EVALUATION OF THE HEPATOPROTECTIVE EFFECTS OF TOCOTRIENOLS AND THE RELATIONSHIP BETWEEN NON-ALCOHOLIC FATTY LIVER AND BRAIN WHITE-MATTER LESIONS

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To My Beloved Elisa

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αTTP	Alpha-tocopherol Transfer Protein	52
AECUSM	Animal Ethics Committee Of Universiti Sains Malaysia	77
ALD	Alcoholic Liver Disease	9
ALP	Alkaline Phosphatase	18
ALT	Alanine Transaminase	2
ANOVA	Analysis of Variance	56
ApoB	Apolipoprotein B	54
AST	Aspartate Transaminase	2
BMI	Body Mass Index	54
C-rp	C-reactive Protein	54
CCl_4	Carbon Tetrachloride	9
CI	Confidence Interval	57
CT	Computed Tomography	8
FLAIR	Fluid Attenuated Inversion Recovery	33
$GdCl_3$	Gadolinium Trichloride	9
GGT	Gamma-glutamyl Transpeptidase	15
HDL	High-density Lipoprotein	37
HFF	High Fat-high Fructose	77
HFF-T3	High-fat-high Fructose Plus Tocotrienols	77
HMG-CoA	3-hydroxy-3-methylglutaril-coenzyme A	15
HPLC	High-performance Liquid Chromatography	54
IL-6	Interleukyn-6	10
IU	International Unit	24
LDL	Low-density Lipoprotein	37

LOQ	Limit of Quantification	55
LP(A)	Lipoprotein A	54
MRI	Magnetic Resonance Imaging	8
MSG	Monosodium Glutamate	74
NAFLD	Non-alcoholic Fatty Liver Disease	2
NASH	Non-alcoholic Steatohepatitis	2
OR	Odds Ratio	63
PET	Positron Emission Tomography	32
PPAR-α	Peroxisome Proliferator-activated Receptor Alpha	10
PPAR-γ	Peroxisome Proliferator-activated Receptor Gamma	18
SLD	Suspected Liver Disorder	47
TC	Total Cholesterol	37
TG	Triglycerides	9
TNF-α	Tumour Necrosis Factor-alpha	10
WHO	World Health Organisation	1
WML	White-matter Lesions	25

PENILAIAN KESAN PERLINDUNGAN HATI OLEH TOKOTRIENOL DAN HUBUNGAN HATI BERLEMAK BUKAN-ALKOHOLIK DENGAN LUKA BAHAN-PUTIH OTAK

ABSTRAK

Penyakit hati berlemak bukan-alkoholik adalah salah satu masalah hati yang paling biasa terdapat seluruh dunia. Namun, tiada data mengenai prevalen atau kadar berlakunya penyakit ini bagi Malaysia boleh diperolehi daripada kajian sistematik. Dalam kajian ini, 180 orang dewasa lelaki dan wanita yang didapati sedikit hiperkolesterolemik semasa memasuki kajian ini telah disaring menggunakan imbasan ultrasound untuk menentukan kehadiran hati berlemak bukan-alkoholik. Kadar didapati tinggi dengan hampir 60% daripada jumlah peserta didiagnosis menghidapi penyakit hepatik ini. Penyakit hati berlemak bukan-alkoholik melibatkan penyusupan lemak ke dalam hati dan buat masa ini, tiada rawatan farmakologikal tersedia ada.

Penyaringan tersebut adalah sebahagian daripada proses perekrutan bagi kajian pembutaan-dua kali plasebo-terkawal yang bertujuan untuk menilai aktiviti tokotrienol dalam rawatan penyakit hati berlemak bukan-alkoholik. Tokotrienol, yang diekstrak daripada minyak kelapa sawit, adalah ahli kumpulan vitamin E.

Penilaian kesan tokotrienol untuk rawatan penyakit hati berlemak bukan-alkoholik telah dijalankan dengan membandingkan ekogenisiti hati pada tahap dasar dengan

pada akhir kajian. Hiperekogenisiti hati adalah berkadar terus dengan kandungan lemak.

Kajian ini pada mulanya telah merekrut 87 orang dewasa berhiperkolesterolemik yang telah dibahagikan secara rawak kepada dua kumpulan rawatan: samada plasebo (n=44) atau tokotrienol 400 mg/hari (n=43) selama satu tahun. Terdapat enam puluh empat subjek pada akhir kajian ini, 30 dalam kumpulan tokotrienol dan 34 dalam kumpulan plasebo.

Keberkesanan rawatan telah ditunjukkan, iaitu subjek yang dirawat dengan tokotrienol menunjukkan kadar penyembuhan (p=0.014; OR = 3.250; 95% CI = 1.117-9.456) dan penambahbaikan (p=0.021; OR=2.857; 95% CI=1.029-7.934) yang signifikan.

Aktiviti tokotrienol untuk menghalang penyakit hati berlemak bukan-alkoholik daripada bermula telah dinilai seterusnya dalam model haiwan. Satu diet steatogenik *ad hoc* telah diformulasi dan diberikan kepada mencit.Kajian histologi telah menunjukkan bahawa haiwan yang diberikan diet steatogenik telah mendapat penyakit hati berlemak bukan-alkoholik, manakala haiwan yang diberikan diet yang sama tetapi ditambah dengan tokotrienol tidak mendapat penyakit tersebut.

Hati berlemak bukan-alkoholik merupakan komponen hepatik bagi sindrom metabolik dan berkongsi faktor-faktor risiko yang sama dengan komponen serebral bagi sindrom tersebut, iaitu luka bahan-putih otak. Luka bahan-putih otak nampak sebagai hiperintensiti pada imbasan resonan magnetik dan mewakili bahagian infark subklinikal.

Imbasan otak bagi 172 subjek yang telah disaring bagi kehadiran penyakit hati berlemak bukan-alkoholik telah diambil. Hubungan antara luka bahan-putih otak dan

penyakit hati berlemak bukan-alkoholik yang dibuktikan dengan ultrasound telah ditunjukkan bagi kali pertamanya di kalangan orang dewasa berusia lebih 50 tahun (Chi-square=6.778; $p<0.01$).

EVALUATION OF THE HEPATOPROTECTIVE EFFECTS OF TOCOTRIENOLS AND THE RELATIONSHIP BETWEEN NON-ALCOHOLIC FATTY LIVER AND BRAIN WHITE-MATTER LESIONS

ABSTRACT

Non-alcoholic fatty liver disease is one of the most common liver disorders worldwide. However, no data from systematic studies are available for Malaysia on prevalence or rate of this disease.

