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The epidemiology of non-alcoholic fatty liver disease and its connection with cardiovascular disease: role of endothelial dysfunction

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Abstract. – The non-alcoholic fatty liver disease is considered a predominant hepatopathy worldwide and a component of metabolic syndrome. It represents a risk factor for the development of cardiovascular diseases, independently of the presence of diabetes mellitus, hypertension and obesity. For this reason, nowadays an epidemiological analysis and a research of the causes that correlate non-alcoholic fatty liver disease and cardiovascular pathologies, are extremely useful. There are important epidemiological variations in relation to various geographical areas, and depending on different population groups, the prevalence of this pathology changes. Epidemiological analysis for non-alcoholic fatty liver disease shows its remarkable relevance and diffusion, especially in Western areas; therefore immediate interventions are necessary for its prevention, diagnosis and therapy. Endothelial dysfunction could be the joining link between non-alcoholic fatty liver diseases and cardiovascular disease risk. Indeed, their correlation should be researched in the alterations that metabolic hepatopathies are able to induce on endothelial function and viceversa. For this reason, the scientific community may research new therapeutic strategies for non-alcoholic fatty liver disease, by intervening on the early stage of the pathology and blocking endothelial dysfunction.

Key Words:
Non-alcoholic fatty liver disease, Endothelial dysfunction, Cardiovascular disease, Epidemiology, Endocan.

Introduction

The non-alcoholic fatty liver disease (NAFLD) is becoming the most predominant hepatopathy in the near future[1]. It is characterized by a pathological fat accumulation in hepatocytes > 5% in the liver tissue, in absence of alcohol consumption, drug intake, and viral hepatopathy[2]. This last point is very important because the pathogenesis of this lipid accumulation is completely different from pathogenesis of other nosological entities, such as drug induced steatohepatitis, chemotherapy associated steatohepatitis, and hepatitis C virus-related steatosis, and it significantly lies on insulin resistance[3]. Insulin resistance may be able to intervene on all mechanisms responsible for lipid accumulation in hepatocytes: hepatic lipogenesis increase, a decrease of lipid export in the liver, a decrease of hepatic fatty acid oxidation, the increase of adipocyte lipolysis[3-7].

Depending on histology, NAFLD can be distinguished into a simple lipid accumulation in hepatocytes, named non-alcoholic fatty liver (NAFL), and into a condition characterized by a cytolitic damage supported by an inflammatory process based on an imbalance of oxidoreductivecell potential, named non-alcoholic steatohepatitis (NASH). Even though factors responsible for the passage from first to second condition are not yet clear, scientific community believes that a basic genetic predisposition together with a negative contribution of different environmental causes, is able to trigger lipid peroxidation and proinflammatory cytokine production, such as interleukin (IL)-6, tumour necrosis factor alfa and IL-1 beta, which support the pathology and also cause the possible progression to more advanced stages, including cirrhosis and hepatocellular carcinoma[8-15]. In this regard, NAFLD currently represents the second most common cause of hepatocellular carcinoma development, as well as the second most frequent indication for liver transplantation, probably becoming the first cause by 2020[1,16-19]. Since NAFLD is a pathology without any symptom, with the exception of advanced stages, its incidence and/or prevalence is largely underestimated. The lack of specific pharmacological therapies, easier to obtain when the diagnosis is done, complicates the achievement of the

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consent for the execution of parenchymal biopsy, that is the diagnostic gold standard. Moreover, nowadays the number of specialist medical consultations related to this pathology is very reduced, because routine analysis execution, performed in order to determine transaminase level, often produces negative response despite the presence of the disease, since there is no correlation between liver damage and aspartate aminotransferase/alanine aminotransferase levels in NASH [20,21].

NAFLD is part of metabolic syndrome and is considered a risk factor for the development of cardiovascular diseases independently of the presence of diabetes, hypertension and obesity[22,23]. Indeed, the most of deaths that occur in patients with NAFLD could be due to cardiovascular causes[24].

