinal tract, food is digested, and glucides, fatty acids (FAs), and amino acids are absorbed and transported to the liver through the portal venous system. In the hepatocytes, glucose is polymerized as glycogen and converted into FAs chains or amino acids. The free FAs are esterified with glycerol-3-phosphate to generate triacylglycerol (TAG). TAG is secreted into the blood as very low-density lipoprotein (VLDL) and/or stored in lipid particles in the adipocyte. Aminoacids are used to synthesize proteins; glucose is metabolized to provide energy, and/or other bioactive molecules. During exercise, energy substrates (e.g. glucose and TAG) are immediately secreted into the blood and catabolized by muscular tissue. Adipocyte produces and releases non-esterified fatty acids (NEFAs) and glycerol via lipolysis, muscle breaks down glycogen and proteins and releases lactate and alanine.

Alanine, lactate, and glycerol are delivered to the liver and used as precursors to synthesize glucose (gluconeogenesis). NEFAs are oxidized in mitochondria through FA oxidation and create ketone bodies (ketogenesis)[8]. However, dysfunction of metabolism and/or liver signaling causes and/or predisposes to NAFLD and/or type 2 diabetes[8].

Biological Features of HCC and Chronic Liver Disease

HCC is accounting approximately 600,000 deaths annually worldwide[9]. It is well-known that hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, and NAFLD are the main risk factors for hepatocarcinogenesis[10]. It is known that in HBV X protein (HBx) transgenic mice HCC develops within one year after birth[11]. Similarly, HCV core transgenic mice exhibit hepatic steatosis several months after birth and dramatically develop HCC[12]. Moreover, the incidence either metastatic (from colorectal cancer) and/or primary HCC in patients with a concomitant metabolic syndrome or NASH has been increasing[13]. It is now widely considered that accumulation of genetic and/or epigenetic alterations transforms healthy cells into cancer cells carrying unique properties acquired, such as self-sustained proliferative signaling[14].

Therefore, further understanding of molecular mechanisms causative of hepatocarcinogenesis and validation of most efficient therapeutic approaches remain the most important challenges. Current progress in molecular biology enabled characterization of cancer cells, allowing the designing of new molecules for targeted therapy. Sorafenib, an oral tyrosine kinase inhibitor (TKI), works by suppressing tumor growth and angiogenesis by inhibiting the RAS/RAF/MAPK signaling pathway and tyrosine kinase (TK) receptors including vascular endothelial growth factor receptor (VEGFR)[15].

In addition to TK pathways, has been recently recognized that deregulation of crucial signaling pathways like as p53/RB, Wnt/β-catenin and PI3K/PTEN/Akt/mTOR, plays an important role in the development and evolution of HCC[16]. It is considered that unusual activation or inactivation of these pathways is attributable to somatic alterations such as mutations, changes in copy numbers, and chromosomal rearrangements[17].

Among them, mutations of TP53 and β-catenin have been recognized for a long time as a frequent genetic alteration in about 33% and 22% of HCC samples respectively[18]. CLD defines an extensive range of syndromes characterized by liver fat storage (mostly triglycerides) exceeding 5% of liver mass, without noteworthy alcohol consumption.

NAFLD is life-threatening condition and may remain effortless or progress into severe steatosis, lobular inflammation, and hepatocyte damage/apoptosis with activation of fibrogenesis[18].

Natural Products with Antioxidant Effects

The employ of some botanicals (plants and fruits) has played basic roles in human healthcare. Currently, about 75% of world's population uses long-established medicine for healthcare, which has origin mainly by botanicals[19].

Many clinical trials on CAM in cancer patients have shown high beneficial effects[20]. These pharmacological properties could be ascribed to the existence of chemical substances that are biologically active, called phytochemicals[21]. In the current literature, several studies that have investigated the benefits of different phytochemicals against liver disease are reported.

Among the majority cited examples we report preferentially: the St. Mary thiestle (Sylimarin, Sylichristrine); the betalain pigments (betanin and indicaxanthin); the anthocyanins (cranberries, *Prunus spinosa*); resveratrol. All of these have generally been known by their chemoprotective properties against cancer[22]. All of the medicinal plants, and the consumption of certain fruits, have demonstrated different effects on liver functions. A small number of these natural antioxidants are listed in Table I. In addition to these studies, empirical data for the treatment of liver diseases accounting the employ of natural remedies has a long history.