In the present study 180 walk-in mildly hypercholesterolemic adults of both genders were screened using ultrasound imaging to determine the presence of non-alcoholic fatty liver. The rate of this hepatic disease was found to be high, since it was diagnosed in almost 60% of the participants. Non-alcoholic fatty liver disease consists of fat infiltration in the liver, at present no pharmacological treatment is available.

The screening was part of the recruitment process for a double-blind placebo-controlled study aimed at evaluating the activity of tocotrienols for the treatment of non-alcoholic fatty liver disease. Tocotrienols, that are extracted from palm oil, are members of the vitamin E family.

Evaluation of the effects of tocotrienols for treatment of non-alcoholic fatty liver disease was performed by comparing liver echogenicity at baseline and at conclusion of the study. Liver hyperechogenicity is proportional to the fat content.

The study initially recruited 87 hypercholesterolemic adults that were randomised in two groups of treatment: either placebo (n=44) or tocotrienols 400 mg/day (n=43) for one year. Sixty-four subjects concluded the study, 30 in the tocotrienol group and 34 in the placebo group.

The efficacy of the treatment was demonstrated, with subjects treated with tocotrienols presenting a significant rate of cure (p=0.014; OR = 3.250; 95% CI = 1.117-9.456) and amelioration (p=0.021; OR=2.857; 95% CI=1.029-7.934) compared to placebo.

The activity of tocotrienols in preventing the onset of non-alcoholic fatty liver disease was further evaluated in an animal model. A steatogenic *ad hoc* diet was formulated and administered to mice. Histology study demonstrated that animals administered with the steatogenic diet developed non-alcoholic fatty liver disease, whereas animals with identical diet plus tocotrienols did not develop the disease.

Non-alcoholic fatty liver represents the hepatic component of the metabolic syndrome and share the same risk factors with the cerebral component of such syndrome: the brain white-matter lesions. Brain white-matter lesions are seen as hyperintensities upon magnetic resonance imaging and represent areas of subclinical infarcts.

The brains of 172 of the subjects that were screened for presence of non-alcoholic fatty liver disease were imaged. Association between brain white-matter lesions and ultrasound proven non-alcoholic fatty liver disease was demonstrated for the first time in adults above 50 years of age (Chi-square=6.778; p<0.01).

CHAPTER 1 : INTRODUCTION

1.1 NON-ALCOHOLIC FATTY LIVER DISEASE

Contemporary affluent society is affected by ailments mostly related to its own excesses and lifestyle. The World Health Organisation (WHO) reported that about 1.5 billion adults worldwide, about one in four persons, are overweight including about 500 million obese accounting for about 2.6 million obesity-related deaths yearly (WHO, 2010a).

Increased intake of processed food, diet high in fats and sugars and reduced physical activity have caused the development of a series of health abnormalities that have driven medical scientists to name several, apparently unrelated, diseases as components of the metabolic syndrome (Reaven, 1988). The metabolic syndrome is a group of potentially deadly, mostly asymptomatic or silent conditions that comprise obesity, hyperglycaemia, arterial hypertension, dyslipidaemia, cardiovascular diseases and fatty liver diseases, that are affecting increasing percentages of the world population. A recent review regarding the prevalence of metabolic syndrome reported a range between 12% and 30% of the world population, with yearly increasing incidence (Grundy, 2008), being in agreement with the 2010 WHO data on obesity. A 12-year retrospective study on a Canadian population investigating the incidence of serious liver events, such as cirrhosis, liver failure and liver transplantation, in over 2 million individuals reported a 2-fold increase in risk in diabetes compared to matched-control cases, with 8.19 and 4.17 cases per 10,000 population/year, respectively (Porepa *et al*, 2010).

It was not until the ‘80s that fatty infiltration of the liver, previously considered solely related to excessive alcohol consumption, was distinguished in alcoholic and non-alcoholic by Ludwig and colleagues at the Mayo Clinic (Ludwig *et al*, 1980). Until then, non-drinker patients presented with fatty liver have been considered as drinkers in denial. From a pathological point of view, alcoholic and non-alcoholic fatty liver are superimposable (James and Day, 1998), thus explaining the earlier misconceptions about the disease.

Non-alcoholic fatty liver disease (NAFLD) is defined as a spectrum of chronic hepatic disorders, in absence of significant intake of alcohol, involving fatty infiltration of the liver or steatosis of increasing severity varying from macro-vesicular steatosis to steatohepatitis and initial fibrotic stage (Medina *et al,* 2004). Macro-vesicular steatosis is a condition involving fatty infiltration in more than 5% of the liver parenchyma, below this threshold it is considered as minimal findings or focal fatty liver (Allard *et al*, 2008). Non-alcoholic steatohepatitis (NASH) is the most severe expression of NAFLD and is characterised by micro- and macro-vesicular steatosis, inflammation, hepatocytes ballooning and, in certain cases, initial stage of fibrosis (Gramlich *et al*, 2004). Presence of NAFLD is usually hinted by the presence of inexplicably and persistently elevated liver transaminases levels, namely alanine transaminase (ALT) and aspartate transaminase (AST), or is an accidental finding upon routine ultrasound examinations (Bedogni *et al*, 2005).

1.1.1 Natural History of NAFLD

Until a decade ago, simple steatosis has been regarded as a benign condition, when it was shown, in a cohort study that included 772 histological specimens with NAFLD diagnosis, that there was higher liver-related deaths in subjects with NASH and advanced fibrosis than in those diagnosed with simple steatosis. However, when the overall causes of death were evaluated no significant difference was found between the two groups. Neoplasm was the most common cause of death, while cardiovascular disease-related deaths were equal to that which were liver-related, both being in second place (Matteoni *et al*, 1999). Indeed, these findings discredited the perception of steatosis being a harmless condition. Since then, several studies considered the progression of NAFLD from steatosis and steatohepatitis up to hepatocellular carcinoma and/or the overall causes of death in these patients. Eksted and colleagues (2006), in their 14-year follow up study of 129 subjects, reported a significantly increased mortality in patients diagnosed with NASH compared to matched controls. Moreover, 41% of NASH patients progressed to advanced fibrosis; development of cirrhosis and hepatocellular carcinoma were also reported. However, the most common cause of death in these patients was not liver related, but was once again a cardiovascular event (Ekstedt *et al*, 2006). Such findings indirectly corroborated a previously reported 5-year prospective study of cardiovascular events investigating the relationship between cardiovascular disease and ultrasound-proven NAFLD. It was shown that NAFLD was positively associated with increased risk of cardiovascular events in 248 type II diabetic patients (Targher *et al*, 2005).

