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RESEARCH ARTICLE

Hyperhomocysteinemia is a key for aggravation of liver injury in non-alcoholic fatty liver disease associated with cardiovascular disease in rats

Heibashy, M.I.A. and Mazen, G.M.A.

Biological Applications Department, Nuclear Research Center, Atomic Energy Authority, Egypt.

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***Corresponding Author**

Heibashy, M.I.A

Abstract

Homocysteine has emerged as a novel independent marker of risk for the development of cardiovascular disease (CVD). Nonalcoholic fatty liver disease (NAFLD) is consistently associated with features of the metabolic syndrome, a condition carrying a high risk of cardiovascular events. The objective of this study was to investigate the correlation between non-alcoholic fatty liver and risk of cardiovascular disease. Also, this study evaluated the level of serum homocysteine and their association with the disease severity in rats fed high fructose diet. The obtained results revealed a significant ($p<0.05$) increase in carbohydrate profile (glucose, insulin & insulin resistance index) in NAFLD associated CVD rat groups dependent on the fructose intake as compared to their normal control ones. Lipid profile (cholesterol, triglycerides, HDL, LDL, VLDL & atherogenic index) were significantly ($p<0.05$) elevated in treated rats (fructose rat groups) when compared to their corresponding control group. Furthermore, liver profile (ALT, AST, GGT, L-FABP & haptoglobin) and cardiac profile (CK, LDH, H-FABP, myoglobin & homocystein) were significantly ($p<0.05$) increased throughout the whole experimental period in treated rats. The levels of inflammatory cytokines (CRP, TNF-α, IL-6 & IL-1β) were increased in animal groups which treated by fructose during the whole experimental periods (1, 2 & 3 months). On the other hand, NAFLD associated with CVD rats groups showed a significant ($p<0.05$) decrease in adiponectin level. These data suggested that high fructose diet induced NAFLD associated with CVD and confirmed the strict association between increased homocystein level, NAFLD and pronouncing of cardiovascular disease which reflected the degree of damage occur in both liver and heart.

Introduction

Homocysteine (HCY) is a sulfur containing amino acid that is formed as an intermediate in methionine metabolism in the liver (Stead et al., 2000). Hyperhomocysteinemia is regarded as an independent risk factor for cardiovascular diseases (CVD); it has been implicated in diverse cardiac pathological conditions including coronary heart disease, acute myocardial infarction and sudden cardiac death (de Carvalho et al., 2013). However, it remains elusive whether HCY itself is a causative risk factor or merely a biomarker of cardiovascular disesae (Smulders & Blom, 2011). Furthermore, hyperhomocysteinemia unleashes mediators of inflammation, increases production of intracellular superoxide anion causing oxidative stress, reduces intracellular level of nitric oxide and induces endoplasmic reticulum stress, all of which can explain many processes of Hcy promoted cell injury and may result also in insulin resistance (IR) (Shoelson et al., 2006). Although, hyperhomocysteinemia and insulin resistance

are both associated with cardiovascular disease, data on the association between homocysteine levels and IR syndrome have provided conflicting results (Vaya et al., 2011).

Insulin resistance (IR) plays an important role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of IR or metabolic syndrome (Polyzos et al., 2009). NAFLD is a disease characterized by abnormal fat accumulation in the liver and the condition can progress into more serious liver disease, such as non alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and more rarely, liver carcinoma (Adams & Angulo, 2005). The metabolic syndrome has received considerable attention over the last decades, as the cluster of its associated diseases significantly increases the risk of cardiovascular disease mortality (Eckel et al., 2005); it is also known as cardiometabolic syndrome. Although, type II diabetes (TIID), obesity and elevated plasma triacylglycerols are well-known contributors to cardiovascular morbimortality, NAFLD has been recently suspected as playing a role in the development of CVD, independently of traditional risk factors (Targher & Arcaro, 2007).

Dietary fructose is a major candidate for causing NAFLD. Unlike glucose, fructose ingestion can rapidly cause fatty liver in animals, in association with the development of leptin resistance (Shapiro et al., 2008), microvascular disease (Cirillo et al., 2009) and vascular inflammation (Glushakova et al., 2008). Recent data suggested that the increase of fructose consumption led to remarkable elevations in the fat mass, *de novo* lipogenesis, inflammation and pronouncing of induces insulin resistance associated with postprandial hypertriglyceridemia, particularly in overweight individuals (Stanhope & Havel, 2009). Moreover, Ouyang et al. (2008) indicated that the development of NAFLD may be associated with excessive dietary fructose consumption. Whether increased fructose consumption correlates merely with the development of NAFLD or promotes the transition from NAFLD to NASH and more advanced stages of liver damage remains unclear.

Therefore, the objective of this work was to investigate the association between hyperhomocysteinemia and the pronouncing of non-alcoholic fatty liver disease associated with the elevation risk of cardiovascular disease.

Material and Methods

Sixty male albino rats (*Rattus rattus*) weighing 200±20 g were used in this study. The animals were housed in regular cages situated in the Animal House at Biological Applications Department, Nuclear Research Center, Atomic Energy Authority, Egypt. Rats were maintained on standard rat chow diet and were given tap water to drink *ad libitum*.

At the beginning of the study, rats were randomly divided into four groups according to the type of diet (n=15 in each group). The first group was fed on a control diet according to NRC (1977) as described in table (1). The other three groups were fed on the same compositions as the control diet, except that corn starch was replaced with an equal amount of 20% fructose & 10% lard (Low fructose diet) for 2nd group, 40% fructose &10% lard (Mid fructose diet) for 3rd group and 60% fructose &10% lard (High fructose diet) for 4th group respectively for one, two and three months according to Du Vigneaud & Karr (1925) and approved by Pooranaperundevi et al. (2010) (Table 1). At the completion of each experimental period, the fasting animals were anaesthetized and blood from individual rats was collected by cardiac puncture. Sera were separated and divided into considerable aliquots to avoid the effects of repeated thawing and freezing. All specimens were stored at -40°C until use.

Table (1): Composition of the normal and fat diets

Nutrient	Standard normal diet	Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
Corn starch	60 %	40 %	20 %	zero %
Soybeans	18 %	18 %	18 %	18 %
Sucrose	10 %	zero %	zero %	zero %
Corn oil	4.5 %	4.5 %	4.5 %	4.5 %
Cellulose	4.5 %	4.5 %	4.5 %	4.5 %
Vitamin & mineral mixture	3 %	3 %	3 %	3 %
Fructose	zero %	20 %	40 %	60 %
Lard	zero %	10 %	10 %	10 %

Serum glucose, cholesterol, triglycerides and HDL-cholesterol levels were determined colorimetrically using commercial kits from Human, Germany. LDL-cholesterol, VLDL-cholesterol and atherogenic index were calculated by the following equation:
LDL (mg/dL) = Total cholesterol – Tiglyceride/5 + HDL (Assman et al., 1984).
VLDL (mg/dL) = Total serum triglyceride/5 (Friedewald et al., 1972).
Atherogenic Index = Total serum triglyceride / Total serum HDL-C (Pandya et al., 2006).

The homeostatic model assessment (HOMA) value as a measure of IR was calculated using the following formula: fasting insulin (μU/L) × fasting glucose (mmol/L)/22.5 according to Matthews et al. (1985).

The activities of lactate dehydrogenase (LDH), creatin kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) were measured by using commercial kinetic kits (Prolabo, France).

Moreover, serum insulin, leptin and angiotensin II levels were estimated by radioimmunoassay (RIA) specific kit for rats using solid phase component system purchased from ICN Pharmaceuticals Inc, USA. However, serum resistin, adiponectin, endothelial-1 (ET-1), C- reactive protein (CRP), interlukin 1β (IL-1β), interlukin- 6 (IL-6) and tumor necrosis factor-α (TNF-α) levels were assayed using commercial ELISA (Sandwich Immunoassay Technique) specific kit for rats (Immuno-Biological Laboratories Co., Ltd. USA). Haptoglobin, myoglobin levels were assayed using commercial ELISA (Sandwich Immunoassay Technique) specific kit for rats (ALPCO, Ltd. USA).

In addition, the levels of rat homocysteine (Hcy) were assayed by the aid of ELISA (Sandwich immunoassay technique) using commercial kit (CUSABIO, China). Also, rat H-FABP and L-FABP quantitative tests based on a solid phase enzyme-linked immunosorbent assay (ELISA) developed by Life diagnostics Inc. West. Chester PA, USA.

Statistical differences between the means were assessed by analysis of variance (ANOVA) followed by Duncan's multiple range test according to Duncan (1955) and Snedecor & Cochran (1982) using a computer program (Costate). Values of $P<0.05$ were considered statistically significant.

