AMERICAN UNIVERSITY OF BEIRUT

# THE ROLE OF PHOSPHORUS IN THE DEVELOPMENT AND PROGRESSION OF HIGH FAT DIET INDUCED NAFLD IN RATS.

by:
MARIAM MOHAMMAD ALI ELHALABI

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submitted in partial fulfillment of the requirements
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# AN ABSTRACT OF THE THESIS OF

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The term non-alcoholic fatty liver disease (NAFLD) refers to the range of diseases characterized by fatty infiltration of the liver ranging from benign steatosis, to the more severe steatohepatitis, or cirrhosis. Interest is raised on the relationship between dietary phosphorus and NAFLD since a recent study showed that dietary Pi restriction plays an important role in the development of fatty liver disease and hyperlipidemia induced by a high-cholesterol diet through regulation of lipid metabolism–related gene expression in the liver. Likewise, hypophosphatemia has been reported in studies of large numbers of individuals with metabolic syndrome. Therefore, it was hypothesized that phosphorus seems to embody the missing link between the metabolic derangements that occur in metabolic syndrome and NAFLD. The aim of this study is to investigate the role of dietary phosphorus on the development and progression of NAFLD in rats maintained on a high fat diet.

Forty-four six week-old male Sprague-Dawley rats were used for the experiment. Rats were randomly allocated into 5 groups and had free access to water and fed ad libitum either a control diet (20% protein, 10% fat by weight) or a high fat diet (20% protein, 40% fat by weight) with different concentrations of phosphorus (0.0178, 0.075, 0.15, and 0.3%) for 8 weeks. Blood was collected for plasma analysis and livers were excised for histology.

Results showed that upon feeding male Sprague-Dawley rats phosphate restricted diets (0.0178% and 0.075% P); rats had significant weight loss, low intake, and low energy efficiency than rats fed a P sufficient diet. In addition, male Sprague-Dawley rats upon administration of high fat diet tend to induce microvesicular steatosis, and mild macrovesicular steatosis and lobular inflammation. However, effect of P manipulation could not be detected.

Although rats developed microvesicular steatosis, mild macrovesicular steatosis, and lobular inflammation; however, effect of P manipulation could not be detected.

Keywords: NAFLD, Phosphorus, Sprague-Dawley rats, High fat diet.

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# ABBREVIATIONS

| | |
|---|---|
| 1,25 (OH)2 vitamin D3 | 1,25- dihydroxy vitamin D3 |
| 2,3-DPG | 2,3-diphosphoglycerate |
| ALT | Alanine aminotransferase |
| AMPK | Adenosine monophosphate kinase |
| AMP | Adenosine monophosphate |
| Apo | Apolipoprotein |
| AST | Aspartate aminotransferase |
| ATP | Adenosine triphosphate |
| β | beta |
| BIS | BioImpedance Spectroscopy |
| BMI | Body mass index |
| cAMP | Cyclic adenosine monophosphate |
| CM | Chylomicron |
| DNL | De novo lipogenesis |
| EDF | Epididymal Fat |
| EDTA | Ethylene diamine tetra-acetic acid |
| ER | Endoplasmic reticulum |
| et al. | and others |
| FGF23 | Fibroblast growth factor 23 |
| FFAs | Free fatty acids |
| FFM | Fat free mass |
| FM | Fat mass |
| GLUT4 | Glucose transporters 4 |
| H&E | Hematoxylin and eosin |

| | |
|---|---|
| HDL | High density lipoprotein |
| HF | High fat |
| HFCS | High fructose corn syrup |
| HPO4 -2 | Monohydrogen phosphate |
| H2PO4 -1 | Dihydrogen phosphate |
| IR | Insulin resistance |
| IRS | Insulin receptor substrate |
| Kcal | kilo calories |
| LDL | Low density lipoprotein |
| MetS | Metabolic syndrome |
| mg | Milligram |
| mg/dl | Milligram per deciliter |
| mM | Milli molar |
| NAFLD | Non-alcoholic fatty liver disease |
| NASH | Non-alcoholic steatohepatitis |
| NAS | Non-alcoholic fatty liver disease Activity Score |
| NaPi2b | Sodium phosphate cotransporter 2b |
| NEFA | Non-esterified free fatty acid |
| NIDDK | National Institute of Diabetes and Digestive and Kidney Diseases |
| NPT2a and NPT2c | Sodium-dependent phosphate transporters |
| OGTT | Oral glucose tolerance test |
| OTC | Over the counter |
| $P_i$ | Inorganic phosphate |
| P | Phosphate |
| PI-3-k | Phospho-inositide-3-kinase |
| PKB | Protein kinase B |

| | |
|---|---|
| PTH | Parathyroid hormone |
| RDA | Recommended daily allowance |
| ROS | Reactive oxygen species |
| SFA | Saturated fatty acid |
| SREBP-1c | Sterol regulatory element binding protein 1c |
| TG | Triglyceride |
| TNF-α | Tumor necrosis factor α |
| UL | Tolerable Upper level |
| VLDL | Very low density lipoprotein |

# CHAPTER I

# INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an increasingly common disease state and a major public health concern owing to its high prevalence worldwide and potentially serious sequelae. The term non-alcoholic fatty liver disease (NAFLD) refers to the range of diseases characterized by fatty infiltration of the liver ranging from benign steatosis, to the more severe steatohepatitis (NASH), or cirrhosis; in the absence of significant ethanol consumption or viral infection (Guturu et al, 2012). Apart from weight loss, there are currently no effective therapies for NAFLD (Kawano and Cohen, 2013). Therefore, research efforts are focusing on understanding the underlying pathophysiology of hepatic steatosis with the expectation to identify novel therapeutic targets.