A study on the relationship between carotid intima-media thickness and NAFLD in 125 patients, with normal to slightly elevated liver enzymes and 250 controls

demonstrated that NAFLD patients have a significantly higher risk of cardiovascular event with an Odds Ratio of 6.9 (Fracanzani *et al*, 2008). In an unrelated study focussed on carotid intima-media thickness as risk factor for cardiovascular events in an adult Malaysian population, showed that non-insulin dependent diabetics had higher carotid intima-media thickness compared to controls (Yunus *et al*, 2006). The combined findings from Fracanzani (2008) and Yunus (2006) hinted higher risk of cardiovascular complications, in particular a cerebrovascular event such as stroke, in adults with metabolic syndrome-associated disease as a common risk factor.

Furthermore, NAFLD has been positively associated with insulin resistance and hyperinsulinemia in non-diabetic patients with mild hypercholesterolaemia, and chronically elevated transaminases, independently of patient's body weight (Marchesini *et al*, 1999). It is now accepted that about 12-40% of diagnosed steatosis progresses to NASH, with or without initial fibrosis within 8-13 years (de Alwis and Day, 2008). Risk of progression from NASH to advanced liver fibrosis was shown to be significantly higher in obese patients, even in absence of elevated hepatic transaminases values (Garcia-Monzon *et al*, 2000). Furthermore, in a 10-year prospective study on 152 patients with biopsy-proven NASH, it was shown that cirrhosis due to NASH can lead to hepatocellular carcinoma in about 14% of the case (Sanyal *et al*, 2006). On the other hand, previous follow-up studies reported a much lower occurrence of NASH-related hepatocellular carcinoma. Hui and colleagues (2003) surveyed the incidence of hepatocellular carcinoma in patients with hepatitis C-associated cirrhosis and NASH-associated cirrhosis. They concluded their 7-year follow-up study without reporting any case of hepatocellular carcinoma amongst the 23 NASH-associated cirrhotic patients, conversely the hepatitis C patients presented

eight cases of hepatocellular carcinoma. Liver failure, on the other hand, was reported as the most common cause of death amongst NASH patients, totalling to 5 cases out of 23 (22%) patients. Low incidence of hepatocellular carcinoma cases were reported in a study of 42 obese NASH patients, with median follow-up of 5-year, showing progression from NASH to cirrhosis, with only one case of hepatocellular carcinoma and one case of cardiovascular event (Powell *et al*, 1990). However, patients sample size could be considered as a severe limitation to the studies of Hui and Powell.

A study on progression and survival rate of NAFLD-related cirrhosis or cryptogenic cirrhosis in obese (n=27) and lean patients (n=10) found the obese subject to have liver failure in 41% of the patients within two years of disease detection and an astonishing 30% of hepatocellular carcinoma within the same follow-up period. Lean subjects were also affected, but to a lower extent, by liver failure (20%) and no cases of hepatocellular carcinoma were recorded (Ratziu *et al*, 2002).

A visual summary of the natural history of NAFLD is shown in Fig 1.1.