Currently, there are more than 7 billion people worldwide. About 1,5 billion is malnourished, and more than double is overfed and obese, in fact obesity is now considered as a real epidemic. This observation is relevant because this social category represents that with a higher prevalence of NAFLD [25,26]. With regard to hepatopathy etiology, in the near future a complete reversal of the ratio between viral and metabolic hepatopathies will probably occur, with a clear prevalence of metabolic ones[1]. Because of the high rate of morbility and mortality with a survival, estimated at ten years, of 60-70% of patients with NASH, international scientific community has wondered whether a screening plan for NAFLD identification were necessary[27-29]. Each screening plan should be direct towards significant and potentially lethal diseases, that are common in the general population, have a well identified natural history, benefit from an early therapeutic approach, are diagnosed in simple and cheap way. NAFLD has two of the features above mentioned, especially in relation to the diffusion of pathological process in world population. This led to believe that in 2015 there were no prerequisites to start a screening plan for this pathology, even if this concept should be probably reassessed in the light of epidemiological data, development of well defined treatments, and new diagnostic procedures that will be observable in the near future[29-31]. This review aims to the recent epidemiological developments, as well as the connection between NAFLD and cardiovascular diseases, taking into consideration a possible joining link of these two entities: endothelial dysfunction (ED).

Epidemiology

Geographical Distribution

Whatever consideration about incidence and prevalence of NAFLD in the general population corresponds to an estimate of real ones, probably because of a large number of patients affected by the pathology who do not know to be diseased. There are remarkable epidemiological variations in relation to geografichal areas analysed, and in this case, the prevalence of the disease changes if we consider specific population groups.

In North America the prevalence of NAFLD in the general population is between 27% and 34%, whereas the prevalence of NASH is between 3% and 5%[2,28,32-34]. However, if we consider the high-risk population groups, in these areas NAFLD prevalence grows exponentially, precisely 75-92% in obese subjects and 60-70% in diabetic ones[34-40]. This problem has a higher importance if we consider the diffusion of obesity and diabetes in the American population[41]. Indeed, one-third of the population consists of overweight subjects or obese people showing an increase of type 2 diabetes mellitus incidences doubled in the last decade. These data seem to be even more disconcerting in view of the fact that this increase is mainly related to an increased diffusion of these pathological conditions in young and pediatric population, that have a higher life expectancy. Consequently, in the absence of an efficient therapeutic intervention, in the near future it will be possible to observe an increase in the prevalence of disease advanced stages and its hepatic and extrahepatic complications[42]. Indeed, both obesity and type 2 diabetes mellitus, are considered risk factors for the development of NASH and fibrosis[2,28,38,43].

In Europe, the estimated NAFLD prevalence affects, on average, one-fourth of the general population among the different nations, including areas with a higher prevalence in Balkan Peninsula (40-45%)[44-47]. In Asia it is possible to observe, on average, a prevalence between 15% and 20% with a variable distribution among China, Japan, Korea, Malaysia, Indonesia and Sri Lanka, from 3 to 10 percentage points more or less[24,47-52]. The increase of prevalence among obese subjects and diabetic ones in Europe and Asia, shows what it has already been highlighted for the United States. Therefore, it is possible to observe the existence of a gradient of NAFLD prevalence in the general population, with higher rates in the Western areas of the

Earth, which gradually decrease if we consider the rates of the Eastern countries. This is probably related to more factors: environmental factors, lifestyle and drastic modifications in diet (i.e., meat consumption in Western countries and fish consumption in Eastern ones), as well as genetic factors transmittable among native people.

Age, Sex, Ethnicity and Genes

In the analysis of world population, a lot of studies highlighted some connections among NAFLD prevalence, age, sex and ethnicity. According to some authors, people in advanced age and male gender have a higher risk of developing NAFLD, independently of presence or absence of metabolic syndrome[53-58]. In male gender, it is possible to observe an increase of the risk of NAFLD in the transition between young and median age, until 50, threshold beyond which a progressive decline occurs[59,60]. Whereas female gender has a higher risk of developing NAFLD from the age of 45-50, with a progressive decline after 70[59-61]. Moreover, the age could have an influence on the risk of progression of the disease from NAFL to NASH, until the development of fibrosis in advanced stages and hepatic and extrahepatic complications related to the pathology[43,62,63]. Furthermore, it has been hypothesized that, in women, oestrogens may have a protective role towards liver fibrosis development: in this way the risk of fibrosis may increase in relation to age progression in the male gender, and it is higher if compared to the risk in women of the same age until 50. From this threshold on, a reduction of this difference may occur, becoming weak after menopause[64].