NAFLD is the hepatic manifestation of the metabolic syndrome (Adams et al., 2009). Concern is raised on the relationship between dietary phosphorus and NAFLD since it is required in ATP production. Moreover, a recent study showed that dietary Pi restriction plays an important role in the development of fatty liver disease induced by a high-cholesterol diet through the regulation of lipid metabolism-related gene expression in the liver (Tanaka et al., 2013). Likewise, hypophosphatemia has been reported in studies of large numbers of individuals with metabolic syndrome (Haglin et al, 2001; kalaitzidis et al, 2005). Therefore, it was hypothesized that phosphorus seems to

embody the missing link between the metabolic derangements that occur in the metabolic syndrome and NAFLD.

In the present study male Sprague-Dawley rats will be given a high fat diet to induce NAFLD. Lard which is rich in saturated fatty acid has been proven to promote severe endoplasmic reticulum stress, inflammation, hepatic steatosis and insulin resistance (Zhao et al., 2013). Therefore, lard was chosen to be used as the source of fat in the high fat groups. At the same time rats will be given varying concentrations of phosphorus supplementation to investigate the role of dietary phosphorus in the development and progression of NAFLD in rats.

# CHAPTER II

# LITERATURE REVIEW

## A. Phosphorus

### *1. Phosphorus Overview:*

Phosphorus is an essential macromineral, ranks second to calcium in abundance in human tissues. Approximately 700g of phosphorus exist in adult tissues, of which about 85% is present in the skeleton and teeth as calcium phosphate crystals, and the remainder (15%) exists in the metabolically active pool in every cell in the body and in the extracellular fluid compartment (Mahan and Escott-Stump, 2004).

The intracellular:extracellular ratio is estimated to be 100:1. Most intracellular phosphate exists as organic phosphate compounds such as creatine phosphate and adenosine triphosphates (ATP), the body's main reservoir of biochemical energy for a wide variety of physiological processes (Gaasbeek and Meinders, 2005). Of the 1% that is present in the extracellular fluid, 10–20% is protein bound and 5% is complexed with cations such as calcium, magnesium, and sodium. The remaining 85% exists as HPO4 -2 and H2PO4 -1. It is the extracellular (1% of total body phosphate) phosphate that is measured by routine laboratory analysis and thus it does not always reflect the total body phosphate stores (Gattineni and Baum, 2012). In human plasma or serum, phosphorus exists in the form of inorganic phosphorus or phosphate ($P_i$), lipid phosphorus, and phosphoric ester phosphorus (Berndt and Kumar, 2008).

## *2. Food Sources and bioavailability*

Dietary phosphorus comes from three different sources which are thought to have different bioavailability and physiologic effects: 1) organic phosphorus: naturally occurring as components of cell membranes, tissue structures, and phospho-proteins; 2) inorganic phosphorus: in active and inactive ingredients in OTC medications, dietary supplements and food enrichment/fortification and water, and 3) largely inorganic phosphorus as added food ingredient for specific approved functions needed in food processing and as phosphorus salts (Calvo and Uribarri, 2013).

In general, good sources of protein are also good sources of phosphorus. Meat, poultry, fish, and eggs are excellent sources. Milk and milk products are good sources, as are nuts and legumes, cereals and grains (Mahan and Escott-Stump, 2004). Phosphorus content of major raw (non-refined) foods like cereals, pulses, and meat is around 1 mg P/kcal (Obeid, 2013).

Grain products and legumes are high protein sources of phosphorus; however its bioavailability differs from that of meat and milk food categories since phosphorus is present as phytate in the outer coating of cereal grains and seed coats of legumes and is less bioavailable unless it is degraded by phytases in the intestine or during processing (Schlemmer et al, 2009). The method of food preparation have an effect on the total phosphorus content and bioavailability from multigrain products and legumes. For example, boiling can remove some phytate (Cupisti, 2006), as well as the soaking or rehydration of dried beans and other legumes (Vohra and Satyanarayana, 2003), and

the leavening of bread with yeasts (Fretzdorff and Brummer, 1992) that produce phytase which increases bioavailability of phosphorus (Schlemmer et al, 2009).

### *3. Recommendations and Current Intakes*

The present recommended daily allowance (RDA) for phosphorus is 700 mg and the tolerable upper intake levels (UL) is 4 g/d for adult males and females, and for the elderly more than 70 years of age is 3 g/d (Food and Nutrition Board, 1997). The average daily adult phosphate intake is about 1–1.5 g (Gattineni and Baum, 2012) which is higher than the RDA but lower than the UL. Approximately 20–30% of the dietary phosphorus for most age and sex groups come from milk and milk products. In addition, meat, poultry and fish are an equally important source of phosphorus, accounting for another 20-30% of the daily intake all of which are high protein foods (Calvo and Park, 1996).

Obeid (2013) raised a concern regarding the remarkable increase in the contribution of low phosphorus commodities to total energy intake especially refined cereals, oils, sugars, and sweeteners such as high fructose corn syrup (HFCS) which contain negligible amounts of phosphorus. These commodities provide more than 50% of the food supply (kcal/capita/day) in most countries (Obeid, 2013).

### *4. Phosphorus Functions:*

Phosphorus plays a critical role in cellular biology since many cellular processes require phosphorus in one form or another and include:

- Nucleic acid synthesis and metabolism
- Energy metabolism (ATP production)
- Cellular signaling by phosphorylation of proteins
- Membrane integrity

- Muscle function
- Enzyme activity
- Lipid metabolism
- Bone mineralization (Berndt and Kumar, 2008)
- Release of oxygen from hemoglobin as phosphate is a component of 2,3 diphosphoglycerate.
- Important urinary and blood acid base buffer (Gattineni and Baum, 2012).

**5. *Phosphorus Regulation in The Body:***

Total serum phosphorus concentrations range between 89 and 149 mg/l (2.87–4.81 mM), and inorganic phosphorus (Pi) concentrations between 25.6 and 41.6 mg/l (0.83–1.34 mM) (Gaasbeek and Meinders, 2005). Phosphorus balance involves the absorption of dietary phosphorus in the intestine, distribution in body fluids and tissues especially bones, and excretion mainly by the kidneys (Tani et al., 2007). Figure 1 illustrates the control of phosphorus balance.