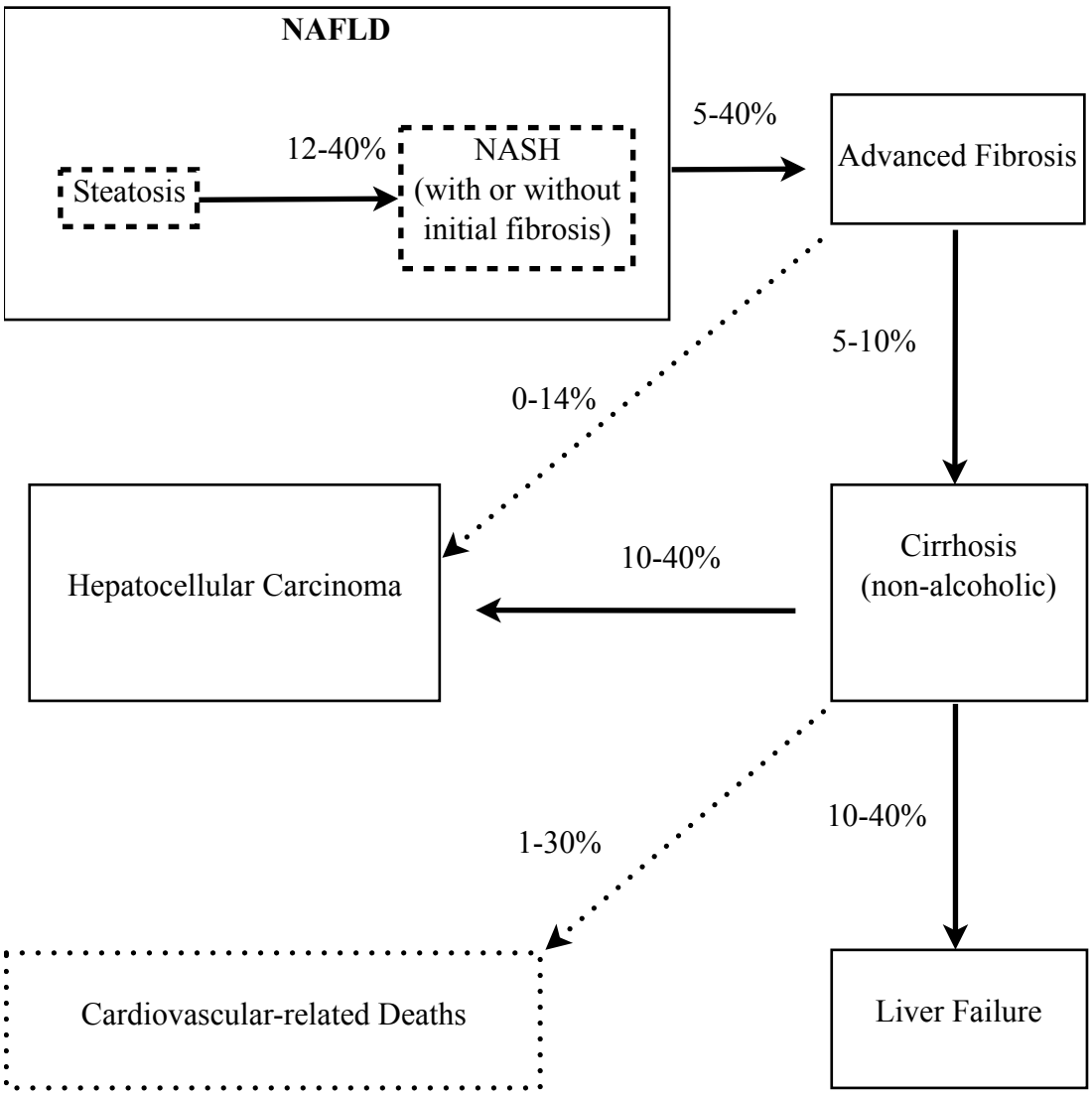

Fig. 1.1: Natural history of NAFLD and its pathological progression

1.1.2 Epidemiology and Susceptibility to NAFLD

NAFLD is considered the most frequently encountered chronic liver disease worldwide and the primary cause of elevated liver function test results after hepatitis and alcohol (Collier, 2006). Estimates of NAFLD prevalence range from 10% to 30% of the general adult population (Neuschwander-Tetri, 2001; Cave *et al*, 2007), a tenth of which is expected to meet the diagnostic criteria for NASH (Salt, 2004). Majority of the epidemiological studies refer to NAFLD diagnosed by ultrasound and/or liver function tests. Ethical and cost considerations prevent large epidemiological studies for histology-proven NASH (Targher *et al*, 2005).

Prevalence estimations are complicated by the severity range of NAFLD, being asymptomatic simple steatosis with normal liver function test largely undetected or only diagnosed upon an incidental ultrasonography examination (Bedogni *et al*, 2005). The absence of specific biomarkers for NAFLD further complicates acquisition of objective prevalence data of the disease. Commonly, cryptogenic elevated liver function test results are the first hint for the presence of NAFLD, in certain cases accompanied by discomfort of the upper-right abdominal quadrant and fatigue, independently of severity of the disease (Newton *et al*, 2008). The prevalence of NAFLD increases in selected populations with increased susceptibility risks. When populations of patients with concomitant risk factors associated with NAFLD, such as obesity, hyperglycaemia, dyslipidaemia are considered, the prevalence grows significantly. Data from an Italian sub-cohort study found the presence of NAFLD in 257 normal-weight adults to be about 16%, while in obese adults grew to nearly 76%, a 4.6-fold increased risk (Bellentani *et al*, 2000). This value is in agreement with smaller reported studies in obese subjects. Matteoni and

colleagues (1999) diagnosed NAFLD in 70% of 132 obese patients. Similarly, Angulo and co-investigators (1999) reported presence of NAFLD in 60% of 144 obese adults.

Diabetes, hyperglycaemia and insulin resistance are strongly associated with NAFLD and this is reflected in a prevalence of the disease of nearly 70% in diabetics (Tolman *et al*, 2007). Even though obese diabetics show higher prevalence rate of NAFLD, non-obese diabetics are not immune to the disease. Moreover, significant differences in prevalence of ultrasound-proven NAFLD in type I and type II diabetes exist. A study found that type I diabetics had a NAFLD prevalence of 24%, whereas in type II diabetics, the prevalence was increased to 80% (Gaiani *et al*, 2009).

A study on 105 severely obese patients found that prevalence of NASH increased with the type and number of concomitant metabolic abnormalities. In patients without diabetes and arterial hypertension, less than 10% was diagnosed with NASH, in patients with arterial hypertension, NASH was found to be increased to 30%, in diabetics 60% and in diabetics with arterial hypertension to be above 80%. Thus, predictive factors for NASH in severely obese adults were found to be arterial hypertension, insulin resistance and diabetes, besides ALT levels. Interestingly, alcohol consumption was not associated with NASH. Indeed, a moderate consumption of alcohol, up to two standard drinks per day, was described as protective against NAFLD (Dixon *et al*, 2001).

Exclusion of concomitant liver-related diseases, particularly viral hepatitis, is helpful for the diagnosis of NAFLD. However, the severity of NAFLD should be assessed at first with ultrasound examinations and subsequently, if appropriate, with a liver biopsy. Magnetic resonance imaging (MRI) and computed tomography (CT) scan are

capable of diagnosing NAFLD, however cost and/or length of the examination, together with comparable levels of specificity and sensitivity with ultrasound examinations do not favour MRI and CT diagnostic techniques over ultrasonography (Mehta *et al*, 2008).

1.1.3 Pathogenesis of NAFLD

Little is known about the aetiology of NAFLD, even though several mechanisms have been postulated for the progression from simple steatosis to NASH. Letteron and colleagues (1996) showed that administration of ethanol, tetracycline, chlortetracycline, demeclocycline, amineptine, amiodarone, pirprofen or valproate (all of them recognised as steatogenic compounds) caused microvescicular hepatic steatosis. Increased hepatic synthesis and release of free-fatty acids led to steatosis *via* lipid peroxidation. This pathway that led to microvesicular deposition of fat, in form of triglycerides (TG) in the liver, was found to be independent of the steatogenic compound used. It was hypothesised that the mere presence of intra-hepatic fat might be the initial step for the development of steatohepatitis (Letteron *et al*, 1996).

A study on alcoholic liver disease (ALD) proved that animals fed with ethanol developed liver injury, but when lipid peroxidation was prevented with the administration of gadolinium trichloride ($GdCl_3$), there was a reduction of inflammation and necrosis (Arteel, 2003), being accord to Letteron's study that lipid peroxidation was involved. $GdCl_3$ has been proven to reduce hepatic fibrosis induced by carbon tetrachloride (CCl_4) in rats through depletion of Kupffer cells (a type of cells present in the liver). Administration of CCl_4 caused oxidative stress, that

induced an increased response by Kupffer cells with consequent release of cytokines (Rivera *et al*, 2001). Similar mechanism to CCl_4 has been known for ethanol abuse (Hoek and Pastorino, 2002).

Similarities in the pathogenesis of alcoholic and non-alcoholic liver injuries were studied in mice fed control diet plus ethanol, methionine-choline deficient diet, ethanol plus methionine-choline deficient diet or control diet (Gyamfi *et al*, 2008). It was shown that ethanol, methionine-choline deficient diet and the combination of ethanol and methionine-choline deficient diet caused oxidative stress, with production of reactive oxygen species that led to the inhibition of the peroxisome proliferator-activated receptor-alpha (PPAR-α). Ethanol causes lipid peroxidation of membrane phospholipids and damages the methionine-adenosyl transferase, followed by depletion of methionine (Colell *et al*, 1998).