Result and Discussion

Non-alcoholic fatty liver disease (NAFLD) is strongly associated with type II diabetes mellitus and cardiovascular disease (CVD). It is characterized by insulin resistance and mitochondrial dysfunction. Fructose-fed rat model develops an insulin-resistance syndrome with a very similar metabolic profile to the human condition, including hyperinsulinemia, insulin resistance, hypertriglyceridemia and decreased HDL cholesterol (Chiu et al., 2014). The literature data diverged widely regarding to the characterization of metabolic syndrome disorders in rats. These differences are attributable mainly to differences among experimental protocols, such as the variability in the composition of diets, duration of dietary treatments, age and species of animals (de Castro et al., 2013). To minimize these biases, the current investigation was designed to study several biochemical parameters of adult rats submitted to different fructose diets are most often used to induce disturbances related to metabolic syndrome.

In the current work, treated rat groups with different fructose diets (Low, mid & high) recorded significant ($p<0.05$) increase in carbohydrate profile (glucose, insulin and HOMA-IR) as compared to their corresponding control rats group (Table 2). One possible explanation for insulin resistance in fructose-fed rats is a downregulation of insulin receptors with lower insulin receptor mRNA levels in skeletal muscle but not kidney. This effect has been found to be associated with a decrease in insulin-stimulated glucose utilization and a reduction in insulin sensitivity (Catena et al., 2003). Kannappan and Aduradha (2009) showed that high fructose diet was able to impair insulin sensitivity and glucose tolerance. This finding was confirmed by the observed hyperglycemia, hyperinsulinemia and elevated HOMA, and these data matched with those of Yadav et al. (2009).

Increase in the fructose load to the liver, could elicit rapid responses that ultimately influence hepatic gene expression, glucose disposal and insulin action. This may be attributed to the fact that fructose metabolism bypasses the regulatory step catalyzed by phosphofructokinase-1. Fructose-1-phosphate is split by aldolase B into glyceraldehyde and dihydroxyacetone phosphate. Both of them can be converted to glyceraldehyde-3-phosphate. Thus, the fructose molecule is metabolized into 2 triose phosphates that bypass the main rate-controlling step in

glycolysis, 6-phosphofructokinase. So, fructose continuously enters the glycolytic pathway resulting in hyperglycemia (Mayes, 1993).

Table (2): Disturbance in carbohydrate profile as results of liver injury in non alcoholic fatty liver disease associated with cardiovascular diseases in rats.

		Standard normal diet	NAFLD + CVD		
			Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
Glucose (nmol/L)	One month	$6.171 \pm 0.26^{A}{}_{a}$	$7.884 \pm 0.41^{B}{}_{a}$	$9.117 \pm 0.62^{C}{}_{a}$	$12.014 \pm 0.88^{D}{}_{a}$
	Two months	$6.166 \pm 0.24^{A}{}_{a}$	$9.624 \pm 0.67^{B}{}_{b}$	$11.589 \pm 0.86^{C}{}_{b}$	$15.839 \pm 0.97^{D}{}_{b}$
	Three months	$6.180 \pm 0.27^{A}{}_{a}$	$11.061 \pm 0.82^{B}{}_{c}$	$15.212 \pm 0.93^{C}{}_{c}$	$19.112 \pm 1.04^{D}{}_{c}$
Insulin (µU/L)	One month	$26.932 \pm 1.07^{A}{}_{a}$	$29.556 \pm 1.16^{B}{}_{a}$	$37.113 \pm 1.32^{C}{}_{a}$	$42.817 \pm 1.45^{D}{}_{a}$
	Two months	$26.897 \pm 1.02^{A}{}_{a}$	$34.429 \pm 1.29^{B}{}_{b}$	$41.692 \pm 1.41^{C}{}_{b}$	$50.772 \pm 1.63^{D}{}_{b}$
	Three months	$26.916 \pm 1.04^{A}{}_{a}$	$40.027 \pm 1.37^{B}{}_{c}$	$48.225 \pm 1.56^{C}{}_{c}$	$59.565 \pm 1.78^{D}{}_{c}$
IR (HOMA)	One month	$7.387 \pm 0.31^{A}{}_{a}$	$10.356 \pm 0.54^{B}{}_{a}$	$15.038 \pm 0.74^{C}{}_{a}$	$22.862 \pm 0.92^{D}{}_{a}$
	Two months	$7.371 \pm 0.29^{A}{}_{a}$	$14.726 \pm 0.69^{B}{}_{b}$	$21.474 \pm 0.89^{C}{}_{b}$	$35.741 \pm 1.11^{D}{}_{b}$
	Three months	$7.392 \pm 0.32^{A}{}_{a}$	$19.677 \pm 0.81^{B}{}_{c}$	$32.604 \pm 1.04^{C}{}_{c}$	$50.596 \pm 1.25^{D}{}_{c}$

- Means bearing different superscripts (A,B,C&D) within the same row are differ significantly ($P<0.05$).
- Means bearing different subscripts (a,b&c) within the same column are differ significantly ($P<0.05$).

Moreover, Stanhope & Havel (2008) reported that visceral adiposity is known to be increased by high fructose diet. It is associated with insulin resistance as a result of the direct delivery of portal blood flow from visceral fat to the liver releasing free fatty acids (FFAs). The greater lipolytic capacity of visceral than peripheral adipocytes releases more FFAs to the portal circulation. Furthermore, when visceral adipocytes enlarge, they become more insulin resistant than smaller adipocytes. Increased amounts of FFAs directly affect insulin signaling, diminish glucose uptake in muscle and induce gluconeogenesis in the liver (Mlinar et al., 2007).

There is considerable evidence supporting the ability of high fructose diets to promote hepatic lipogenesis because it provides unregulated amounts of lipogenic substrates acetyl-CoA and glycerol-3-phosphate which in turn stimulates *de novo* lipogenesis, *via* increases the synthesis, esterification, secretion and accumulation of cholesterol and fatty acids in hepatocytes (Yadav et al., 2009). In this work, the consumption of fructose diets (Low, mid & high) in rat groups for 1, 2 & 3 months resulted in hypertriglyceridemia, hypercholesterolemia and increased levels of both LDL, HDL, VLDL and atherogenic index as compared to their corresponding control rats as shown in table (3). These results may be due to the disturbance in the hypothalamus-pituitary-thyroid axis (HPTA), the elevation in the lipid oxidation, the elevation of ceramides levels or/and the decrease of β-oxidation of lipid in the matrix of mitochondria. These results are in agreement with that obtained by D'souza et al. (2012). The authors reported that increment in *de novo* lipogenesis may help in the promotion and increment in the synthesis of ceramides which they are the potentially toxic by-products of excessive lipid accumulation in insulin-sensitive tissues. Ceramides are indeed deemed to be toxic to hepatic function and contribute to liver lipotoxicity associated with the pronouncing of insulin resistance (Promrat et al., 2011). Moreover, Roglans et al. (2002) reported that continuous fructose feeding caused down regulation of PPAR-α, which in turn leads to a reduction in triglycerides catabolism which is involved with LDL, acyl Co-A oxidase and β-oxidation.

Insulin and glucose are known to directly regulate lipid synthesis and secretion. Insulin controls hepatic sterol regulatory element binding protein (SREBP) expression, which is a key transcription factor responsible for regulating fatty acid and cholesterol biosynthesis. SREBP binds to sterol responsive elements (SRE) found on multiple genes and can activate a cascade of enzymes involved in cholesterol biosynthetic pathways, such as HMG-CoA reductase (Brown & Goldstein, 1997) and fatty acid synthase (FAS) (Bennett et al., 1995).

High density lipoprotein (HDL) is the major cholesterol lipoprotein carrier in rats, thus, the elevation of serum HDL could merely be a reflection to the observed the increment of serum T-cholesterol (Girard et al., 2006). Also, insulin resistance decreases lipoprotein lipase activity, the major mediator of VLDL clearance, which may make a smaller contribution to elevate triglycerides levels. VLDL is metabolized to remnant lipoproteins and LDL, both strongly associated with atherosclerotic risk (Semenkovich, 2013).

Table (3): Disturbance in lipid profile as results of liver injury in non alcoholic fatty liver disease associated with cardiovascular diseases in rats.