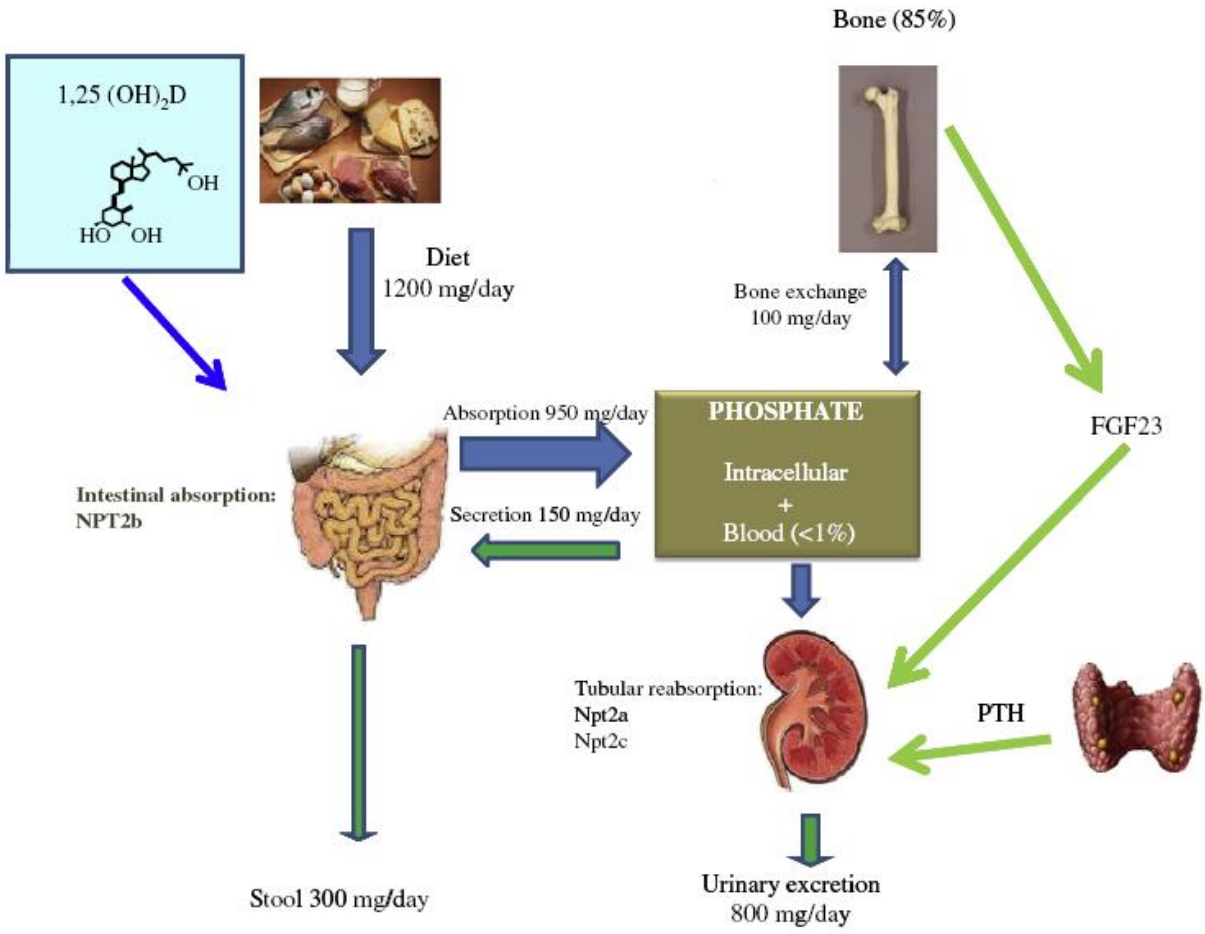



Figure 2.1: Phosphate Homeostasis (Source: Gonzalez-Parra, 2012)

a. Intestinal Absorption

The intestine absorbs about 70% of dietary phosphorus only in the inorganic form. Organic phosphate must be hydrolyzed enzymatically in the gut lumen and released as inorganic phosphate before being absorbed (Gonzalez-Parra, 2012). Phosphate is absorbed from the gastrointestinal tract via 2 pathways: (1) active transcellular route mediated by sodium phosphate cotransporter 2b (NaPi2b) and (2) a

paracellular pathway which is a diffusion-driven non-saturable process in which phosphate transport increases with increasing phosphate in the diet (Gattineni and Baum, 2012).

b. Renal Handling

Once phosphate enters the systemic circulation, free and complexed phosphate is freely filtered at the glomerulus. Under basal conditions, 80–90% of the filtered load of phosphate is reabsorbed by the kidney. Phosphate is primarily reabsorbed by the proximal tubule via brush border membrane sodium-dependent phosphate transporters, NaPi2a and NaPi2c (Virkki et al., 2007). The amount of phosphate reabsorbed by the proximal tubule is hormonally regulated and determines, in subjects with normal renal function or moderately reduced glomerular filtration rate, serum phosphate levels (Prie´ et al, 2009). Dietary phosphate intake, parathyroid hormone (PTH), 1,25 (OH)2 vitamin D3, and fibroblast growth factor 23 (FGF23) are the principal regulators of phosphate reabsorption from the kidney (Gattineni and Baum, 2012).

*i. 1,25- dihydroxyvitamin D3*

Calcitriol, the active form of vitamin D, increases renal NPT2a expression and phosphate reabsorption, as well as intestinal NPT2b expression and phosphate absorption. However, its positive effect on intestinal and renal phosphate absorption is counterbalanced by the stimulation of fibroblast growth factor 23 (FGF23) productions. Calcitriol also modulates bone resorption and bone formation. Vitamin D suppresses synthesis of PTH at the parathyroid gland level via 2 mechanisms: (1) directly repressing its gene and (2) indirectly, by increasing calcium-sensing receptor (CaR) expression and the sensitivity of parathyroid cells to extracellular calcium. Hence,

vitamin D can increase or decrease serum phosphate concentration depending on its balanced effect on parathyroid glands, bone, and intestine (Torres and De Brauwere, 2011).