Oxidative stress, induced by ethanol ingestion or diet high in unsaturated fatty acids (in the ALD or NAFLD models respectively) was known to cause liver damage through the release of pro-inflammatory adipocytokines, in particular the tumour necrosis factor-alpha (TNF-α) (Nanji *et al*, 1995). Inhibition of PPAR-α, associated with ethanol consumption or obesity in humans, caused lipogenesis and intra-hepatic fat deposition. This represents the pathway of the first hits involved in development of steatosis and its subsequent progression to alcoholic- or non-alcoholic-steatohepatitits (Gyamfi *et al*, 2008). Adipose tissue is known to release several products active in carbohydrate and lipid metabolism. In the case of carbohydrate metabolism, the products are leptin and adiponectin, whereas in lipid metabolism, the products are apolipoproteins and pro-inflammatory adipocytokynes such as TNF-α and the pro-fibrogenic interleukyn-6 (IL-6) (Brunt, 2004). Increased release of TNF-

α and reduced release of adiponectin in presence of obesity, interferes with the reactivity of insulin-receptors leading to insulin resistance and promotes the release of long-chain fatty acids (Day, 2002). This might explain the strong association of NAFLD with insulin resistance (Marchesini *et al*, 1999; Tolman *et al*, 2007), with obesity (Matteoni *et al,* 1999; Angulo *et al*, 1999; Bellentani *et al*, 2000) and with the combination of both, insulin resistance/diabetes and obesity (Tolman *et al*, 2007; Gaiani *et al*, 2009). Moreover, hepatocyte injury induced by TNF-α-mediated production of reactive oxygen species has been found to be exacerbated by ethanol administration (Colell *et al*, 1998).

Low levels of insulin delay glucose uptake by adipocytes and inhibit lipolysis in combination with increased release of TNF-α, particularly in overweight or obese individuals, leads to augmented long-chain fatty acids production and consequent increased TG synthesis and deposition in the hepatocytes (Bradbury and Berk, 2004). Thus, insulin resistance and obesity might be considered amongst the first causal insults for the development of steatosis with inflammation caused by increased oxidative stress and lipid peroxidation representing the second hits for the progression from steatosis to NASH (Day, 2006), as shown in Fig. 1.2.

Dietary factors and sedentary lifestyle play a pivotal role in the development of NAFLD. Increased intake of food rich in fats and carbohydrates and reduced physical activity lead to development of obesity, thus initiating the vicious cycle that contributes to NAFLD, diabetes and cardiovascular disease.

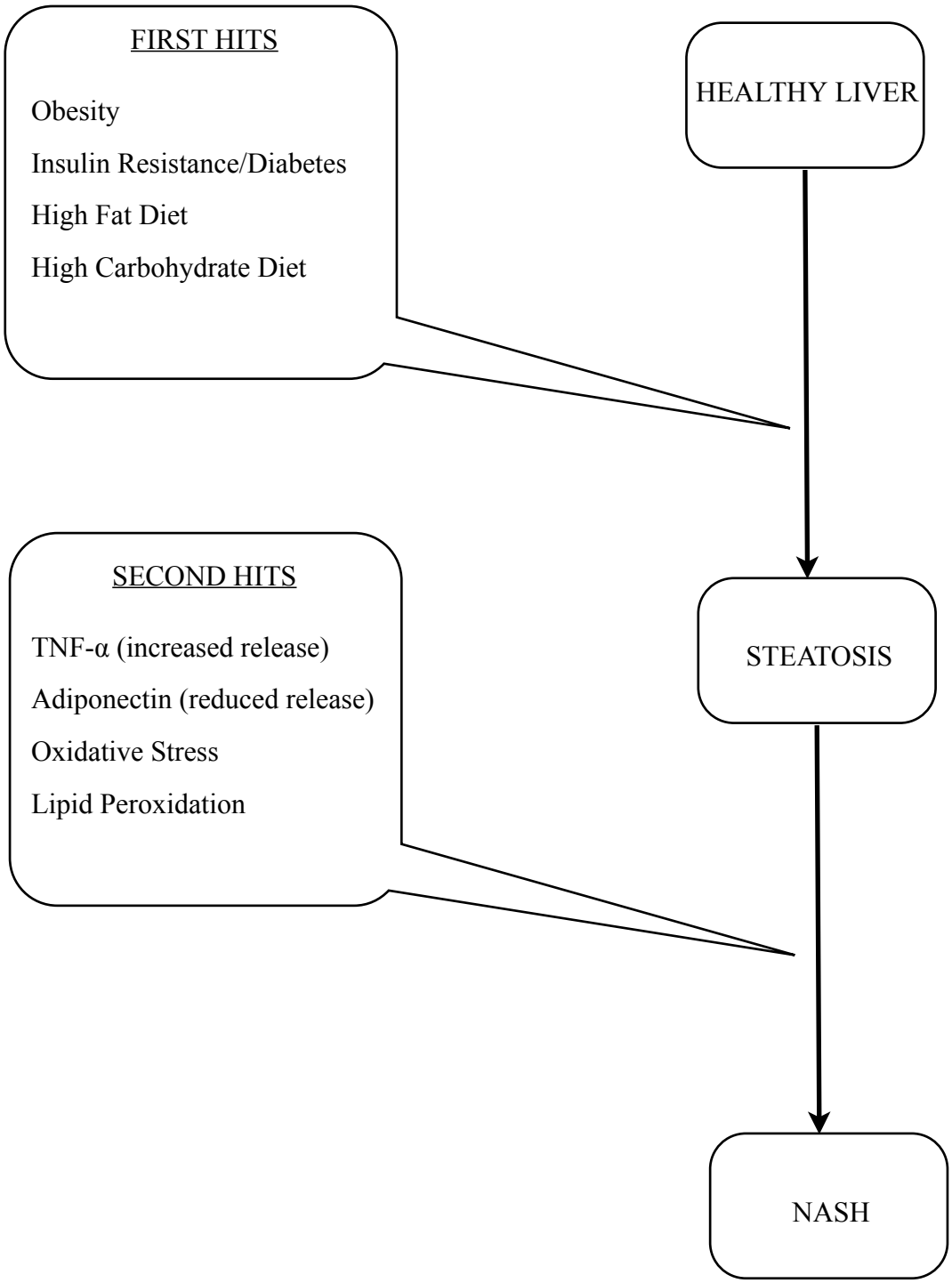

Fig. 1.2: The “Two Hits” theory for onset of steatosis and its progression to NASH

1.1.4 Management and Proposed Treatments of NAFLD

In the quest for the discovery of a suitable treatment for NAFLD, several approaches have been investigated in clinical trials, such as lifestyle modifications, alone or in combination with pharmacological treatments with a number of agents belonging to several therapeutical classes.

Common working hypothesis to all investigations was an intervention on the metabolic conditions that were commonly associated with NAFLD.