This has become an innovative scenario of study, with the principal aim of analyzing the consumption of fruits and traditional medicinal plants by a great number of people and the different phytochemicals that are extracted from these foods. In general, fruits, as well as plants, contain a variety of compounds known as liver-protective, such as glycosides, phenols, lignans, monoterpenes, alkaloids, carotenoids, flavonoids, organic acids, and xanthenes[23]. Among these hepatoprotective botanicals, *Cardus marianum* (also known as St. Mary thistle) is one of the most important plants, with known mechanisms of action for traditional treatment of toxic liver damage. The basic constituents of *Cardus marianum* are a mixture of flavonoids and lignin compounds that make up the main extract called silymarin[24]. Silymarin has been used as a protective treatment in acute and CLD. Its protective property is correlated to several mechanisms, such as inhibiting toxins infiltration into hepatocytes, increasing superoxide dismutase (SOD) activity, increasing glutathione cytoplasmic levels, inhibiting fatty acid peroxidation, and enhancing hepatic protein synthesis. Aller et al[24] reported positive results concerning the effect of Silymarin and Vitamin E in patients with NAFLD.

The authors report the outcome of a pilot study of 36 patients randomized in two group: the group I was treated with Silymarin plus Vitamin E (2 tablet/day), hypocaloric diet (1520 kcal, 52% carbohydrates, 25% lipids and 23% proteins) and exercise for three months. The Group II was treated only with hypocaloric diet. The results are interesting and revealed that treatments adopted improved the hepatic functions. These conclusions were measured by Fatty Liver Index (FLI), and NAFLD-Fibrosis Score. They close that Silymarin can be an alternative and valid therapeutic option especially as a complementary treatment associated with other therapeutic programs[25]. Currently, a similar pilot study adding up Sylimarin and antioxidant agents to conventional treatments is ongoing in an Italian trial on HCC patients and preliminary results were comparable to Aller' s data. This study was planned in two randomized patient groups: group I was treated with a combination of Sylimarin 400 mg/day, Vitamin E 12 mg/day, N-acetyl cysteine 600 mg/day Betaine 600 mg/day and Selenium 81 μg/day (3 tablet/day of *Epatil*®), hypocaloric diet (1500 kcal, 50% carbohydrates, 20% lipids and 25% proteins) and exercise for three months. The Group II was treated only with hypocaloric diet and exercise for three months.

Table I. Natural agents with antioxidant activities used as liver protection.

Agents	Standard dosage (active agents)	Activities in the liver metabolism	Appropriate indication of use
Citrus paradisi	20-50 mg/kg/day (naringenin)		DMN-induced hepatic damage
Citrus paradisi	0.05-0.125 g/L (naringin)		Ethanol-induced hepatic damage
Vaccinium corymbosum	0.6-10 g (proanthocyanidins)		OS-induced hepatic damage
Vaccinium macrocarpon	7 mg/kg (proanthocyanidins)		CCl_4-induced hepatic damage
Vaccinium spp	20 mg/kg/day (proanthocyanidins)		DMN-induced hepatic damage
Vitis vinifera	15%; 5 g/kg (resveratrol)	Antioxidant activities	Ethanol-induced hepatic damage
Opuntia ficus indica f. inermes	20-40 mL/kg,	Antioxidant activities	α-Fetoprotein 1 Ethanol-induced hepatic damage
Matricaria chamomilla	50, 100, 200 mg/kg	Membrane function activity	CCl_4-induced hepatic damage
Silybum marium	25 mg/kg/day (Sylimarin)	Stimulating cell regeneration, cell membrane stabilization	CCl_4-induced hepatic damage
Silybum marium-Aloe vera (ACTIValoe-N931)	85, 170, 340 mg/kg at 48, 24 an 2h before and 6h after the injection (acute);	Attenuated TNF-α levels in NO synthase, in COX-2 and in the expression of mRNA	CCl_4-induced hepatic damage
Silybum marium-*Ginkgo biloba*			NDEA-induced hepatocarcinogenesis
Spirulina platensis	100 µg/kg/day (carotenoids)		$HgCl_2$-; cisplatin CCl_4-induced hepatic damage
Cuban propolis	25, 50, 100 mg/kg (95% ethanol extract)		PCM-induced hepatic damage
Cuban red propolis	5, 10, 25 mg/kg or 25, 50, 100 mg/kg (95% ethanol extract)		Alcohol-; CCl_4- induced hepatic damage
Propolis	100-200 µg/mL (methanol extract)		Cypermethrin D-Ga1N/ TNF-α induced cell death
Prunis spinosa (Trigno)			
β-glucan	5% 4 days-10% 21 days (laminarin)		Lipo-Poly-Saccaride induced hepatic damage
β-glucan	500, 1000, 2000 mg/kg in 3 doses (paramylon)		CCl_4-induced hepatic damage
Vitamin E	12 mg/day	Regulation of TGFβ1 and PPAR activity	Stabilizing hepatocyte lipid-membranes in NASH and NAFLD
N-acetyl cysteine (as source of glutathion)	600 mg/day	Regulation of liver detoxification function	Source of methyl and glucoronil radical for bile eliminations