		Standard normal diet	NAFLD + CVD		
			Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
Cholesterol (mg/dL)	One month	$57.559 \pm 1.54^{A}_{a}$	$74.326 \pm 1.79^{B}_{a}$	$95.227 \pm 1.96^{C}_{a}$	$115.492 \pm 2.11^{D}_{a}$
	Two months	$57.137 \pm 1.51^{A}_{a}$	$90.419 \pm 1.94^{B}_{b}$	$147.129 \pm 2.34^{C}_{b}$	$166.728 \pm 2.66^{D}_{b}$
	Three months	$57.763 \pm 1.59^{A}_{a}$	$126.768 \pm 2.23^{B}_{c}$	$172.601 \pm 2.78^{C}_{c}$	$220.319 \pm 3.07^{D}_{c}$
Triglycerides (mg/dL)	One month	$60.859 \pm 1.63^{A}_{a}$	$82.146 \pm 1.94^{B}_{a}$	$104.412 \pm 2.09^{C}_{a}$	$133.018 \pm 2.44^{D}_{a}$
	Two months	$61.217 \pm 1.65^{A}_{a}$	$111.538 \pm 2.27^{B}_{b}$	$159.879 \pm 2.64^{C}_{b}$	$208.213 \pm 3.21^{D}_{b}$
	Three months	$61.352 \pm 1.66^{A}_{a}$	$152.325 \pm 2.58^{B}_{c}$	$197.653 \pm 2.91^{C}_{c}$	$265.773 \pm 3.48^{D}_{c}$
HDL (mg/dL)	One month	$14.723 \pm 0.64^{A}_{a}$	$16.347 \pm 0.72^{B}_{a}$	$18.659 \pm 0.81^{C}_{a}$	$22.786 \pm 0.91^{D}_{a}$
	Two months	$14.678 \pm 0.63^{A}_{a}$	$19.179 \pm 0.86^{B}_{b}$	$22.527 \pm 0.90^{C}_{b}$	$26.191 \pm 1.06^{D}_{b}$
	Three months	$14.619 \pm 0.61^{A}_{a}$	$23.521 \pm 0.93^{B}_{c}$	$25.854 \pm 0.97^{C}_{c}$	$29.097 \pm 1.14^{D}_{c}$
LDL (mg/dL)	One month	$30.664 \pm 0.78^{A}_{a}$	$41.550 \pm 0.89^{B}_{a}$	$55.686 \pm 1.07^{C}_{a}$	$66.102 \pm 1.18^{D}_{a}$
	Two months	$30.216 \pm 0.75^{A}_{a}$	$48.932 \pm 0.98^{B}_{b}$	$92.626 \pm 1.64^{C}_{b}$	$98.894 \pm 1.77^{D}_{b}$
	Three months	$30.879 \pm 0.81^{A}_{a}$	$72.782 \pm 1.26^{B}_{c}$	$107.216 \pm 1.91^{C}_{c}$	$138.067 \pm 2.29^{D}_{c}$
VLDL (mg/dL)	One month	$12.172 \pm 0.49^{A}_{a}$	$16.429 \pm 0.62^{B}_{a}$	$20.882 \pm 0.68^{C}_{a}$	$26.604 \pm 0.77^{D}_{a}$
	Two months	$12.243 \pm 0.51^{A}_{a}$	$22.308 \pm 0.71^{B}_{b}$	$31.976 \pm 0.80^{C}_{b}$	$41.643 \pm 0.96^{D}_{b}$
	Three months	$12.270 \pm 0.52^{A}_{a}$	$30.465 \pm 0.83^{B}_{c}$	$39.531 \pm 0.91^{C}_{c}$	$53.155 \pm 1.08^{D}_{c}$
Atherogenic Index	One month	$4.134 \pm 0.09^{A}_{a}$	$5.025 \pm 0.19^{B}_{a}$	$5.596 \pm 0.24^{C}_{a}$	$5.838 \pm 0.29^{D}_{a}$
	Two months	$4.171 \pm 0.12^{A}_{a}$	$5.817 \pm 0.28^{B}_{b}$	$7.097 \pm 0.45^{C}_{b}$	$7.950 \pm 0.61^{D}_{b}$
	Three months	$4.147 \pm 0.11^{A}_{a}$	$6.476 \pm 0.37^{B}_{c}$	$7.645 \pm 0.54^{C}_{c}$	$9.134 \pm 0.76^{D}_{c}$

- Means bearing different superscripts (A,B,C&D) within the same row are differ significantly ($P<0.05$).
- Means bearing different subscripts (a,b&c) within the same column are differ significantly ($P<0.05$).

Several studies reported the relationship between elevated serum activities of liver enzymes (e.g., alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) and metabolic syndrome, diabetes and NAFLD. Raised serum activities of liver enzymes independently predicted the future development of metabolic syndrome as well as cardiovascular (CV) events and/or total CV mortality in prospective studies (Haring et al., 2009 and Kim et al., 2014). In the current work, fructose fed rat groups (Low, mid & high) recorded hepatic injury which was evident by significant elevation in the serum activities of ALT, AST and GGT associated with a significant increment in the level of L-FABP and haptoglobin levels as compared to their corresponding normal fed rats groups throughout whole experimental periods (1, 2 & 3 months) (Table 4). These changes may be attributed to the damage occur in hepatocytes. Gamma-glutamyltransferase (GGT) is a subclinical indicator of insulin resistance and it has been suggested that its high serum values represent early evidence for oxidative stress. Moreover, hepatic steatosis is

characterized by a remarkable elevation in the activities of GGT which is also a predictor of coronary artery disease (Meisinger et al., 2006).

Table (4): Disturbance in liver profile as results of liver injury in non alcoholic fatty liver disease associated with cardiovascular diseases in rats.

		Standard normal diet	NAFLD + CVD		
			Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
ALT (U/L)	One month	24.836 ± 0.56 $^{A}_{a}$	32.679 ± 0.78 $^{B}_{a}$	41.315 ± 0.88 $^{C}_{a}$	55.217 ± 1.08 $^{D}_{a}$
	Two months	25.004 ± 0.59 $^{A}_{a}$	42.113 ± 0.91 $^{B}_{b}$	53.749 ± 0.97 $^{C}_{b}$	73.545 ± 1.26 $^{D}_{b}$
	Three months	24.927 ± 0.58 $^{A}_{a}$	65.257 ± 1.06 $^{B}_{c}$	70.334 ± 1.19 $^{C}_{c}$	86.109 ± 1.41 $^{D}_{c}$
AST (U/L)	One month	119.867 ± 1.79 $^{A}_{a}$	132.597 ± 2.13 $^{B}_{a}$	147.652 ± 2.46 $^{C}_{a}$	160.441 ± 2.63 $^{D}_{a}$
	Two months	121.524 ± 1.82 $^{A}_{a}$	151.114 ± 2.52 $^{B}_{b}$	170.003 ± 2.88 $^{C}_{b}$	192.107 ± 3.16 $^{D}_{b}$
	Three months	122.187 ± 1.84 $^{A}_{a}$	166.926 ± 2.71 $^{B}_{c}$	187.221 ± 3.03 $^{C}_{c}$	247.893 ± 3.42 $^{D}_{c}$
GGT (U/L)	One month	15.233 ± 0.44 $^{A}_{a}$	22.437 ± 0.67 $^{B}_{a}$	34.821 ± 0.79 $^{C}_{a}$	40.089 ± 0.86 $^{D}_{a}$
	Two months	15.136 ± 0.41 $^{A}_{a}$	39.225 ± 0.83 $^{B}_{b}$	47.558 ± 0.96 $^{C}_{b}$	58.417 ± 1.17 $^{D}_{b}$
	Three months	15.187 ± 1.23 $^{A}_{a}$	51.773 ± 1.02 $^{B}_{c}$	61.206 ± 1.21 $^{C}_{c}$	72.651 ± 1.43 $^{D}_{c}$
L-FABP (pg/ml)	One month	67.876 ± 0.81 $^{A}_{a}$	82.139 ± 1.07 $^{B}_{a}$	103.779 ± 1.18 $^{C}_{a}$	127.017 ± 1.42 $^{D}_{a}$
	Two months	67.907 ± 0.83 $^{A}_{a}$	110.662 ± 1.31 $^{B}_{b}$	144.422 ± 1.56 $^{C}_{b}$	175.491 ± 2.06 $^{D}_{b}$
	Three months	68.894 ± 0.82 $^{A}_{a}$	153.351 ± 1.72 $^{B}_{c}$	179.582 ± 2.14 $^{C}_{c}$	228.753 ± 2.37 $^{D}_{c}$
Hptoglobin (ng/ml)	One month	7.089 ± 0.22 $^{A}_{a}$	11.412 ± 0.43 $^{B}_{a}$	14.391 ± 0.52 $^{C}_{a}$	16.114 ± 0.59 $^{D}_{a}$
	Two months	7.107 ± 0.24 $^{A}_{a}$	17.579 ± 0.65 $^{B}_{b}$	22.553 ± 0.77 $^{C}_{b}$	25.647 ± 0.92 $^{D}_{b}$
	Three months	7.116 ± 0.24 $^{A}_{a}$	24.083 ± 0.84 $^{B}_{c}$	29.919 ± 1.13 $^{C}_{c}$	36.205 ± 1.41 $^{D}_{c}$

- Means bearing different superscripts (A,B,C&D) within the same row are differ significantly (P<0.05).
- Means bearing different subscripts (a,b&c) within the same column are differ significantly (P<0.05).