Lifestyle Modifications

Hitherto there is no pharmacological treatment for NAFLD and the only recommendations are lifestyle modifications, such as weight loss, diet and physical exercise (American Gastroenterological Association, 2002). Since NAFLD is closely related to obesity, the first logical approach is to tackle the excess of body fat.

A 3-month study in obese Japanese patients had shown that a restricted diet (25 kCal/ kg of ideal body weight) plus physical exercise were helpful in improving liver enzyme levels and histological findings of steatosis, but not inflammation and fibrosis (Ueno *et al*, 1997). Kugelmas and colleagues (2003) compared the effects of diet and exercise (30 min of daily jog) with or without the administration of 800 mg/ day of vitamin E, in two groups of 16 patients each with biopsy-proven NASH. At conclusion of a 12-week period, they found that vitamin E did not to influence the outcomes of the study. Thus, for statistical purposes, they pooled the two groups together and concluded that a weight reduction of about 5% was the single independent predictive factor to induce significant improvements in liver

transaminases (Kugelmas *et al*, 2003). However, histopathological data on repeated liver biopsy were not disclosed.

Petersen and colleagues (2005) proved the benefit of a moderate weight loss (8 kg or 10% reduction in body weight), achieved solely through diet, in sedentary type II diabetic patients with MRI-proven steatosis and normal liver enzymes. All the subjects were found with significant reduction of hepatic fat content at conclusion of the study (Petersen *et al*, 2005).

Orlistat, a drug that prevents fat absorption through inhibiting gastric and pancreatic lipases (Lucas *et al*, 2003), was recently investigated in patients with NASH. An interventional 24-month study on the effects of orlistat, besides diet, in overweight subjects with biopsy-proven NASH was conducted by Harrison and colleagues (2009). Overall, no advantage was seen in the orlistat group compared to the control group, that was under dietary regimen alone, with regard to body weight reduction and histopathological score for NASH. However, Harrison (2009) reported a significant improvement of steatosis in all subjects that lost 10% or more of their body weight, thus confirming earlier findings from Petersen (2005). Hatzitolios and colleagues (2004) reported improvement of liver transaminases levels and liver echogenicity in 3 groups of dyslipidemic patients treated with omega-3, atorvastatin or orlistat. However, the improvements were found to be related to weight loss and not to any of the treatments assigned (Hatzitolios *et al*, 2004).

Lipid Lowering Drugs

The high prevalence of NAFLD in dyslipidaemic patients encouraged researchers to investigate the effects of lipid lowering drugs, such as statins, in NAFLD. Statins are

inhibitors of hepatic 3-hydroxy-3-methylglutaril-coenzyme A (HMG-CoA) reductase, an enzyme converting HMG-CoA into the cholesterol-precursor mevalonic acid (Stancu and Sima, 2001).

Atorvastatin, the most widely prescribed cholesterol lowering drug, was recently investigated in 31 dyslipidaemic patients with biopsy-proven NASH (Hyogo *et al*, 2008). After 24 months of treatment with atorvastatin, all subjects showed significant reduction in liver enzymes and, not surprisingly, in lipid profile. Biopsy which was repeated in 17 out of the 31 patients, showed significant histological changes recorded for steatosis, but not for other NASH parameters, such as ballooning, inflammation and fibrosis. However, 4 out of 17 patients, showed pathology progression. On all 31 patients, CT scan was performed to determine liver density, that is inversely correlated to fat content, and confirmed a decreased presence of fat infiltration, although it was not statistically significant (Hyogo *et al*, 2008). As mentioned earlier, study by Hatzitolios (2004), showed that intervention with atorvastatin was found ineffective in NAFLD.

Another lipid lowering agent, the fibrate gemfibrozil, an activator of PPAR-α, was investigated in a brief prospective study of NASH patients with elevated liver transaminases. After 4 weeks of treatment the 23 patients in the drug group were found to have significantly reduced ALT, AST and gamma-glutamyl transpeptidase (GGT) levels, even though the values were still above their respective upper limit. Whereas, amongst the 23 untreated control patients, no changes were seen (Basaranoglu *et al*, 1999). Liver biopsy was not repeated on these subjects. Thus, it was not possible to evaluate if liver enzymes changes were reflective of actual histological changes.

Insulin Sensitising Drugs

Due to the proven association diabetes-NAFLD and the role played by insulin resistance in the progression of NAFLD (Day, 2002), insulin sensitising drugs have been investigated for the treatment of NAFLD.

The effects of metformin, a biguanide oral antidiabetic drug that suppresses hepatic gluconeogenesis (Kirpichnikov *et al*, 2002) has been investigated in several, mostly open label, trials. The rationale for metformin clinical trials was based on an animal study that reported the efficacy of this drug in reversing fatty liver in genetically engineered obese mice (Lin *et al*, 2000). Shortly after Lin's publication, the first human trial on metformin (as proposed treatment for NASH) versus diet alone in non-diabetic, non-obese subjects was described by Marchesini and colleagues (2001a). A significant reduction of liver volume, assessed using ultrasound, was reported in the metformin group and, to a lower extent, in the control group. ALT levels were reported to be significantly lower at the end of the 4-month study in the metformin group compared to baseline (Marchesini *et al*, 2001a).

Metformin was also investigated in 15 adults patients, including a single diabetic, with slightly elevated (up to 1.5 times the normal values) liver aminotransferases and biopsy-proven NAFLD (Nair *et al*, 2004). Improvement in liver enzymes levels was seen only at the 3-month follow-up, but was not sustained thereafter. At conclusion of 1-year treatment, the results were far from encouraging, an improvement in steatosis and NASH-related inflammation was recorded in only 30% and 20% of patients, respectively (Nair *et al*, 2004).

Contrasting results were obtained by Schwimmer and colleagues (2005), who evaluated metformin administration in 10 obese, non-diabetic paediatric patients in

an open-label study. At baseline all patients had histology-proven NASH with elevated liver transaminases. The liver fat content was measured by MRI, thus no data on inflammation was obtained. At completion of the 24-week investigation, liver enzymes were found to be significantly lower. ALT and AST returned within the normal limits in 40% and 50% of the patients, respectively. Furthermore, hepatic fat significantly decreased in 90% of the children (Schwimmer *et al*, 2005). However liver biopsies were not repeated, thus no data on histology changes were available.