As is known, there is a connection between the risk of developing NAFLD and ethnicity[65]. In the American population there is a clear prevalence of liver steatosis among Hispanic subjects if compared to Caucasian ones, and even less in African Americans[66]. This difference is related to a higher distribution of risk factors responsible for the development of NAFLD in the Hispanic population, if compared to African Americans. Indeed, despite African Americans show a prevalence of obesity and insulin resistance similar toHispanic people, they have a lower frequency of liver steatosis[66]. Additionally, at histology, Hispanic subjects often present NASH signs, including ballooning hepatocytes and Mallory bodies, if compared to Caucasian people and African Americans[67]. In epidemiological analysis, the evaluation of familial predisposition to NAFLD development is also reported. Indeed, there are familial clusters in which the copresence of NAFLD among lineal relatives can reach 39%[68]. Sometimes, the transmission of a mutation developed on a regulator gene of lipid metabolism can occur, which can produce an increase of fatty acid synthesis, hepatic uptake, export decrease, alteration of oxidative metabolism[69,70]. However, monogenic mutations able to determine this disease transmission are very rare in the general population, for this reason it is impossible to exclusively ascribe the role of this familial predisposition to them. Rather, there are allelic variants of some genes, such as *I148M* for the gene patatin-like phospholipase domain-containing 3 (PNPLA3), involved in triglyceride hydrolisis, which are associated with a higher risk of development and progression of the disease[71,72]. The distribution of these allelic variants among different ethnicities could explain, at least in part, their different predisposition to develop this pathology. However, other studies have put in correlation this allelic variant with the development and progression of NAFLD in advanced stages, independently of age, sex and ethnicity[72-74]. Another variant of PNPLA3 gene, *S4531* is associated to a reduced hepatic accumulation of triglycerides. It is more widespread among African Americans than between Caucasian and the Hispanic people, which present a smaller expression[75].

The background of the damage supported by liver lipid accumulation, is the outbreak of an inflammatory reaction related to the generation of reactive oxygen species (ROS). Therefore, the overexpression of genes with proinflammatory activity, supported by *rs12979860 CC* genotype of IL-28B, together with PNPLA3 *rs738409 GG,* is associated to both a higher lobular inflammation and fibrosis[76]. Moreover, the minor activity of superoxide dismutase-2 enzime too, in determining a reduction of ROS concentration is associated to a similar histological response[77]. Other genic variants, related to familial forms of NAFLD in advanced stages are *rs780094* of hepatic glicokinase regulatory protein, *rs2228603* polymorphism of neurocan gene, *rs3750861* polymorphism of Kruppel-like factor-6, involved in the activation of liver stellate cells for the deposit of fibrotic tissue, and *rs58542926* polymorphism of trans-membrane 6 superfamily member 2[78-83]. The study of genes responsible for the development and progression of the pathology may allow the identification of familial groups at risk to be undergone screening tests aimed at obtain-

ing an early diagnosis of the disease, in order to avoid its evolution in more advanced and severe forms, as well as to avoid the development of hepatic or extrahepatic complications.

Endothelial Dysfunction

Endothelium can be considered as an organ that has a key role in vascular homeostasis, through the release of a large number of substances with autocrine and paracrine activity, such as: nitrix oxide (NO), prostacycline, endothelium derived hyperpolarizing factor, endothelin-1, thromboxane A2, prostaglandin A2, platelet activating factor and many others[84]. The processes regulated by these substances include the maintenance of vascular tone, vasal permeability, balance between coagulation and fibrinolysis, as well as subendothelial matrix structuring and proliferation/apoptosis of smooth muscle cells[85]. NO is produced by L-arginine catabolism due to NO-synthase (NOS)[86]. NO production is stimulated by both substances, including acetylcholine, bradykinin, substance P, serotonin, which act on specific receptors and mechanical stimuli: wall shear stress. NO causes a reduction of intracellular calcium concentration in smooth muscle cells and consequently their release[87]. When endothelium is physically and functionally damaged, these homeostatic mechanisms fail, especially those related to NO-dependent vasodilation, determining ED. ED is characterized by a reduction of bioavailability of vasodilator molecules and/or an increase of vasocontrictor stimuli, such as thromboxane A2, prostaglandin H2 and ROS[86]. ROS cause not only vasocontriction, but also NO degradation, reducing its bioavailability and producing a vicious circle that further damages endothelium, making it more predisposed to the onset of cardiovascular disease.