### *ii. Parathyroid Hormone (PTH)*

PTH plays a role in phosphate homeostasis via two opposite effects: (1) it reduces serum phosphate by decreasing its renal reabsorption, and (2) increases it by directly stimulating bone turnover and phosphate release or indirectly by stimulating intestinal phosphate absorption through its stimulatory effect on renal 1α-hydroxylase activity and calcitriol production (Torres and De Brauwere, 2011). Through binding to type 1 PTH receptor in proximal tubular cells, PTH stimulates cAMP synthesis and phospholipase C pathway and decreases renal phosphate transport (Prasad and Bhadauria, 2013). However, the main role of PTH is to maintain constant serum ionized calcium concentration and not phosphate. PTH regulates calcium release from bone and calcium reabsorption in the kidney; in turn ionized calcium concentration controls PTH secretion through the calcium sensor (Prie´ et al, 2009).

### *iii. Fibroblast growth factor 23 (FGF-23)*

FGF-23 is a circulating peptide that is secreted by osteocytes, osteoblast and osteoclast in response to hyperphosphatemia and vitamin D. It decreases renal phosphate reabsorption by lowering sodium phosphate transporters (NPT2a and NPT2c) expression in the tubules, and it reduces calcitriol synthesis by inhibiting 1α hydroxylase and stimulating its catabolizing enzyme 24, 25 hydroxylase. The reduced

calcitriol decreases intestinal NPT2b expression and phosphate reabsorption. FGF-23 may also alter parathyroid gland functions (Prie´ et al, 2009).

A coreceptor, klotho, is necessary for FGF-23 to exhibit bioactivity. The role of klotho in FGF-23 signaling is supported by the observation that klotho knockout mice have a phenotype identical to that of FGF-23 knockout mice (Berndt and Kumar, 2008). Figure 2 summarizes regulatory mechanisms of phosphate homeostasis by the three organs: parathyroid, bone, and kidney thru three feedback loops.

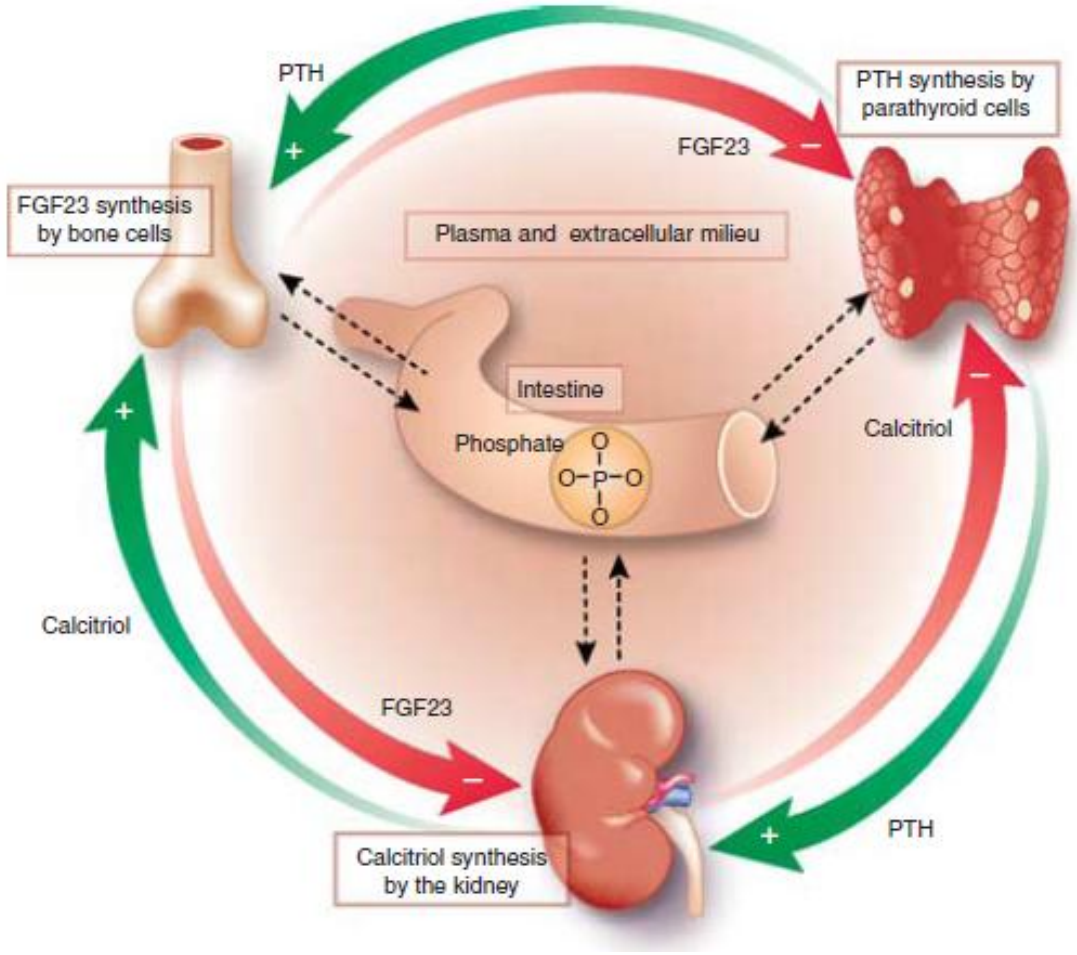



Figure 2.2: Regulatory mechanisms of phosphate homeostasis (Source: Torres and De Brauwere, 2011).