In search of even smaller and more specific cytoplasmic proteins of liver injury, the liver fatty acid binding protein (L-FABP) may be a promising new marker. L-FABP is a 14-kDa cytoplasmic protein that binds long-chain fatty acids (LCFAs) with high affinity (Veerkamp & Maatman, 1995). The putative functions assigned to L-FABP include the desorption of LCFAs from the plasma membrane to the cytoplasm, the promotion of intracellular fatty acid (FA) diffusion (Huang et al., 2002), the targeting of FAs to different metabolic pathways and protection against the cytotoxic effects of free FA (Poirier et al., 2001). This property of L-FABP modulates diverse cellular functions, including fatty acid uptake and intracellular lipid contents and metabolism.

The current study revealed that L-FABP levels were significantly ($p<0.05$) increased in all treated rat groups during the whole experimental periods, suggesting that a compensatory mechanism to release excess lipid as VLDL is promoted in the liver. These data are comparable with previous studies of van Nieuwenhoven et al. (1996). The last authors showed that small cytoplasmic proteins diffuse earlier into the vascular system after cell injury than large proteins. However, because of the abundant content of L-FABP in liver tissue, the magnitude of increment of serum values after liver injury is higher than of ALT, making L-FABP a more sensitive marker. It is also proven to be earlier increased when the day of treatment is taken as reference. This sensitivity could even be enhanced as more frequent blood sampling allows better monitoring of the rise of a serum marker.

NAFLD is commonly associated with the development of hepatic inflammation (Gaemers et al., 2011). So, serum levels of haptoglobin, a well-known acute-phase marker of acute hepatic inflammation were measured in rats

suffered from non-alcoholic fatty liver associated with cardiovascular disease. Data in table (4) recorded significant ($p<0.05$) elevation in haptoglobin concentrations in all treated animals. Also, pro-inflammatory cytokines TNF-α, IL-6, IL-1β and CRP levels were significantly ($p<0.05$) elevated in all rat groups dependent on the fructose dietary intake (Low, mid & high) and time of treatment (1, 2 & 3 months) as compared to control ones (Table 5). These results suggested the development of fibrosis and liver injury in this model of NAFLD. TNF-α is known to activate intracellular signaling molecules, including stress related kinases such as Jun N-terminal kinase and inhibitor kappa beta kinase beta, that make cells resistant to the actions of insulin (Diehl et al., 2005). Furthermore, TNF-α activates harmful proatherogenic pathways partially through the reduction of HDL-cholesterol, elevated expression of cholesterogenic genes, accompanied by an increase in potentially harmful precholesterol metabolites and suppression of cholesterol elimination (Fon Tacer et al., 2007). In addition, TNF-α stimulates hepatic fatty acid synthesis, increases serum triglyceride levels and stimulates VLDL production from liver (van Nieuwenhoven et al., 1996).

IL-6 is considered as a predictor marker of insulin resistance and cardiovascular diseases. It is a multifunctional cytokine that regulates immune responses, mediating the synthesis of several acute phase proteins (such as C-reactive protein) (Papanicolaou et al., 1998). Both TNF-α and IL-6 stimulate hepatic lipogenesis, impair insulin signaling and can alter insulin sensitivity by triggering different key steps in the insulin signaling pathway (Kim et al., 2004). Firstly, the presence of TNF-α favours IL-6 and related suppressor of cytokine signaling-3 (SOCS-3) production which blocks the activity of the insulin receptor and secondly, it inhibits the intracellular insulin signaling pathway by activation of serine phosphorylation of IRS. Finally, high TNF-α level suppress production and activity of adiponectin indicating that both high TNF-α (more insult) and low adiponectin (less protection) levels, however, they act together in the development of insulin resistance (Xirouchakis et al., 2009).

Table (5): Disturbance in cytokines profile as results of liver injury in non alcoholic fatty liver disease associated with cardiovascular diseases in rats.

		Standard normal diet	NAFLD + CVD		
			Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
TNF-α (pg/ml)	One month	5.166 ± 0.008	5.763 ± 0.012	7.224 ± 0.023	10.538 ± 0.034
	Two months	5.172 ± 0.009	6.329 ± 0.017	11.553 ± 0.038	18.474 ± 0.062
	Three months	5.174 ± 0.009	6.988 ± 0.025	16.721 ± 0.051	29.701 ± 0.083
IL-6 (pg/ml)	One month	10.497 ± 0.23	13.177 ± 0.34	15.336 ± 0.38	20.528 ± 0.62
	Two months	10.508 ± 0.25	17.004 ± 0.41	22.219 ± 0.71	27.215 ± 0.80
	Three months	10.419 ± 0.25	20.326 ± 0.52	27.583 ± 0.86	37.614 ± 1.05
IL-1β (pg/ml)	One month	3.874 ± 0.007	5.323 ± 0.012	7.527 ± 0.021	12.618 ± 0.044
	Two months	3.891 ± 0.008	6.479 ± 0.017	10.631 ± 0.037	20.039 ± 0.072
	Three months	3.882 ± 0.008	8.291 ± 0.026	15.191 ± 0.052	31.545 ± 0.081
CRP (ng/ml)	One month	7.289 ± 0.12	8.026 ± 0.16	9.351 ± 0.19	11.127 ± 0.24
	Two months	7.291 ± 0.12	9.658 ± 0.21	11.776 ± 0.27	20.514 ± 0.43
	Three months	7.302 ± 0.13	11.021 ± 0.29	15.479 ± 0.36	27.883 ± 0.51

- Means bearing different superscripts (A,B,C&D) within the same row are differ significantly ($P<0.05$).
- Means bearing different subscripts (a,b&c) within the same column are differ significantly ($P<0.05$).

Elevation in the level of CRP led to promote inflammation and accelerate atherosclerosis *via* increasing the expression of plasminogen activator inhibitor-1 and adhesion molecules in endothelial cells, inhibiting nitric oxide formation and increasing LDL uptake into macrophages (Chapman et al., 2011). All these metabolic abnormalities, common in subjects with NAFLD, have been shown to directly or indirectly promoted atherosclerosis. Also, NAFLD is associated with endothelial dysfunction and coronary artery disease (Targher et al., 2011).

Table (6) revealed that administration of fructose diets (Low, mid & high) to rats resulted in increment the levels of cardiac markers enzymes CK and LDH in serum which reflected the damage occurring in cardiac tissue. These data are consistent with the results of Bhatia et al. (2012). The later authors reported that there are several factors that can explain the elevation of cardiovascular disease risk in NAFLD subjects. Among these factors; the elevation of lipolysis and VLDL secretion (Yadav et al., 2009), the atherogenic lipoprotein profile *via* the increases of small dense LDL fractions and reduced in HDL fractions (Gastaldelli et al., 2007), the hyperglycemia due to hepatic overproduction of glucose and the increment of releasing inflammatory factors such as fibrinogen and C reactive protein (CRP) (Gastaldelli et al., 2007).

Table (6): Disturbance in heart profile as results of liver injury in non alcoholic fatty liver disease associated with cardiovascular diseases in rats.