Evermore, it is recognized the benefit derived from Vitamin E supplementation in NALFD and NASH patients[26]. Vitamin E stabilizes the hepatocyte membranes by protecting the unsaturated fat chains, inhibits the inflammation via Tumour Growth Factor-β1 (TGF-β1) and regulates the expression of PPARγ[26]. Recently, it was demonstrated that an extract from *Moringa Oleifera* promotes the brown remodeling of white adipose tissue inducing thermogenesis and improving metabolic homeostasis by activating Sirtuin-1 (SIRT-1) and PPARγ, respectively[27]. Among the fruits extract, *citrus paradise* plus vitamin D is noteworthy due to the antifibrotic proprieties of naringenin[28,29]. Grapes are rich sources of flavonoids, such as catechins, anthocyanidins and resveratrol. Recently, in Italy, a fluid extract of *Prunus spinosa* is usefully prescribed as a potent antioxidant in cancer patients. This is a noteworthy example of how a popular drink is discovered to have anti-tumor properties[30].

Genotyping HCC and CLD Patients

Combined analytical methods and novel biomarkers contribute to early diagnosis and selection of the suitable treatment for patients with

CLD and HCC. Progress in high processing platforms and molecular biology techniques, such as next-generation sequencing (NGS), unveils the biological features of hepatic cells[31].

These analyses have been conducted on hepatic tissue samples and more recently on the "liquid biopsy". It is non-invasive sampling method that allows the detection of mutations of target genes by capturing the circulating tumor DNA in peripheral blood[32]. This approach is improving the prognosis of patients with advanced HCC. NGS methods allow a cost-effectiveness genome-wide association studies (GWAS) and exome sequencing analyses on a large cohort of patients[33]. In a recent study[18] on 24 HCC samples, it has been verified that the signaling pathways PI3K/RAS, Wnt/β-catenin, p53/cell cycle, oxidative stress and chromatin acetylating have been recurrently altered by somatic mutations or gene deletions.

Authors have found frequent alterations in four genes (PS6KA3, ARID1A, IRF2R and NFE2L2). Focusing, ARID1A, a chromatin-remodeling gene, has been revealed to be frequently mutated in alcohol-related HCC. Whole-genome sequencing of HCC samples has also revealed repeated somatic mutations in quite a lot of genes linked with chromatin regulation, such as ARID1A, ARID1B, ARID2, HIST1H4B17MLL, MLL3, BAZ2B, BRD8, BPTF, and BRE[34]. A mutation in at least one of these chromatin regulator genes has been identified in more than 50% of HCC tissues. The genetic variants of steatosis are not acquired mutations as found in cancer cells, but usually, are heritable germline Single Nucleotide Polymorphisms (SNPs). Currently, it has been identified by using GWAS. These studies[34] have acknowledged diverse SNPs predicting the progression from steatosis to liver fibrosis.