Table2.1: Factors influencing phosphate absorption or reabsorption in the intestine and kidney (Berndt and Kumar, 2008).

| Intestine | Kidney |
|---|---|
| *Factors that increase Pi absorption* | *Factors that increase Pi reabsorption* |
| 1. Reduced dietary intake of phosphate<br>2. Elevated serum 1 ,25-dihydroxyvitamin D | 1. Phosphate depletion<br>2. Parathyroidectomy<br>3. 1 ,25(OH)2D<br>4. Volume contraction<br>5. Hypocalcemia<br>6. Hypocapnia |
| *Factors that reduce Pi absorption* | *Factors that decrease Pi reabsorption* |
| 3. Reduced serum 1 ,25-dihydroxyvitamin D<br>4. Elevated concentrations of calcium salts in intestinal lumen<br>5. MEPE | 7. Phosphate loading<br>8. Parathyroid hormone and cyclic AMP<br>9. Volume expansion<br>10. Hypercalcemia<br>11. Carbonic anhydrase inhibitors<br>12. Dopamine<br>13. Glucose and alanine<br>14. Acid-base disturbances<br>15. Increased bicarbonate<br>16. Hypercapnia<br>17. Metabolic inhibitors<br>18. Arsenate<br>19. FGF-23<br>20. sFRP-4<br>21. MEPE<br>22. FGF7 |

6. **Hypophosphatemia:**

Hypophosphatemia can result from three different mechanisms: (1) decreased intestinal absorption (2) increased renal excretion, (3) or internal redistribution of inorganic phosphate from the extra- to the intra-cellular compartments (Geerse et al., 2010).

Hypophosphatemia does not necessarily mean phosphate deficiency as it may exist with low, normal, or elevated total body phosphate (Gaasbeek and Meinders,

2005). Hypophosphatemia can be acute or chronic. Acute hypophosphatemia may be mild (phosphorus level between 2-2.5 mg/dl), moderate (1-1.9 mg/dl), or severe (<1 mg/dl) and usually occurs in clinical settings such as refeeding syndrome, alcoholism, diabetic ketoacidosis, malnutrition/starvation, and after surgery and in the intensive care unit. Increased morbidity and mortality is manifested in acute hypophosphatemia with phosphate depletion. However, chronic hypophosphatemia is often associated with genetic or acquired renal phosphate-wasting disorders and usually produces abnormal growth and rickets in children and osteomalacia in adults (Felsenfeld and Levine, 2012).

Acute symptoms of hypophosphatemia are the result of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) depletion, which in turn lead to reduced energy stores and impaired oxygen delivery, respectively (Felsenfeld and Levine, 2012). Consequently, impaired energy metabolism will lead to cellular dysfunction in multiple organ systems (Geerse et al., 2010). Although most patients with hypophosphatemia do not develop symptoms, fatal complications have been described. Figure 3 summarizes causes and effects of hypophosphatemia.

a. Diet-induced hypophosphatemia

Animals on a low Pi diet show a reduction in serum Pi concentrations which are associated with a reciprocal increase in circulating plasma calcium concentrations, which results in the inhibition in PTH release, and reduced excretion of Pi by the kidney. After several hours on a low pi diet and reduced serum pi concentrations, an increase in the serum concentrations of 1,25- dihydroxyvitamin D3 (which occurs despite a reduction in PTH) increases the absorption of Pi in the intestine, and also increases renal Pi reabsorption in the proximal tubule (Sommer et al, 2007). Indeed,

Jara et al showed that growing rats given a low-phosphate diet (Phosphate less than 0.05%), with all other dietary constituents kept the same, rapidly produces hypercalcemia, hypercalciuria, hypophosphatemia, and urine virtually free of phosphorus (Jara et al, 1999).

Phosphate restriction causes resistance to the phosphaturic effect of PTH. The adaptive changes of the tubular capacity to reabsorb Pi are paralleled by changes in the active Na+-dependent component of Pi transport at the brush border membrane, with no change in the Na+-independent component of Pi transport (Loghman-Adham, 1996).