In another open label study, with a follow-up of 12 months, metformin, diet or vitamin E were compared in three groups of non-diabetic patients with biopsy-proven NAFLD. Improvement of liver aminotransferases was reported in all patients and it was associated with weight loss, independently of treatment, even though a moderate advantage for metformin was observed (Bugianesi *et al*, 2005). A longer study, with a follow-up of 24 months, on 57 overweight or obese children and 30 controls confirmed that metformin was not superior to lifestyle modifications in improving neither liver enzymes levels nor NAFLD (Nobili *et al*, 2008a). The latest study on metformin was conducted in 26 overweight or obese patients with biopsy-proven NASH and non-clinically significant elevation of liver enzymes. After 48 weeks, a significant improvement in histology findings was detected in about half of the patients. However, in the responders group there was a strong association between improvement and weight loss (Loomba *et al*, 2009).

In summary, no concrete evidence supported the use of metformin, at dose of 1-2 g/ day, in the treatment of NAFLD. However, another class of insulin sensitising agents attracted the attention of many researchers, namely the thiazolidinediones. The thiazolidinediones class of antidiabetics, reduces insulin resistance in the liver and

other tissues through stimulating the peroxisome proliferator-activated receptor gamma (PPAR-γ) and alpha (PPAR-α) (Smith, 2001).

Rosiglitazone, a drug belonging to the thiazolidinediones class, was investigated in a 48-week open-label study of 30 adults with elevated ALT and biopsy-proven NASH. After treatment, significant reduction was found in ALT, GGT, alkaline phosphatase (ALP) and insulin sensitivity levels. Positive results were also found for steatosis, but not for NASH in the 22 patients that underwent repeated liver biopsy. No correlation was found between ALT and histology changes. However, body weight was found to have increased significantly. Serious adverse reactions led to withdrawal of 10% of participants to the study (Neuschwander-Tetri *et al*, 2003).

A long-term assessment of rosiglitazone in 63 patients with histology-proven NASH showed a significant reduction for transaminases levels and steatosis, but not for fibrosis and inflammation compared to placebo during the first year of the double-blinded study (Ratziu *et al*, 2008). Subsequently, 40 of these subjects continued the study, that became a longitudinal open label trial with all patients given rosiglitazone, for a further 24-month period (Ratziu *et al*, 2009). The results indicated no further benefit from a prolonged therapy with rosiglitazone as compared to 1-year treatment. Results obtained with the patients previously on placebo during the 2 years of treatment with the drug, were similar to those obtained during the first year of the study by the original rosiglitazone group (Ratziu *et al*, 2009). No activity on inflammation and/or fibrosis was seen even in the longitudinal study. Thus, the results of the study discredited the hypothesis that long-term administration would have been needed to improve inflammation and/or fibrosis. A median gain weight of 2 kg was reported, with 36% of the patients gaining more than 3 kg. Furthermore,

adverse events occurred among the patients far too frequently to consider rosiglitazone as a well tolerated drug. Adverse events reported were asthenia (36%), muscular cramps (26%), swollen legs (25%), gastrointestinal symptoms (19%), headache (11%) and dyspnea (4%).

Another thiazolidinediones drug, pioglitazone, was investigated in a 16-week open-label clinical trial in 14 type 2 diabetic patients with normal liver function test. This study, focussed mostly on the hepatic glucose metabolism, showed pioglitazone to be beneficial in significantly reducing insulin resistance, fasting plasma glucose and hepatic fat content, determined by MRI. Liver AST and ALT values, even though normal at baseline, were significantly reduced at conclusion of the study. However, body weight significantly increased (Bajaj *et al*, 2003). These positive results on risk factors associated with NAFLD prompted the initiation of several pilot studies. One investigation enrolled 18 non-diabetic, overweight or obese patients with elevated liver enzymes and biopsy-proven NASH. At the end of the 48 weeks of treatment with pioglitazone, the liver function test values were significantly reduced, with normalisation in about 70% of the patients, as well as liver fat content, as measured by MRI. Moreover, repeated biopsy confirmed an overall amelioration of histological parameters, such as steatosis, NASH, inflammation and fibrosis (Promrat *et al*, 2004). Also in this study body weight increased significantly.

The hypothesis that not only insulin resistance is involved in NASH, but also oxidative stress is amongst the contributing elements for the onset of NASH, led to initiation of a pilot trial with a combination therapy of antioxidant and oral antidiabetic drug (Sanyal *et al*, 2004). Twenty non-diabetic patients with histology-proven NASH, were randomised into two arms and administered either vitamin E (as

alpha-tocopherol) alone or pioglitazone plus vitamin E. At conclusion of the 6 months study amelioration in steatosis was recorded for both arms, with a greater advantage for the combination therapy (Sanyal *et al*, 2004). This pilot study was scaled-up to include almost 250 patients and the study design was fine-tuned to identify which agent of the combination therapy had greater influence on the response. Moreover, a placebo group was included besides the vitamin E and the pioglitazone arms (Sanyal *et al*, 2010). The follow-up period was lengthened to 96 weeks and the dose of alpha-tocopherol administered doubled to 800 mg/day. After 2 years of treatment, patients were found with improved steatosis in 31%, 54% and 69% of cases for placebo, vitamin E and pioglitazone, respectively. Hepatic ALT and AST decreased in a comparable manner for vitamin E and pioglitazone, however they rebounded towards baseline levels once the treatments were discontinued. The pioglitazone group gained an average of 5 kg during the study, compared to vitamin E and placebo groups where body weight was maintained at baseline levels (Sanyal *et al*, 2010). The results obtained were essentially negative for pioglitazone treatment which did not lead to any significant level of histological improvement in NASH (primary outcomes), even though an amelioration in the overall NAFLD score (secondary outcomes) was found. However, vitamin E treatment led to significant improvement for both, primary and secondary outcomes in this cohort of non-diabetic patients (Sanyal *et al*, 2010).

Hepatoprotective Agents and Antioxidants

Several hepatoprotective agents, either drugs or supplements, have been investigated for the treatment of NAFLD. Vajro and colleagues (2000) studied ursodeoxycholic

acid, a secondary bile acid used for removal of cholesterol gallstones, in 31 obese children with liver abnormalities diagnosed either by blood chemistry or by ultrasound examination. This open-label study, comparing the effects of ursodeoxycholic acid with or without diet on ALT or ultrasound abnormalities, showed that diet associated with weight loss to be more affective than ursodeoxycholic acid in improving liver abnormalities (Vajro *et al*, 2000).

In another double-blind placebo-controlled, ursodeoxycholic acid was evaluated in 166 patients with biopsy-proven NASH. After two years of intervention, no significant change was associated with the use of ursodeoxycholic acid compared to placebo (Lindor *et al*, 2004).