		Standard normal diet	NAFLD + CVD		
			Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
CK (U/L)	One month	92.764 ± 1.15	134.323 ± 1.48	161.552 ± 1.83	200.189 ± 2.16
	Two months	93.364 ± 1.18	152.478 ± 1.69	211.675 ± 2.27	282.378 ± 3.02
	Three months	93.007 ± 1.17	191.126 ± 1.95	252.993 ± 2.54	337.451 ± 3.89
LDH (U/L)	One month	233.893 ± 2.93	307.652 ± 4.12	353.702 ± 4.53	391.375 ± 4.98
	Two months	236.649 ± 2.97	381.231 ± 4.87	406.253 ± 5.14	452.268 ± 5.51
	Three months	237.127 ± 3.02	420.592 ± 5.37	467.779 ± 5.76	517.113 ± 6.12
Myoglobin (ng/ml)	One month	33.874 ± 0.74	38.547 ± 0.83	40.771 ± 0.91	43.067 ± 0.98
	Two months	33.962 ± 0.77	41.532 ± 0.94	45.549 ± 1.03	49.239 ± 1.21
	Three months	34.018 ± 0.79	46.116 ± 1.07	49.887 ± 1.28	55.651 ± 1.43
H-FAPP (ng/ml)	One month	15.473 ± 0.29	18.643 ± 0.42	21.227 ± 0.49	24.315 ± 0.52
	Two months	15.486 ± 0.28	23.019 ± 0.49	25.583 ± 0.55	32.004 ± 0.71
	Three months	15.501 ± 0.31	27.665 ± 0.59	31.091 ± 0.64	41.852 ± 0.86
Hcy (µmol/L)	One month	10.769 ± 0.23	12.325 ± 0.31	13.899 ± 0.36	15.207 ± 0.48
	Two months	10.812 ± 0.26	14.453 ± 0.40	17.112 ± 0.55	19.548 ± 0.61
	Three months	10.785 ± 0.24	16.211 ± 0.52	20.631 ± 0.69	24.012 ± 0.73

- Means bearing different superscripts (A,B,C&D) within the same row are differ significantly ($P<0.05$).
- Means bearing different subscripts (a,b&c) within the same column are differ significantly ($P<0.05$).

Myoglobin and heart type-FABP (H-FABP) both are early and sensitive markers for myocardial infarction. They are rapidly released following myocardial injury and rapidly cleared from the circulation by the kidneys. Therefore, they are very useful as early markers in the time interval up to approximately 24-36 h after onset of symptoms. Although, myoglobin is currently recommended as preferred early cardiac marker, H-FABP would be a better choice (Alpert et al., 2000). H-FABP has a higher specificity and sensitivity than myoglobin for the detection of myocardial injury (Nakata et al., 2003). Due to the relatively low plasma concentration of H-FABP in healthy individuals and its high tissue content, H-FABP shows sensitivity that even allows the detection of minor myocardial injury in patients with chronic heart failure or unstable angina pectoris (Setsuta et al., 2002). In the current work, it is shown that after cardiac injury, the serum myoglobin and H-FABP levels increased above the references values and can therefore be determined as the earliest markers of cardiac injury.

Homocysteine, a homologue of cysteine with an additional methylene group, is an intermediate in methionine metabolism and is located at the intersection of two metabolic pathways; it is either remethylated back to

methionine or it is irreversibly degraded *via* the transsulfuration pathway to cysteine (Blom & Smulders, 2011). Regarding remethylation pathway, methionine is the precursor of S-adenosylmethionine (SAM), the key methyl donor for phosphotidylcholine synthesis. Low methyl group availability decreases the synthesis of phosphotidylcholine, a major phospholipid required for the assembly and export of very low density lipoprotein (VLDL) from the liver which may lead to hepatic lipid accumulation resulting in steatosis (Obeid & Herrmann, 2009). Regarding transsulfuration pathway, cystathionine beta synthase, the first enzyme involved in the transsulfuration pathway, catalyzes the condensation of HCY with serine to form cystathionine. Cystathionine is subsequently hydrolyzed to form cysteine, which in turn, can be incorporated into protein or used to synthesize the antioxidant GSH (Noll et al., 2011).

Viewing the aforementioned data, hyperhomocysteinemia observed in the fructose fed rat groups throughout whole experimental period (1, 2 & 3 months) may be attributed to the reduction in the specific activity of two key enzymes of Hcy metabolism, namely, methyltetrahydrofolate reductase and cystathionine β synthase (CβS). These results are in accordance with those of Polyzos et al. (2012), who found that plasma Hcy was significantly elevated in cirrhotic patients versus controls.

Hyperhomocysteinemia has been suggested to be associated with cardiovascular diseases as it is proved to cause cytotoxicity, lipid peroxidation, increase platelet aggregation, impairment activation of the coagulation system and stimulation of vascular smooth muscle cell proliferation (Herrmann et al., 2007). Moreover, NAFLD is associated with accelerated atherosclerosis (Targher & Arcaro, 2007) and oxidative stress which is now believed to be an important factor in the development/progression of the condition (Videla et al., 2006). Several studies indicated that the increase of oxidative stress is an important trigger for insulin resistance, which is believed to cause the disturbances in liver lipid metabolism that result in NAFLD. The resulting fat accumulation is then thought to promote further production of reactive oxygen species, thus oxidative stress can be considered to be both a cause and a consequence of hepatic steatosis (Videla et al., 2004). Also, Gulsen et al. (2005) noted that plasma Hcy concentrations were significantly higher in NAFLD patients as compared to healthy subjects and reported that the Hcy is a good predictor of progression to NASH.

Other key players in the pathogenesis of NAFLD which associated with cardiovascular disease are the so called adipokines (leptin, resistin and adiponectin) secreted by the adipocyte that infiltrate the adipose tissue in insulin-resistant states. Visceral adipose tissue represents a preferential source of adipokines and cytokines potentially acting on the liver tissue. The adipokines exert both metabolic and immune functions and mediates the metabolic cross-talk between adipose tissue, muscle and liver. In fact, levels of adiponectin are decreased in NAFLD patients and inversely related to hepatic insulin resistance and hepatic fat content (Bugianesi et al., 2005), degree of inflammation (Hui et al., 2004) and extent of fibrosis in the liver (Musso et al., 2005).

Pathogenesis of NAFLD involves hyperleptinemia, which is also related to atherosclerosis. Leptin is a hormone that regulates appetite and metabolism. Also, it has been found to have many biological effects on various systems and may be the signal that integrates vascular, metabolic and neuroendocrine responses (Leung & Kwan, 2008). Moreover, insulin has a direct influence on the synthesis and secretion of leptin. It is thought that leptin participating in glycol-metabolism and fat-metabolism in liver mainly depends on the regulation of gene expression of phosphoenolpyruvic acid and efficiency of glyconeogenesis which stimulates liver intake of lactic acid and genesis of hepatic glycogen (Kolaczynski et al., 1996).

As shown in table (7), hyperleptinemia was recorded in all rat groups of NAFLD associated with CVD fed on different percent of fructose (Low, mid & high) when compared to their corresponding control animals. These results are in agreement with that obtained by Angulo et al. (2004) and Basaranoglu et al. (2013). The authors reported that plasma leptin levels have been positively associated with cardiovascular complications in humans and this effect has been observed independent of BMI and traditional cardiac risk factors. According to Machado et al. (2012), leptin level increases progressively with increasing severity of hepatic steatosis *via* the stimulated release of cytokines such as tumor necrosis factor-α, interferon-γ, interferon-18 and tumor growth factor-β1.

Resistin is primarily secreted by monocytes or macrophages in humans (Curat et al., 2006). On one hand, obesity-associated inflammation stimulates the adipocytes themselves to produce inflammatory mediators, on the other hand, these inflammation mediators aggravate inflammation and thus, increases resistin secretion and *vice versa*.

This vicious circle causes and maintains macrophages infiltration into adipose tissue (Zhou et al., 2007). Data in table (7) showed that serum resistin level was increased in all NAFLD associated with CVD rat groups throughout the whole experimental period (1, 2 & 3 months). These results are in parallel with that obtained by Bokarewa et al. (2005). They reported that resistin regulates hepatic glucose and lipid metabolism and act as a mediator of hepatic insulin resistance in animal models. Furthermore, resistin has proinflammatory properties. It enhances interleukin 6 and TNF-α production and both of them are increased in NAFLD patients (Bokarewa et al., 2005).

Table (7): Disturbance in hormonal profile as results of liver injury in non alcoholic fatty liver disease associated with cardiovascular diseases in rats.