NAFLD and Endothelial Dysfunction

NAFLD and its association with other known risk factors is now considered both a marker of cardiovascular diseases and a pathological manifestation able to carry out a pathogenetic role towards cardiovascular diseases[88-91]. Nowadays, among death causes in patients with NAFLD stand out cardiovascular pathologies, for this reason an intervention in the "metabolic epidemic era" is necessary in order to reduce the number of deaths[24]. The connection between NAFLD and cardiovascular diseases may be researched in the alterations that metabolic hepatopathies are able to induce on endothelial function, independently of other cardiovascular risk factors. Carotid intima-media tickness alterations, atherosclerosis, coronary calcification and low coronary flow reserve are also associated with NAFLD, and their severity in the pejorative sense is directly correlated to the severity of histological liver damage, defined by lobular inflammation and fibrosis extent[22,92-95]. ED evaluation is one of the most recent research areas in the field of NAFLD, and its evaluation may be essential to define patients with a higher risk of developing cardiovascular diseases. Indeed, a study by Rubinshtein et al[96] that included 270 symptomatic outpatients with unexplained chest pain, low-risk findings during stress testing and/or the absence of new obstructive lesions by an invasive coronary angiogram, demonstrated an association between ED, evaluated by peripheral arterial tonometry, and the onset of adverse cardiovascular events, such as: cardiac death, myocardial infarction, revascularization or cardiac hospitalization, during the seven years of follow-up. Actually, the association between ED and NAFLD was already highlighted in 2005, in a study, that demonstrated a significant reduction of flow mediated dilatation (FMD) in NAFLD patients if compared to controls, after adjusting for sex, age, body mass index, and insulin resistance[97]. Back then, even though NAFLD started to be considered a significant problem of hepatology, it did not have the same epidemiological relevance that we live nowadays. In 2013, Colak et al[98] highlighted in an observational case-control study, a reduction of FMD in NAFLD patients if compared to controls. These data did not correlate with classic risk factors for cardiovascular diseases, neither with the presence nor the absence of metabolic syndrome, and this difference was mainly marked in patients with NASH.

In 2015 Long et al[99], with a study in a large community-based sample (n. 2284) of patients without apparent cardiovascular diseases, highlighted the association of NAFLD, as defined by decreased liver attenuation on multidetector computed tomography, and abnormalities in both the microcirculation and ED. NAFLD correlated with measures of microvascular dysfunction: fingertip peripheral arterial tonometry ratio, baseline brachial artery mean flow velocity, and baseline peripheral artery pulse amplitude after adjusting for cardiovascular and metabolic risk factors. The possible explanation of this ill-fated association could derive from the fact that NAFLD, inducing proinflammatory cytockine production and low-grade inflammation, would lead to an

inefficiency of mechanisms that underlie functional endothelial homeostasis[100]. However the pathogenetic connection between NAFLD and ED maybe also inversely considered, as different studies demonstrated: ED would be able to induce and worsen metabolic hepatopathy, producing a self-feeding vicious circle.

Some investigations have highlighted how ED may be considered an early alteration in metabolic hepatopathy that develops before the on set of whatever cardiovascular disease or structural alteration of vasal wall[101]. In a paper by Pasarìn et al[102], it has been demonstrated how mice fed for 30 days with a "Cafeteria diet" (including 65% of calorie intake made up of fats, especially saturated ones) precociously developed ED, before the development of structural endothelial alterations, inflammation and liver fibrosis. They studied hepatic microcirculation through an *ex-vivo* liver perfusion model, eliminating the possible extrahepatic effects on endothelial functionality. They observed an *ex-vivo* portal perfusion pressure increase in the liver of mice fed for 30 days with Cafeteria diet, compared to those fed with conventional diet; there was a reduction of this difference using NO, therefore the most likely hypothesis is that it was due to an increase of vasal tone[102]. Moreover, they evaluated the vasodilator response to acetylcholine stimulus (ED standardized evaluation method) that was clearly lower in mice fed for 30 days with Cafeteria diet than in mice fed with conventional diet[102]. Lastly, they measured phosphorylated protein kinase B and phosphorylated NOS (active forms of proteins) levels, which were lower in mice fed for 30 days with Cafeteria diet than in mice fed with conventional diet[102]. Regarding this last observation, the researchers concluded that an early phase of ED development was the onset, induced by Cafeteria diet, of liver endothelial insulin resistance: insulin administration, that physiologically increases the amount of active NOS, did not cause any effect in hepatic capillaries of mice fed with Cafeteria diet, compared to the increase obtained in mice fed with conventional diet.

A suitable NO production due to the correct operation of NOS stops hepatic stellate cells activation, prevents sinusoidal thrombosis, a known progression mechanism of liver cirrhosis, and it is essential for the correct process of liver regeneration[102-108].