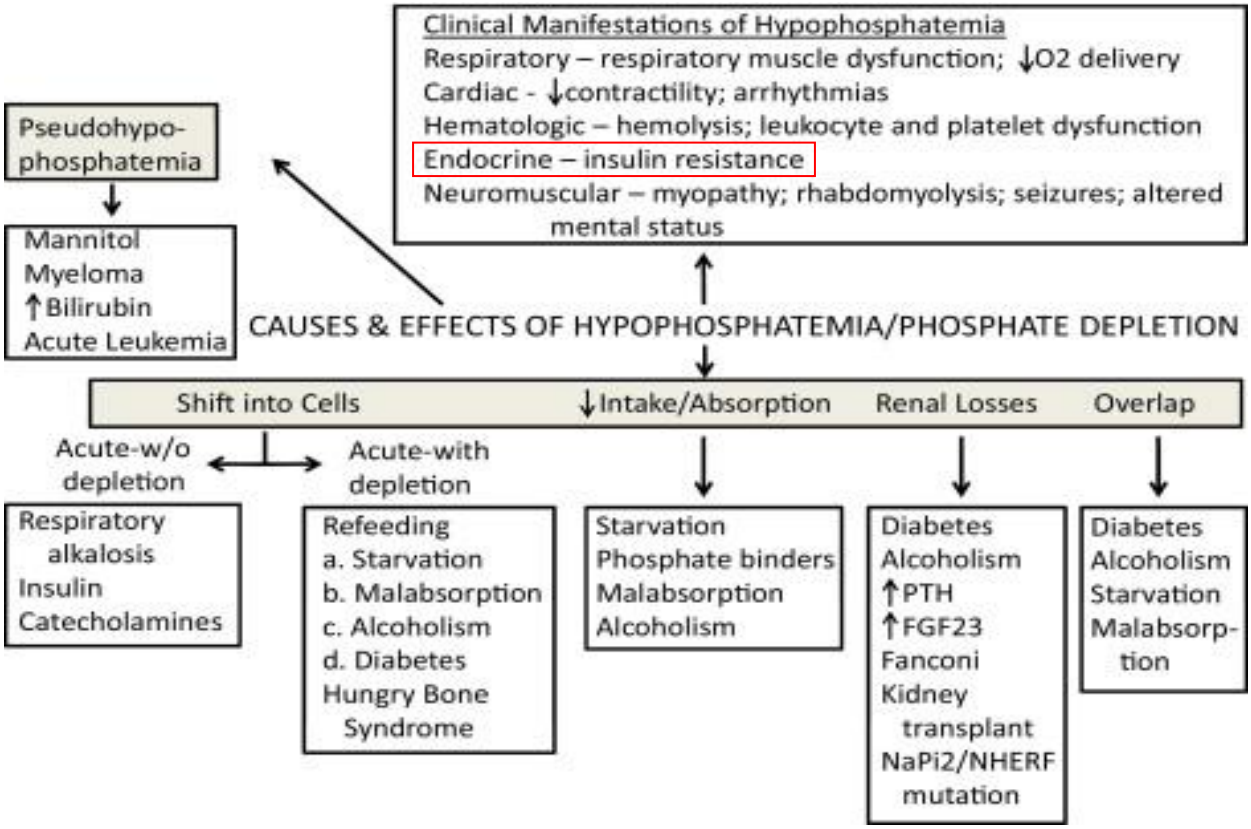



Figure 2.3: Causes and effects of hypophosphatemia (Felsenfeld and Levine, 2012).

Our interest in hypophosphatemia is its effect on insulin and insulin resistance; and its relation to the metabolic syndrome.

**B. Hypophosphatemia and the metabolic syndrome (MetS):**

***a. Metabolic syndrome:***

According to the Adult Treatment Panel III guidelines, MetS is based on the presence of 3 or more of the following criteria: hypertension, impaired glucose tolerance, abdominal obesity, and dyslipidemia involving hypertriglyceridemia and decreased high-density lipoprotein cholesterol values (JAMA, 2001).

***b. Disturbance in Phosphate Metabolism in Metabolic Syndrome:***

In 2001, Häglin presented a hypothesis suggesting that low serum phosphate is the cause of the disturbed metabolism in the metabolic syndrome. He based his hypothesis on the fact that serum phosphate is an important component of energy metabolism; therefore, a reduction of serum inorganic phosphate levels could contribute to the pathogenesis of the MetS by leading to disturbances in energy metabolism resulting in insulin resistance, hyperglycemia, disturbed lipid metabolism, increased weight, and hypertension (Häglin, 2001).

More recently, hypophosphatemia has been reported in studies of large numbers of individuals with metabolic syndrome.

- Haglin et al (2001) studied 2,752 consecutive patients (1,190 men, 1,562 women) admitted between 1986 and 1996 to a patient education centre. They found that a low plasma inorganic phosphate was associated with high BMI, high blood glucose, high systolic and diastolic blood pressures, but low serum high-density lipoprotein; key features of the MetS (Haglin et al, 2001).
- A different study by Kalaitzidis et al (2005) on 254 persons diagnosed with the metabolic syndrome based on Adult Treatment Panel III guidelines; showed

that patients with metabolic syndrome had significantly lower plasma Pi levels than controls (i.e. subjects with less than 3 criteria served as controls). They also found that the fractional excretion of phosphate was similar in both groups; therefore, it was hypothesized that hypophosphatemia in patients with MetS is attributable to decreased dietary intake and internal redistribution of inorganic phosphate (Kalaitzidis et al, 2005).

- In a prospective study, Gudmundsdottir et al investigated the relationship between serum phosphate and the metabolic syndrome in a group of middle-aged hypertensive and normotensive men during a 20-year follow-up. Individuals with the lowest serum phosphate levels had the highest number of risk factors according to the components of the MetS. These findings may suggest a role of low serum phosphate in the development of the metabolic syndrome (Gudmundsdottir et al, 2008).

In fact, it is well known that insulin resistance and compensatory hyperinsulinemia are key factors in the pathogenesis of MetS, and that the metabolic syndrome is a state of sympathetic nerve hyperactivity (De Pergola et al, 2008), which in turn may contribute to the intracellular shift of phosphate.

## C. Hypophosphatemia and Insulin Resistance:

### a. *Relation between Phosphorus, glucose, and insulin:*

An intimate relationship exists between glucose and phosphate metabolism. It has been known for a long time that insulin-dependent glucose uptake into target cells is accompanied by translocation of phosphate from the extracellular into the intracellular space (Nowicki et al, 1996). Epidemiological data show that, in healthy

men, serum phosphate is inversely correlated with serum glucose levels (Ljunghall et al, 1979). Experimental studies have also revealed that transport of glucose and glucose metabolism are correlated with hypophosphataemia (Davis et al, 1979).

Inorganic phosphate is needed in phosphorylation of carbohydrate intermediates in glycolysis and glycogenesis (Marshall et al., 1979). Inorganic phosphate stimulates the activity of hexokinase which is the first step in glucose metabolism that helps to trap glucose within the cell and hence, helps in glucose transport (Ditzel and Lervang, 2010). In fact, phosphate depletion decreases glycolysis and glucose transport and uptake by skeletal muscles (Davis 1979). Xie et al. (2000) demonstrated that rats fed with a Pi-deficient diet showed enhanced endogenous glucose production which is triggered by an increase in cAMP resulting from stimulated liver gluconeogenesis and glycogenolysis concentration. These changes indicate that the liver contributes to impaired glucose homeostasis in Pi-deficient states (Xie et al., 2000).

***b. Association of serum phosphate with Insulin Secretion and Sensitivity:***

It is of interest that hypophosphatemia is widely reported to be associated with insulin resistance. Several researchers found that hypophosphatemia has been shown to increase insulin resistance and induce glucose intolerance (Lindgarde and Trell, 1978; DeFronzo and Lang, 1980; Nowicki et al, 1996; and Haap et al, 2006). In fact, phosphate plays an essential role in insulin signaling and receptor affinity (Lizcano and Alessi, 2002). Haap et al (2006) showed that serum phosphate was positively correlated with insulin sensitivity calculated from the OGTT, and that this effect stayed to be

significant after adjusting for the covariates gender, age and percentage of body fat (Haap et al, 2006).