		Standard normal diet	NAFLD + CVD		
			Low fructose diet (20%)	Mid fructose diet (40%)	High fructose diet (60%)
Leptin (ng/ml)	One month	2.791 ± 0.006	3.126 ± 0.011	4.038 ± 0.022	4.509 ± 0.029
	Two months	2.803 ± 0.005	3.869 ± 0.017	4.968 ± 0.036	5.398 ± 0.043
	Three months	2.797 ± 0.006	4.457 ± 0.024	5.721 ± 0.051	6.046 ± 0.062
Resistin (ng/ml)	One month	3.419 ± 0.015	3.879 ± 0.022	4.402 ± 0.029	5.107 ± 0.048
	Two months	3.428 ± 0.017	4.223 ± 0.031	4.976 ± 0.045	6.014 ± 0.061
	Three months	3.433 ± 0.018	4.886 ± 0.039	5.516 ± 0.053	6.897 ± 0.069
Adiponectin (ng/ml)	One month	8.692 ± 0.043	8.101 ± 0.047	7.513 ± 0.053	6.917 ± 0.056
	Two months	8.683 ± 0.041	7.322 ± 0.052	6.847 ± 0.058	6.126 ± 0.053
	Three months	8.677 ± 0.041	6.654 ± 0.048	6.008 ± 0.055	5.523 ± 0.062
Ent-1 (pg/ml)	One month	0.392 ± 0.004	0.527 ± 0.011	0.682 ± 0.014	0.841 ± 0.017
	Two months	0.393 ± 0.004	0.886 ± 0.018	1.035 ± 0.022	1.378 ± 0.033
	Three months	0.395 ± 0.004	1.209 ± 0.027	1.387 ± 0.004	1.701 ± 0.038
Angiotensin II (pmol/L)	One month	1.752 ± 0.003	1.984 ± 0.008	2.478 ± 0.017	2.698 ± 0.019
	Two months	1.758 ± 0.004	2.367 ± 0.014	2.901 ± 0.022	3.117 ± 0.021
	Three months	1.756 ± 0.003	2.539 ± 0.019	3.328 ± 0.023	3.661 ± 0.026

- Means bearing different superscripts (A,B,C&D) within the same row are differ significantly ($P<0.05$).
- Means bearing different subscripts (a,b&c) within the same column are differ significantly ($P<0.05$).

Adiponectin is one of the important adipocytokines in the pathogenesis of NAFLD. Adiponectin has been shown to decrease *de novo* fatty acid synthesis and enhance fat oxidation (Musso et al., 2008). Decreased adiponectin is associated with insulin resistance and hyperlipidaemia and low level of adiponectin was shown in NAFLD independent of the components of the metabolic syndrome (Targher et al., 2004). Importantly low levels of adiponectin are also shown to be associated with CVD (Pischon et al., 2004). The low adiponectin levels in NAFLD accompanied with CVD rats groups which fed on fructose low, mid and high were recorded in this study. These data may represent a pathogenic mechanism leading to alteration in the hepatocyte lipid metabolism and fat accumulation (Table 7). These results are supported by report of Bajaj et al. (2004), who found an inverse correlation between adiponectin levels and liver fat content. In fact, high adiponectin levels have been reported to protect against both alcoholic and nonalcoholic fatty liver disease in mice by reducing fatty acid synthesis through inhibition of acyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) expression and activity (Xu et al., 2003). The reduction of ACC activity decreases the malonyl-CoA level, which is known to inhibit carnitine palmitoyltransferase I (CPT-I) activity and fatty acid oxidation. Therefore, the reduction of adiponectin in NAFLD could be due to increase in fatty acid synthesis, accumulation of triglycerides and decrease fatty acid oxidation (Lo´pez-Bermejo et al., 2004).

Also, adiponectin exerts potent anti-inflammatory effects, as documented in experimental studies where authors demonstrated that reduces TNF-α production in response to various stresses in plasma, adipose tissue, vascular wall, heart and liver (Ujiie et al., 2006). In addition, antagonizes several of the inflammatory effects of TNF-α can facilitate the removal of early apoptotic cells by macrophages and modulate the processes of inflammation and autoimmunity (Ouchi et al., 2003).

As regard to endothelial-1 and angiotensin-II, the significant ($p<0.05$) increase in the levels were detected herein through the whole experimental time. Jepsen et al. (2003) postulated that endothelial dysfunction and increased cardiovascular risk were expected in NAFLD, a condition strictly associated with metabolic syndrome. In addition, angiotensin-II, the main peptide of the renin–angiotensin system, is a true cytokine that regulates cell growth, inflammation and fibrosis (Ruiz-Ortega et al., 2001). It plays an important role in the pathophysiology of various cardiovascular disorders, such as hypertension and atherosclerosis. It has been reported that blockade of the renin–angiotensin system increases adiponectin concentrations in patients with essential hypertension (Furuhashi et al., 2003).

In conclusion, hyperhomocysteinemia and the elevation of L-FABP in non-alcoholic fatty liver disease could be used as independent predictor of cardiovascular disease severity in NAFLD rats. However, further studies are needed to determine more clearly and explanations.

References

Adams, L.A. and Angulo, P. (2005). Recent concepts in non-alcoholic fatty liver disease. Diabetic Med. 22: 1129-1133.

Alpert, J.S.; Thygesen, K.; Antman, E. and Bassand, J.P. (2000). Myocardial infarction redefined-A consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. J. Am. Coll. Cardiol. 36: 959-969.

Angulo, P.; Alba, L.; Petrovic, L.; Adams, L.; Lindor, K. and Jesen, M. (2004). Leptin, insulin resistance and liver fibrosis in human nonalcoholic fatty liver disease. J. Hepatol. 41(6): 943- 949.

Assman, G.; Jabs, H.; Kohnert, U.; Note, W. and Schriewer, H. (1984). Enzymatic determination of LDC-Cholesterol. Clin. Chem. Acta. 140: 77-83.

Bajaj, M., Suraamornkul, S., Piper, P., Hardies, L., Glass, L., Cersosimo, E., Pratipanawatr, T., Miyazaki, Y. and DeFronzo, R. (2004). Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients. J. Clin. Endocrinol. Metab. 89: 200 - 206.

Basaranoglu, M.; Basaranoglu,G.; Sabuncu, T. and Sentürk, H. (2013). Fructose as a key player in the development of fatty liver Disease. World J. Gastroenterol. 19(8): 1166-1172.

Bennett, M.; Lopez, J.; Sanchez, H. and Osborne, T. (1995). Sterol regulation of fatty acid synthase promoter coordinate feedback regulation of two major lipid pathways. J. Biol. Chem. 270: 25578-25583.

Bhatia, L.S.; Curzen, N.P.; Calder, P.C. and Byrne, C.D. (2012). Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? Eur. Heart J. 33: 1190–1200.

Blom, H.J. and Smulders, Y. (2011). Overview of homocysteine and folate metabolism with special references to cardiovascular disease and neural tube defects. J. Inherit. Metab. Dis. 34: 75-81.

Bokarewa, M.; Nagaev, I.; Dahlberg, L.; Smith, U. and Tarkowski, A. (2005). Resistin, an adipokine with potent proinflammatory properties. *J. Immunol. 174:* 5789-5795.

Brown, M. and Goldstein, J. (1997). The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89: 331–340.

Bugianesi, E.; Pagotto, U. and Manini, R. (2005). Plasma adiponectin in nonalcoholic fatty liver disease is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. J. Clin. Endocrinol. Metab. 90: 3498–504.

Catena, C.; Cavarape, A.; Novello, M.; Giacchetti, G. and Sechi, L. (2003). Insulin receptors and renal sodium handling in hypertensive fructose-fed rats. Kidney Int. 64(6): 2163-71.

Chapman, M.; Ginsberg, H.; Amarenco, P.; Andreotti, F.; Boren, J.; Catapano, A.; Descamps, O.; Fisher, E.; Kovanen, P. and Kuivenhoven, J. (2011). Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. Eur. Heart J. 32: 1345–1361.

Chiu, S.; Sievenpiper, J.; de Souza, R.; Cozma, A.; Mirrahimi, A.; Carleton, A.; Ha, V.; Di Buono, M.; Jenkins, A.; Leiter, L.; Wolever, T.; Don-Wauchope, A.; Beyene, J.; Kendall, C. and Jenkins, D. (2014). Effect of fructose on markers of non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of controlled feeding trials. Eur. J. Clin. Nutr. 68: 416-423.

Cirillo, P.; Sautin, Y.; Kanellis, J.; Kang, D.; Gesualdo, L. and Nakagawa, T. (2009). Systemic inflammation, metabolic syndrome and progressive renal disease. Nephrol. Dial. Transplant. 24: 1384-1387.

Curat, C.; Wegner, V.; Sengenès, C.; Miranville, A.; Tonus, C.; Busse, R. and Bouloumie, A. (2006). Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia.* 49: 744-747.

de Carvalho, S.; Muniz, M.; Siqueira, M.; Siqueira, E.; Gomes, A.; Silva, K.; Bezerra, L.; D'Almeida, V.; de Oliveira, C. and Pereira, L. (2013). Plasmatic higher levels of homocysteine in Non-alcoholic fatty liver disease (NAFLD). Nutr. J. 12: 37.

de Castro, U.; dos Santos, R.; Silva, M.; de Lima, W.; Campagnole-Santos, M. and Alzamora, A. (2013). Age-dependant effect of high-fructose and high-fat diets on lipid metabolism and lipid accumulation in liver and kidney of rats. Lipid in Health and Dis. 12: 136-146.