These current advances have contributed in a little time to clarify the NAFLD and NASH pathogenesis and may soon translate these genetic markers into clinical application[35]. A recent study has been focused on six patatin-like phospholipase domain-containing 3 (PNPLA3) variants, Lipin 1 (LPIN1), peroxisome proliferator-activated nuclear receptors (PPAR), superoxide dismutase 2 (SOD2), insulin receptor substrate-1 (IRS-1), Kruppel-like factor 6 (KLF6) and transmembrane 6 superfamily member 2 (TM6SF2).

Taken together, these SNPs define an individual genotype-haplotype with major/minor relative risk for progression in NASH and NALFD (Table II)[36].

PNPLA3 is a major determinant of biological and ethnicity-related variety in liver lipid tissues development. It is deeply involved in hepatic fatty acid transformation and VLDL secretion[37]. The gene variant rs738409, causing the aminoacidic substitution Iso148Met, is a prognostic factor for predicting the progression of steato-hepatitis in NALFLD[38].

LPIN1, a gene coding for a homonym phosphatidate phosphatase enzyme that is extremely expressed in the liver and adipose tissue, and is implicated in the synthesis of phospholipids downstream of the step catalyzed by PNPLA3. LPIN1 is co-activator and controller of adipogenesis process and metabolism[39]. It is, also, required for the normal metabolic trafficking between liver and adipose tissue. LPIN1 variants (rs13412852) have been linked with several causes of the metabolic syndrome, including elevation body mass, increasing insulin levels, and responsiveness to insulin sensitizers[39]. Noteworthy, the LPIN1 rs13412852 TT genotype is protective towards NAFLD in a child population[35]. These data suggest that LPIN1 AA genotype influence to progressive NASH/NAFLD at pediatric age by interfering with lipid metabolism. Although, an independent and deeply validation of these results is required.

PPARγ, catabolize fatty acids and it activated form prevents the buildup of triglycerides. PPAR is a molecular target for long chain fatty acids, fibrates, and eicosanoids[40].

PPARγ is extremely expressed in adipose tissue and liver, regulates adipocyte differentiation, FFA uptake, and storage[41]. The well-known SNP variant Pro12Ala, which causes loss-of-function in PPARγ, induces a weak reduction of transcriptional activity due to decreased DNA-binding affinity. Moreover, a loss-of-function PPARα variant Leu162Val does not influence the development of CLD and/or risk of NAFLD. These SPNs have also been related to a reduction of PPARγ activity in adipocytes as well as decreased adiponectin release and insulin resistance[41].

SOD2 is a key component of antioxidant protection system against mitochondrial superoxide radicals. SNP rs4880 C47T variations have been correlated in NASH and diabetes[42]. This SNP coding Ala16Val substitution in SOD2 and produces a β-sheet secondary configuration instead of the expected α-helix structure. As results, SOD decreases the transport efficiency into the mitochondria and modifies the antioxidant barrier against free oxygen radicals.

Table II. SNPs determining an individual genotype-aplotype with major/minor relative risk for progression in NASH and NALFD.

SNP gene	Genotype	Impact on steatosis risk	Impact on NASH risk	Impact on NALFD risk
rs3750861 KLF6 IVS1–27 G>A	Wild-type G/G	↑	↑	↑↑
	Heterozygote G/A	↑	↑	↑
	Homozygote A/A	↓	↓	↓
rs13412852 LPIN1	Wild-type C/C	↑	↑↑	↑↑
	Heterozygote C/T	↑	↑	↑
	Homozygote T/T	↓	↓	↓
rs738409 PNPLA3 Iso168Met	Wild-type C/C	–	–	–
	Heterozygote C/G	↑↑	↑↑	↑↑
	Homozygote G/G	↑↑↑	↑↑↑	↑↑↑
rs4880 SOD2 Ala16Val	Wild-type C/C	–	–	–
	Heterozygote C/T	↑	–	↑
	Homozygote T/T	↑	–	↑↑
rs58542926 TM6SF2 Lys167Glu	Wild-type C/C	↓	–	↓
	Heterozygote C/T	↑	–	↑
	Homozygote T/T	↑	–	↑↑

Annotation: the relative risk is relative correlated to Caucasian population. Legend ↑: Weak increment of the relative risk; ↑↑: Moderate increment of the relative risk; ↑↑↑: High increment of the relative risk; –: Haplotype without impact on relative risk; ↓: Protective haplotype relative to general population.