In vitro studies showed that pancreatic islets of phosphate-depleted rats had low ATP levels, elevated cytosolic calcium, impaired glycolytic activity, and impaired insulin secretion; however, these effects were corrected after phosphate supplementation (Castillo et al., 1982). These findings are consistent with the results of Zhou et al (1991) who reported that glucose-stimulated insulin secretion was lower in pancreatic islets from rats fed a phosphate-depleted diet. A defective closure of ATP-dependent potassium channels which is a process needed for activation of voltage-sensitive calcium channels which permit calcium influx into islets and thus a rise in cytosolic calcium and subsequent insulin secretion, might be related to impaired synthesis of ATP due to phosphate depletion. Thus, insulin secretion might be impaired during phosphate depletion due to an abnormality in one or more of these processes (Zhou et al., 1991).

## D. NAFLD

### 1. *Definition*

Non-alcoholic fatty liver disease (NAFLD) is an increasingly common disease state and a major public health concern owing to its high prevalence worldwide and potentially serious sequelae. The term non-alcoholic fatty liver disease (NAFLD) refers to the range of diseases characterized by fatty infiltration of the liver ranging from benign steatosis, to the more severe steatohepatitis (NASH), or cirrhosis; in the absence of significant ethanol consumption or viral infection (Guturu et al, 2012). NAFLD is the hepatic manifestation of metabolic syndrome and a risk factor for type 2 diabetes

mellitus, dyslipidemia and hypertension (Adams et al, 2009). The term NASH was coined in 1980 after the now famous report of Ludwig et al (1980). However, it was not until the 1990s, that the prevalence and increasing incidence of the condition brought it into the attention. Moreover, the identification of NAFLD paralleled the alarming increase in body mass index in the American population (Oh et al, 2008).

Apart from weight loss, there are currently no effective therapies for NAFLD (Kawano and Cohen, 2013). Therefore, research efforts are focusing on understanding the underlying pathophysiology of hepatic steatosis with the expectation to identify novel therapeutic targets.

### *2.Epidemiology*

The prevalence of NAFLD is up to 30% in developed countries and nearly 10% in developing nations, making NAFLD the most common liver condition in the world (Briohny et al, 2011). However, the actual prevalence of NAFLD remains unknown and hard to determine due to the lack of population-based studies and reliable noninvasive screening tools. Moreover, the disease definition and modalities used for diagnosis are not standardized (Oh et al, 2008). Nonetheless, estimates of the prevalence of NAFLD range widely from 11 to 46 % (Kawano and Cohen, 2013) with approximately 70% obese and diabetic patients also having NAFLD (Jianga et al., 2013).

The prevalence of NASH is even more difficult to determine as large population-based studies are not possible given that a liver biopsy is required for diagnosis (Oh et al, 2008). The rapid increase in the incidence of NAFLD might be

explained by the current epidemic of obesity and metabolic syndrome, which are manifested at the hepatic level as NAFLD (Guturu et al, 2012). Non-alcoholic steatohepatitis may progress to cirrhosis and liver-related death (mostly from hepatocellular carcinoma) in ~25% and ~10% of cases respectively (Tessari et al., 2009). To our knowledge, there are no data in the literature about the prevalence of NAFLD in Lebanon or the Middle East; however, it is expected to be high due to the high prevalence of obesity. Results from a national population-based study in Lebanon showed high prevalence rates of overweight and obesity (Sibai et al., 2003).

***3.Symptoms and Signs***

Most patients with NAFLD (48–100%) are asymptomatic, and the disease is often discovered incidentally during routine laboratory examination when a hepatic panel reveals an elevated ALT level. Some symptoms might occur but they are usually nonspecific: vague right upper quadrant abdominal pain, fatigue, and malaise are the most common of these nondescript symptoms. However, pruritus, anorexia, and nausea may develop in rare cases. Moreover, jaundice, ascites (abdominal distension), gastrointestinal bleeding, and confusion (encephalopathy) are all indicative of advanced liver disease (decompensated cirrhosis), occurring in the late stages of NASH. Up to 75% of patients were reported with hepatomegaly in several studies (Sass et al., 2005).

***4.Diagnosis***

a. Biochemical studies

i. Liver function tests

The most common and often the only laboratory abnormality found in patients with NAFLD is mild to moderate elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Sass et al., 2005). Although this has not been universally observed, hepatic transaminase levels tend to be elevated more in the presence of NASH compared to simple steatosis. Furthermore, hepatic transaminase levels tend to fall as fibrosis progresses in individuals with NAFLD. Therefore, hepatic transaminases are not reliable for determining the presence or absence of NASH or fibrosis in an individual subject (Briohny et al, 2011). In addition, the AST to ALT ratio is more valuable than individual parameters, and is usually <1 in patients with no or minimal fibrosis, though this may reverse with the development of cirrhosis (Jianga et al., 2013).

ii. Lipid profile

Although lipid concentrations may be normal or elevated, many patients have the atherogenic dyslipidemia associated with the metabolic syndrome. The characteristic lipid profile of subjects with NAFLD and NASH includes high triacylglycerol, low HDL, and increased small, dense LDL (Koruk et al., 2003).

b.Histology

The diagnosis of hepatic steatosis and steatohepatitis is not yet possible without liver biopsy which is known as the “Gold standard” for diagnosis of NAFLD

(Oh et al, 2008). It is defined histologically by the presence of fatty change, inflammation and fibrosis (Wierzbicki et al., 2012).

The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-Sponsored NASH Clinical Research Network (CRN) developed a histological scoring system for use in clinical trials of NASH (Kleiner et al., 2005). The Non-alcoholic fatty liver disease Activity Score (NAS) and Fibrosis Score were developed and validated by the CRN to determine the histological severity of NAFLD. The scoring system comprised 14 histological features and ranges from 0 to 8 and is defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2). Other nine features were recorded as present or absent. Fibrosis Score was defined from 0 to 4. A NAS of 0–2 is not NASH and a score of 5 is usually NASH (Kleiner et al., 2005).