In another study, Miyao et al[109] have highlighted that structural variation of sinusoids, observed beyond the fourth week from the beginning of the experiment, underlies the process that causes the inflammation and fibrosis development. Indeed, they demonstrated the way sinusoidal capillarization in mice, in L-amino acid-defined diet model of NAFLD, precedes the development of inflammation and consequently the passage from NAFL to NASH, as well as fibrosis[109]. The theory of researchers is that the structural change of sinusoids, the loss of fenestrations and the acquisition of continual basal membrane can be associated with a functional change of endothelial tissue that, becoming dysfunctional, would be able to carry out a role that promotes the activation of immune cells, with the onset of inflammation, and hepatic stellate cells for the deposit of extracellular matrix.

The most suggestive hypothesis is to manage to intervene on ED process, through its early identification, with the use of next-generation medicines, which may lead to the interruption of pathogenetic chain that leads to the progression of hepatocellular damage.

Serum Markers of Endothelial Dysfunction

A possible joining link between NAFLD and cardiovascular diseases has therefore been identified in ED. For this reason, the development of new diagnostic methods able to measure ED should be necessary, in order to predict cardiovascular risk in NAFLD patients. In this regard, for ED evaluation it is possible to use invasive methods (intravascular injection of acetylcholine and the measurement of vasodilation caused by this neurotransmitter) and non-invasive methods, that are economically unsustainable for ED screening (FMD), up to dosage of ED serum markers.

Elsheikh et al[110] have tried to correlate NAFLD and presence of coronary artery disease (CAD) to the level of some ED serum markers. Sixty-six patients with NAFLD (diagnosed by ultrasonography and fatty liver index) and CAD (evaluated by coronary angiography) were enrolled. The evaluated serum markers were:

Endocan (ESM-1): a soluble proteoglycan, discovered by Lassalle and co-workers in 1996, produced by endothelial cells[111]. It is released by damaged endothelial cells in response to proinflammatory and angiogenetic stimuli[112,113].

High mobility group box 1 (HMGB1): a molecule that has a role in the regulation of inflammatory response by endothelial cells, inducing the dysfunction[114,115]. However, on the contrary, other

scientific proofs demonstrate the role of this molecule in repair process and endothelial homeostasis[116].

Anti-endothelial cells antibodies: antibodies directed against endothelial cells; they would increase endothelial expression of leukocyte adhesion molecules, pro inflammatory cytokine production and their apoptosis[117-119].

ESM-1 was higher in NAFLD patients with CAD than in controls, and its level directly correlated with CAD degree, observed by arteriography. ESM-1, together with hyperlipemia, was significantly associated with an increased risk of cardiovascular diseases in NAFLD patients.

HMGB1 levels were lower in patients with NAFLD and CAD than in NAFLD patients without CAD. However, the researcher observed that the correlation between HMGB1/ESM-1 ratio was significantly reduced in NAFLD patients with CAD if compared to controls. A possible explanation of this outcome, as the authors hypothesized, could be the alteration of balance between damage (measured by ESM-1 levels) and endothelial repair (measured by HMGB1 levels). Nevertheless, since the role of this molecule is not clear in endothelial physiopathology, future researches are necessary in order to confirm the predictive role in ED. Finally, no significant difference about anti-endothelial cells antibodies levels between NAFLD patients with CAD and controls was observed.

In summary, currently the most reliable serum markers for ED evaluation are ESM-1 serum levels and HMGB1/ESM-1 ratio.

Conclusions

Epidemiological analysis for NAFLD shows, year by year, the huge relevance of this pathology, economically and socially. The large diffusion of risk factors for NAFLD in Western areas, implicates the necessity of an immediate intervention for the prevention, diagnosis and therapy of this pathology, because it has also been identified as an independent cardiovascular risk factor. A greater awareness of physiopathological steps that lead to the correlation between NAFLD and cardiovascular diseases, allows us to highlight a new scientific scenario, which is ED may be considered as an early phase of pathogenetic process that leads to death due to cardiovascular pathologies in these patients[24]. Therefore, ED identification in patients with simple steatosis, with low-cost methods, may provide extra information about patient prognosis, in addition to the evaluation of conventional risk factors, as regards both cardiovascular system and liver. For this reason, the scientific community should research new therapeutic strategies for NAFLD, and intervene on an early stage of the pathology, blocking ED. This approach could determine a strong impact on cardiovascular in these patients, with a clear advantage with regard to public health.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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