This process is a significant pathophysiological mechanism for development and evolution forward CLD and co-morbidities like diabetes[43].

KLF6 is a transcription factor ubiquitously expressed, well-known as a direct early gene in activated Hepatic Stellate Cells after liver injury and as a tumor suppressor gene in a wide range of tissues including the liver[44]. An SNP IVS1-27G>A (rs3750861) inside functional intron, creates a novel binding site for the splicing factor SRp40, and promotes unusual splicing of KLF6 into aggressive shortened isoforms. Among these isoforms, KLF6 splice variant 1 (KLF6-SV1) reduces differentiation in neoplastic cells *in vivo*, while, in the main time promotes cell growth[45].

Also, KLF6 rs3750861 G>A (SV-1) genotype is associated to fibrosis progression in NASH and is considered as a predictor for NAFLD histological stage[45]. To date, the activity of KLF6 and KLF-SV1 in non-neoplastic cells are still unknown.

The TM6SF2 gene variant rs58542926 C>T causes amino acid substitution Lys167Glu, interfering with VLDL releasing, which could be stimulated in early NAFLD[46]. This SNP has been linked with the increasing of progression in NASH by lipids storage inside hepatic cells[47]. Furthermore, case-control studies demonstrated that the polymorphic variant Lys167 alone can't be causative of fibrogenesis but it has been supported by other genetic variants related to inflammation, insulin signaling, oxidative stress and iron metabolism[47].

Also, other candidate gene variants correlated to the evolution of NAFLD have been revealed to be involved in liver fat synthesis, storage uptake and transport. Among these, it has been reported the Fatty Acid Transport Proteins type 2 and 5 (FATP) plays a fundamental function in mediating cellular FFA uptake[48].

FATP5 gene encodes a multifunctional protein, which increases the hepatic very long-chain fatty acids uptake, and also has bile-CoA ligase activity. Interestingly, the rs56225452 FATP5 gene variant has been related with high transaminase levels in a cohort of patients with steatosis and severity NAFLD[48].

Conclusions

The current upgrading in genetics providing outstand opportunities to identify diagnostic and predictive markers for HCC and liver chronic diseases.

Hepatic cells acquire gene mutations before transforming into cancer cells[49]. These mutations could be serving either as predictive markers and/or as targets for drugs as TKIs[50]. Also, genetic markers may be a tool to identify patients who will benefit from this targeted therapy, and exclude patients at elevated risk to develop severe toxicity[51]. The gene variants correlated to NASH and NAFLD are inherited SNPs, as confirmed by studies on twins.

These studies[52,53] correlated heritable genetic trait to elevated hepatic transaminase levels and high lipid accumulation in the absence of alcohol abuse or viral hepatitis.

Since the 75% of risk factors for CLD are food-related, healthy national programs worldwide promote the daily use of antioxidant nutrients[54]. In the complex, these substances have diverse properties that allow the hepatic fat storage and the increase of peripheral lipolysis due to hyperinsulinemia. Furthermore, the progression of NASH has been typically attributed to the activation of inflammation, and may be connected to a multiplicity of circumstances: oxidative stress at the outlay of hepatocytes, caused by free radicals created during FFAs oxidation; increasing intestinal permeability produce inflammation triggered by endotoxin engaging Toll-like receptor-4 in Kupffer cells and hepatocytes and correlated cytokines released by the hepatic stellate cells[55]; no less important, quantitative and qualitative changes in gut microbiota[56].

All these environments lead in the closing stages to inflammation, cellular damage, and activation of fibrogenesis in the liver. Taken together, these dysregulated metabolic pathways play a crucial role in HCC and liver chronic disease development. Promising in the future, we believe that the right features of these challenges are based on a multidisciplinary treatment approach, to rationalize the costs of these treatments due to aimed-interventions.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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