Diehl, A.; Li, Z.; Lin, H. and Yang, S. (2005). Cytokines and the pathogenesis of non-alcoholic steatohepatitis. Gut. 54(2): 303–306.

D'souza, A.; Beaudry, J.; Szigiato, A.; Trumble, S.; Snook, L.; Bonen, A.; Giacca, A. and Riddell, M. (2012). Consumption of a high-fat diet rapidly exacerbates the development of fatty liver disease that occurs with chronically elevated glucocorticoids. Am. J. Physiol. Gastrointest. Liver Physiol. 15: 302(8): G850-63.

Du Vigneaud, V. and Karr, W. (1925). Carbohydrate utilisation. Rate of disappearance of D-glucose from the blood J. Biol. Chem. 66: 281-300.

Duncan, D.B. (1955). Multiple range and multiple F-tests. Biometr., 11: 1-42.

Eckel, R.; Grundy, S. and Zimmet, P. (2005). The metabolic syndrome. Lancet 365: 1415-1428.

Fon Tacer, K.; Kuzman, D.; Seliskar, M.; Pompon, D. and Rozman, D. (2007). TNF-alpha interferes with lipid homeostasis and activates acute and proatherogenic processes. Physiol Genomics. 31(2): 216–227.

Friedewald, W.; Lewy, R. and Fredrickson, D. (1972). Estimation of low-density lipoprotein cholesterol in plasma, without use of the preparative centrifuge. Clin. Chem. 18: 499-504.

Furuhashi, M.; Ura, N.; Higashiura, K.; Murakami, H.; Tanaka, M.; Moniwa N, Yoshida, D. and Shimamoto, K. (2003). Blockade of the renin-angiotensin system increases adiponectin concentrations in patients with essential hypertension. Hypertension 42: 76–81.

Gaemers, I.; Stallen, J.; Kunne, C.; Wallner, C.; van Werven, J.; Nederveen, A. and Lamers, W. (2011). Lipotoxicity and steatohepatitis in an overfed mouse model for non-alcoholic fatty liver disease. Biochim. Biophys. Acta. 1812: 447-458.

Gastaldelli, A.; Cusi, K.; Pettiti, M.; Hardies, J.; Miyazaki, Y.; Berria, R.; Buzzigoli, E.; Sironi, A.; Cersosimo, E.; Ferrannini, E. and Defronzo, R. (2007). Relationship between hepatic/visceral fat and hepatic insulin resistance in non-diabetic and type 2 diabetic subjects. Gastroenterology 133: 496-506.

Girard, A.; Madani, S.; Boukortt, F.; Cherkaoui-Malki, M.; Belleville, J. and Prost, J. (2006). Fructose-enriched diet modifies antioxidant status and lipid metabolism in spontaneously hypertensive rats**.** Nutrition, 22:758-766.

Glushakova, O.; Kosugi, T.; Roncal, C.; Mu, W.; Heinig, M.; Cirillo, P.; Sánchez- Lozada, L.; Johnson, R. and Nakagawa, T. (2008). Fructose induces the inflammatory molecule ICAM-1 in endothelial cells. J. Am. Soc. Nephrol. 19: 1712-1720.

Gulsen, M.; Yesilova, Z.; Bagci, S.; Uygun, A.; Ozcan, A.; Ercin, C.; Erdil, A.; Sanisoglu, S.; Cakir, E.; Ates, Y.; Erbil, M.; Karaeren, N. and Dagalp, K. (2005). Elevated plasma homocysteine concentrations as a predictor of steatohepatitis in patients with non-alcoholic fatty liver disease. J. Gastroenterol. Hepatol. 20:1448-55.

Haring, R.; Wallaschofski, H.; Nauck, M.; Dörr, M.; Baumeister, S. and Völzke, H. (2009). Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. Hepatol. 59:1403-1411.

Herrmann, W.; Herrmann, M. and Obeid, R. (2007). Hyperhomocysteinaemia: a critical review of old and new aspects. Curr. Drug Metab. 8: 17-31.

Huang, H.; Starodub, O.; McIntosh, A.; Kier, A. and Schroeder, F. (2002). Liver Fatty Acid-binding Protein Targets Fatty Acids to the Nucleus. Real time confocal and multiphoton fluorescence imaging in living cells. J. Biol. Chem. 277: 29139-29151.

Hui, J.; Hodge, A.; Farrell, G.; Kench, J.; Kriketos, A. and George, J. (2004). Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatol. 40: 46-54.

Jepsen, P.; Vilstrup, H.; Mellemkjaer, L.; Thulstrup, A.; Olsen, J.; Baron, J. and Sorensen, H. (2003). Prognosis of patients with a diagnosis of fatty liver: a registry-based cohort study. Hepatogastroenterology 50: 2101-2104.

Kannappan, S. and Anuradha, C. (2009). Insulin sensitizing actions of fenugreek seed polyphenols, quercetin & metformin in a rat model and metformin in rat model**.** Indian J. Med. Res. 129:401-408.

Kim, H.; Higashimori, T.; Park, S.; Choi, H.; Dong, J. and Kim, Y. (2004). Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action *in vivo*. Diabetes 53(4): 1060-1067.

Kim, S.; Choi, Y.; Huh, B.; Park, S.; Lee, E.; Cho, Y. and Huh, K. (2014). Nonalcoholic fatty liver disease is associated with increased carotid intima-media thickness only in type 2 diabetic subjects with insulin resistance. J. Clin. Endocrinol. Metab. 99: 1879-1884.

Kolaczynski, J.; Nyce, R.; Considine, R.; Boden, G.; Nolan, J.; Henry, R.; Mudaliar, S.; Olefsky, J. and Caro, J. (1996). Acute and chronic effects of insulin on leptin production in humans: Studies *in vivo* and *in vitro*. Diabetes 45: 699- 701.

Leung, Y. and Kwan, C. (2008). Dual vascular effects of leptin via endothelium: hypothesis and perspective. Chin. J. Physiol. 51: 1–6.

Lo´pez-Bermejo, A.; Botas, P.; Funahashi, T.; Delgado, E.; Kihara, S.; Ricart, W. and Ferna´ndez-Real, J. (2004). Adiponectin, hepatocellular dysfunction and insulin sensitivity. Clin. Endocrinol. 60: 256-263.

Machado, M.; Coutinho, J.; Carepa, F.; Costa, A.; Proenca, H. and Cortez-Pinto, H. (2012). How adiponectin, leptin and ghrelin orchestrate together and correlate with the severity of nonalcoholic fatty liver disease. Eur. J. Gastroenterol. Hepatol. 24: 1166-1172.

Matthews, D.; Hosker, J. and Rudenski, A. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 412: 9-28.
Mayes, P. (1993). Intermediary metabolism of fructose**.** Am. J. Clin. Nutr. 58(Suppl):754-765.

Meisinger, C.; Döring, A.; Schneider, A. and Löwel, H. (2006). Serum gamma-glutamyltransferase is a predictor of incident coronary events in apparently healthy men from the general population. Atherosclerosis 189:297-302.

Mlinar, B,; Marc, J.; Janež, A. and Pfeifer, M. (2007). Molecular mechanisms of insulin resistance and associated diseases. Clin. Chim. Acta. 375:20-35.

Musso, G.; Gambino, R.; Biroli, G.; Carello, M.; Faqa, E.; Pacini, G.; De Michieli, F.; Cassader, M.; Durazzo, M.; Rizzetto, M. and Pagano, G. (2005). Hypoadiponectinemia predicts the severity of hepatic fibrosis and pancreatic beta-cell dysfunction in nondiabetic nonobese patients with nonalcoholic steatohepatitis. Am. J. Gastroenterol. 100: 2438-46.

Musso, G.; Gambino, R.; De Michieli, F.; Durazzo, M.; Pagano, G. and Cassader, M. (2008). Adiponectin gene polymorphisms modulate acute adiponectin response to dietary fat: possible pathogenetic role in NASH. Hepatology, 47(4): 1167–1177.

Nakata, T.; Hashimoto, A.; Hase, M.; Tsuchihaski, K. and Shimamoto, K. (2003). Human heart-type fatty acid binding protein as an early diagnostic and prognostic marker in acute coronary syndromes. Cardiology 99: 96-104.

Noll, C.; Lacraz, G.; Ehses, J.; Coulaud, J. Bailbe, D.; Paul, G. and Portha, B. (2011). Early reduction of circulating homocysteine levels I Goto-Kakizaki rat, a spontaneous nonobese model of type 2 diabetes. Biochim. Biophys Acta. 1812: 699-702.

NRC National Research Counal (1977). Nutrient requirements of domestic animals, nutrient requirements of rat. National Academy of Science, Washington, DC, U.S.A.