Mice fed choline-deficient diet have been reported to develop hepatitis similar to human hepatitis (Grattagliano *et al*, 2000). Phosphatidylcholine, a choline-conjugated phospholipid and essential membrane component, is commonly employed for improving general liver health and as such was investigated in type 2 diabetic patients with ultrasound-proven NAFLD. In an open-label study conducted by Poongothai and colleagues (2005), phosphatidylcholine appeared to be beneficial in reducing the liver function markers and ameliorating the hepatic echotexture. Liver function markers such as ALT and AST showed a significant reduction within two months of treatment, meanwhile GGT showed a significant reduction after six months of treatment. Moreover, it was reported that the amelioration of the NAFLD score was directly related to the baseline severity, thus patients with more severe initial score were more likely to see a greater improvement compared to their milder counterparts (Poongothai *et al*, 2005). The suggested mechanisms of action for phosphatidylcholine was its cholesterol lowering activity. However, the authors

could not exclude that an improved glycaemic control in these patient played a role in improving the overall study outcomes.

Another approach to NAFLD treatment was based on the use of antioxidants, due to hepatic lipid peroxidation (Letteron *et al*, 1996) and increased oxidative stress levels observed in subjects with NAFLD (Haque and Sanyal, 2002). Vitamin E, one of the most potent antioxidant, has been tested in a number of clinical trials over the last decade.

Lavine (2000) treated 11 obese paediatric patients, with elevated liver transaminases and ultrasound-proven NAFLD, with synthetic alpha-tocopherol. The dose employed ranged from 400 mg to 1200 mg a day. The study lasted between 4 and 10 months, at conclusion of which reduction of ALT and AST values was found to be significant. compared to baseline. However, ultrasound examination was not repeated (Lavine, 2000).

Efficacy of alpha-tocopherol was investigated in a study that included 12 adults with NASH and 10 adults with steatosis (Hasegawa *et al*, 2001). All patients were given a 6-month entry dietary therapy and were later treated with 300 mg/day of alpha-tocopherol for another 6-month period. Common to all patients were impaired glucose tolerance, hyperlipidemia and elevated liver markers. At conclusion of the study, it was found that the 6 months diet therapy has no effect on the liver enzymes in the NASH patients, but a normalisation of the values was recorded only after alpha-tocopherol treatment was initiated. On the other hand, patients with steatosis benefited from the diet more than from alpha-tocopherol treatment. In this group of patients, a normalisation of the liver enzymes was noted after dietary therapy and remained stable after the alpha-tocopherol treatment. Biopsy performed in 9 out of

12 patients in the NASH group showed amelioration of steatosis, inflammation and fibrosis (Hasegawa *et al*, 2001).

A paediatric, single-blind placebo-controlled investigation involving 28 obese children with similar inclusion criteria of that of Lavine (2000) was also carried out using vitamin E (alpha-tocopherol). Vitamin E was given at a dose of 400 mg/day for the initial 2 months and subsequently reduced to 100 mg/day for 3 months. Controlled diet was given to both, the vitamin E and placebo groups. The two groups showed similar results in terms of ALT reduction as well as ultrasound examination (Vajro *et al*, 2004). No clinical improvement was attributed to alpha-tocopherol.

Harrison and co-investigators (2003) conducted a prospective, randomised trial on 45 subjects with NASH assigned to receive vitamin E and vitamin C or placebo for 6 months. The dose of both vitamins given was 1000 mg daily. When liver biopsy was repeated, the treatment with vitamins was found to have improved the diagnosis of NASH compared to baseline, but the changes were not significant as compared to placebo. Moreover, there was no improvement in ALT levels and/or inflammation (Harrison *et al*, 2003).

As previously discussed, the open-label study of Kugelmas (2003), showed that vitamin E plus lifestyle modification did not produce any significant outcomes as compared to lifestyle modification alone in NAFLD. Also, the study by Sanyal *et al* (2004) about a combination therapy of pioglitazone and alpha-tocopherol *versus* alpha-tocopherol alone, that showed amelioration of NASH in both arms, have been previously discussed.

A study that enrolled 90 NAFLD paediatric patients investigating combined antioxidant treatment with alpha-tocopherol (600 IU/day) plus ascorbic acid (500 mg/day) or placebo was reported by Nobili *et al* (2008b). Obese children were prescribed tailored hypocaloric diets, whereas isocaloric personalised diets were prescribed to children with normal weight. All patients were advised to increase physical activity. Even though only 53 out of 90 children completed the 2-year study, it was claimed sufficient for concluding that lifestyle modifications were associated with an overall improvement in the NAFLD score and that the antioxidant therapy did not provide any significant advantage (Nobili *et al*, 2008b).

Despite the contradicting therapeutic results obtained with alpha-tocopherol so far and in light of the previously discussed 3-way study by Sanyal's group (2010) on pioglitazone, vitamin E or placebo in NASH patients, treatment of NAFLD with vitamin E is hitherto considered the most promising.

1.2 BRAIN WHITE-MATTER LESIONS

Brain white-matter abnormalities were first and briefly described at the end of nineteenth century by Binswanger in the contest of a clinical case of progressive decline of brain functionalities. Soon after, Alzheimer, in his description of similar cases, attributed these white-matter changes to arteriosclerosis of the brain vessels (Pantoni and Garcia, 1995). Until the introduction of CT scan and MRI, white-matter changes could only be ascertained during autopsy. These recent imaging techniques provided the opportunity to detect white-matter changes or white-matter lesions (WML) without having to wait for autopsy. Thus, it became evident that not only patients afflicted by Alzheimer's disease had WML, but also other pathologies could be associated with WML. A correlation study between histopathological changes and the hyperintensity signals obtained from MRI scans of deceased elderly, confirmed the presence of actual abnormalities in the brain white-matter (Grafton *et al*, 1991). However, no indication on the pathogenesis of WML was obtained.

Being an asymptomatic condition and to avoid the designation of a new pathology for patients, the term leuko-araiosis was coined (Hachinski *et al*, 1987). Leuko-araiosis derives from the Greek words *leuko* (white) and *araiosis* (rarefaction). Immunohistochemical investigation in three groups of patients with cardiovascular disease, Alzheimer's disease and control, concluded that the blood-brain barrier is altered to higher extent in subjects with cardiovascular disease than Alzheimer's disease, suggesting this alteration might be involved in the development of WML. Moreover, it was noted that Alzheimer's disease subjects had lesser changes, both in number and severity, than cardiovascular disease subjects, indicating the possibility of separate pathological pathways (Tomimoto *et al*, 1996).