Obeid, R. and Herrmann, W. (2009). Homocysteine and lipids: S-adenosylmethionine as a key intermediate. FEBS Lett. 583: 1215-25.

Ouchi, N.; Kihara, S. and Funahashi, T. (2003). Obesity, adiponectin and vascular inflammatory disease. Curr. Opin. Lipidol. 14: 561-566.

Ouyang, X.; Cirillo, P.; Sautin, Y.; McCall, S.; Bruchette, J. and Diehl, A. (2008). Fructose consumption as a risk factor for non-alcoholic fatty liver disease. J. Hepatol. 48: 993-999.

Pandya, N.; Santani, D. and Jain, S. (2006). Antioxidant activity of ezetimibe in hypercholesterolemic rats. Indian J. Pharmacol. 38: 205.

Papanicolaou, D.; Wilder, R.; Manolagas, S. and Chrousos, G. (1998). The pathophysiologic roles of interleukin-6 in human disease. Ann. Intern. Med. 128: 127–37.

Pischon, T.; Girman, C.; Hotamisligil, G.; Rifai, N.; Hu, F. and Rimm, E. (2004). Plasma adiponectin levels and risk of myocardial infarction in men. J. Am. Med. Assoc. 291(14): 1730-1737.

Poirier, H.; Niot, I.; Monnot, M.; Braissant, O.; Meunier-Durmort, C.; Pineau, T.; Wahli, W.; Willson, T. and Besnard, P. (2001). Differential involvement of peroxisome proliferator-activated receptors in fibrate and fatty acid mediated inductions of the gene encoding liver fatty acid binding protein in the liver and the small intestine. Biochem. J. 355: 481- 488.

Polyzos, S.; Kountouras, J. and Zavos, C. (2009). Nonalcoholic fatty liver disease: the pathogenic roles of insulin resistance and adipocytokines. Curr. Mol. Med. 72: 299-314.

Polyzos, S.; Kountouras, J.; Patsiaoura, K.; Katsiki, E.; Zafeiriadou, E.; Deretzi, G.; Zavos, C.; Gavalas, E.; Katsinelos, P.; Mane, V. and Slavakis, A. (2012). Serum homocysteine levels in patients with nonalcoholic fatty liver disease. Ann. Hepatol. 11(1): 68-76.

Pooranaperundevi, M.; Sumiyabanu, M.; Viswanathan, P.; Sundarapandiyan, R. and Anuradha, C. (2010). Insulin resistance induced by a high-fructose diet potentiates thioacetamide hepatotoxicity. Singapore Med. J. 51(5): 389-98.

Promrat, K.; Longato, L.; Wands, J. and de la Monte, S. (2011). Weight loss amelioration of non-alcoholic steatohepatitis linked to shifts in hepatic ceramide expression and serum ceramide levels. Hepatol Res 41:754–762.

Roglans, N.; Sanguino, E.; Peris, C.; Alegret, M.; Nazquez, M.; Adzet, T.; Diaz, C.; Hernandez, G.; Laguna, J. and Sanchez, R. (2002). Atorvastatin treatment induced peroxisome proliferator activated receptor alpha expression and decreased plasma non esterified fatty acids and liver triglyceride in fructose fed rats. J. Pharmacol. Exp. Ther. 302: 232-39.

Ruiz-Ortega, M.; Lorenzo, O.; Suzuki, Y.; Ruperez, M. and Egido, J. (2001). Proinflammatory actions of angiotensins. Curr. Opin. Nephrol. Hypertens. 10: 321-329.

Semenkovich, C.F. (2013). Insulin resistance and artherosclerosis. J. clin. Invest. 116(7): 1813- 1822.

Setsuta, K.; Seino, Y.; Ogawa, T.; Arao, M. and Miyatake, Y. (2002). Use of cytosolic and myofibril markers in the detection of ongoing myocardial damage in patients with chronic heart failure. Am. J. Med. 113: 717-22.

Shapiro, A.; Mu, W.; Roncal, C.; Cheng, K.; Johnson, R. and Scarpace. P. (2008). Fructose induced leptin resistance exacerbates weight gain in response to subsequent high-fat feeding. Am. J. Physiol. Regul. Integr. Comp. Physiol. 295: R1370-1375.

Shoelson, S.; Lee, J. and Goldfine, A. (2006). Inflammation and insulin resistance. J. Clin. Invest. 116: 1793-801.

Smulders, Y. and Blom, H. (2011): The homocysteine controversy. J. Inherit. Met. Dis. 34: 93-99.

Snedecor, G.W. and Cochran; W.G. (1982). Statistical Methods. 7th ed., Twa State University Press, Ames, Lowa, USA.

Stanhope, K. and Havel, P. (2008). Fructose consumption: potential mechanisms for its effects to increase visceral adiposity and induce dyslipidemia and insulin resistance**.** Curr. Opin. Lipidol. 19:16-24.

Stanhope, K. and Havel, P. (2009). Fructose consumption: considerations for future research on its effects on adipose distribution, lipid metabolism, and insulin sensitivity in humans. J. Nutr. 139: 1236S-1241S.

Stead, L.; Brosnan, M. and Brosnan, J. (2000). Characterization of homocysteine metabolism in the rat liver. Biochem. J. 350: 685-92.

Targher, G. and Arcaro, G. (2007). Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. Atherosclerosis. 191: 235-240.

Targher, G.; Bertolini, L.; Scala, L.; Poli, F.; Zenari, L. and Falezza, G. (2004). Decreased plasma adiponectin concentrations are closely associated with nonalcoholic hepatic steatosis in obese individuals. Clin. Endocrin. 61(6): 700-703.

Targher, G.; Zoppini, G. and Day, C. (2011). Risk of all-cause and cardiovascular mortality in patients with chronic liver disease. Gut 60: 1602–1604.

Ujiie, H.; Oritani, K.; Kato, H.; Yokota, T.; Takahashi, I.; Maeda, T.; Masair, H.; Ichii, M.; Kamada, Y.; Tamura, S.; Kihara, S.; Funahashi, T.; Tomiyama, Y. and Kanakura, Y. (2006). Identification of amino-terminal region of adiponectin as a physiologically functional domain. J. Cell Biochem. 98(1): 194-207.

Van Nieuwenhoven, F.; Musters, R.; Post, J.; Verkleij, G.; van der Vusse, G. and Glatz, J. (1996). Release of proteins from isolated neonatal rat cardiomyocytes subjected to simulated ischemia or metabolic inhibition is independent of molecular mass. J. Mol. Cell Cardiol. 28(7): 1429- 1434.

Vaya, A.; Carmona, P.; Badia, N.; Perez, R.; Mijares, A. and Corella, D. (2011). Homocysteine levels and the metabolic syndrome in a mededitерrean population: a case control study. Clin. Hemorheol Microcirc. 47: 59-66.

Veerkamp, J. and Maatman, R. (1995). Cytoplasmic fatty acid-binding proteins: Their structure and genes. Progress lipid Res. 34(1): 17-52.

Videla, L.; Rodrigo, R.; Araya, J. and Ponichik, J. (2006). Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease. Trends Mol. Med. 12: 555-558.

Videla, L.; Rodrigo, R.; Orellana, M.; Fernandez, V.; Tapia, G.; Quiñones, L.; Varela, N.; Contreras, J.; Lazarte, R.; Csendes, A.; Rojas, J.; Maluenda, F.; Burdiles, P.; Diaz, J.; Smok, G.; Thielemann, L. and Poniachik, J. (2004). Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. Clin. Sci. 106:261-268.

Xirouchakis, E.; Manousou, P.; Tsartsali, L.; Georgopoulos, S. and Burroughs, A. (2009). Insights into the pathogenesis of NAFLD: The role of metabolic and pro-inflammatory mediators. Ann. Gastroenterol. 22(1): 24-33.

Xu, A.; Wang, Y.; Keshaw, H.; Xu, L.; Lam, K. and Cooper, G. (2003). The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. J. Clin. Invest. 112: 91–100.

Yadav, H.; Jain, S.; Yadav, M.; Sinha, P.; Prasad, G. and Marotta, F. (2009). Epigenomic derangement of hepatic glucose metabolism by feeding of high fructose diet and its prevention by Rosiglitazone in rats. Digest Liver Dis. 41: 500-508.

Zhou, L.; Sell, H.; Eckardt, K.; Yang, Z. and Eckel, J. (2007). Conditioned medium obtained from in vitro differentiated adipocytes and resistin induce insulin resistance in human hepatocytes. *FEBS Lett*. 581: 4303-4308.