***5.Pathophysiology***

NAFLD represents the hepatic manifestation of the metabolic syndrome and is strongly associated with obesity, hypertension, dyslipidaemia and insulin resistance (Briohny et al, 2011). The pathogenesis of NASH has not been completely elucidated. A "two-hit hypothesis" was suggested by Day et al. to explain the pathogenesis and progression of NAFLD. Although this theory has recently been challenged, the first hit causes accumulation of excess triglycerides in the liver leading to simple steatosis and the second hit causes the steatosis to progress to inflammation and fibrosis (Guturu et al, 2012). Hepatic lipid accumulation results from an imbalance between lipid availability (from circulating lipid uptake or de novo lipogenesis) and lipid disposal

(via free fatty acid oxidation or triglyceride-rich lipoprotein secretion) and eventually triggers lipoperoxidative stress and hepatic injury (Musso et al, 2009).

It is very important to understand the complex molecular mechanisms involved in the pathophysiology of NAFLD to develop new therapeutic targets for this disease. Although the mechanisms underlying disease progression remain unclear, insulin resistance and obesity-related inflammation are thought to play a key role, along with possible genetic, dietary and lifestyle factors (Nseir et al, 2010). The interaction of lipogenesis, fatty acid oxidation, inflammation, endoplasmic reticulum stress and hepatic insulin resistance contribute to the pathogenesis of NAFLD/NASH (Wierzbicki et al., 2012).

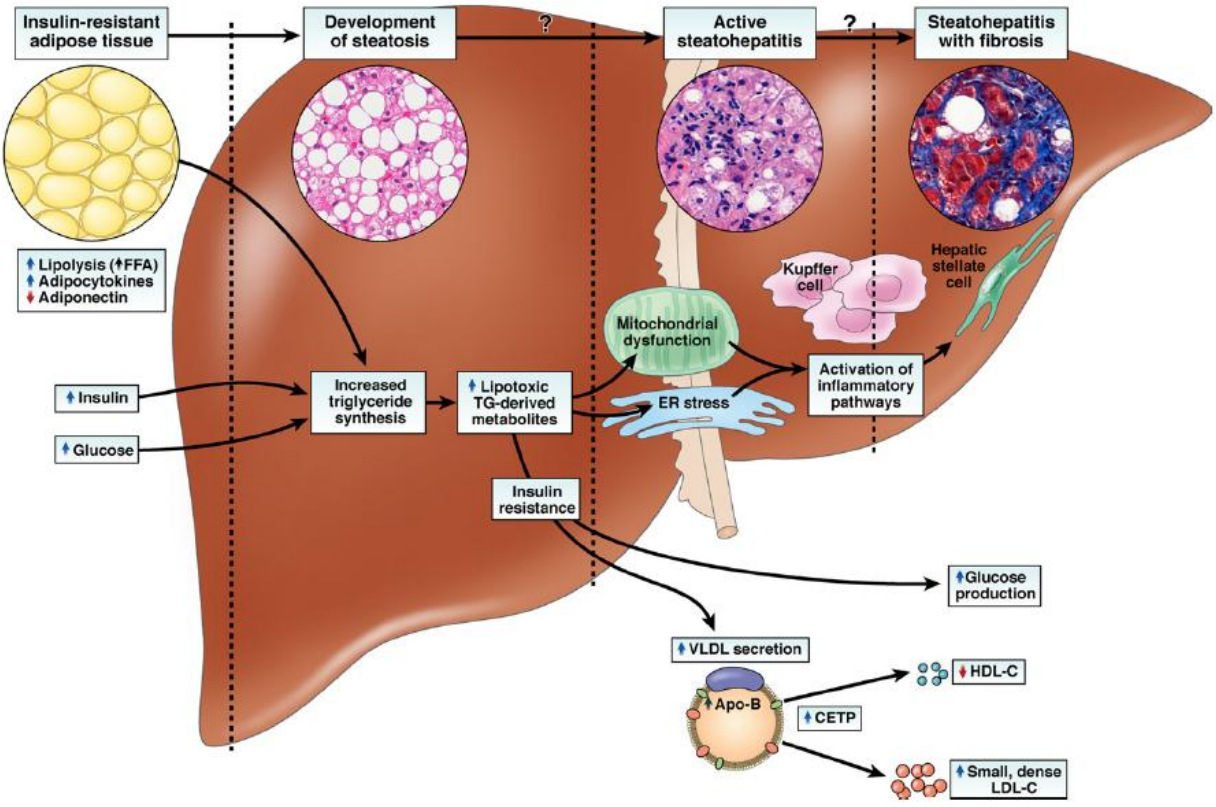



Figure 2.4: Schematic representation of the pathophysiology of NASH (Source: Cusi, 2012)

a. The role of insulin resistance and free fatty acids

Insulin, an anabolic hormone, plays a vital role in mediating carbohydrate and lipid homeostasis in the liver by stimulating glycogen synthesis, lipogenesis, lipoprotein synthesis, suppression of gluconeogenesis/glycogenolysis and very low density lipoprotein secretion in the fed state (Fatani et al, 2011). Therefore, the integrity of hepatic insulin signaling is essential for the action of insulin in hepatocytes, and a defect in protein kinase expression may cause insulin resistance with subsequent induction of hepatic insulin resistance-related clinical disorders, NAFLD (Fatani et al, 2011).

Insulin resistance is the primary liaison between NAFLD and the metabolic syndrome, which is defined as visceral obesity, hypertension, insulin resistance (IR) or diabetes, and dyslipidemia (Oh et al, 2008). Indeed, it is widely reported that insulin resistance plays an important role in the pathogenesis of NAFLD, and that patients with NAFLD have IR which results in impaired suppression of lipolysis from the adipose tissue leading to increased levels of circulating non-esterified or free fatty acids (FFAs) (Ibrahim, 2011; Schwenger and Allard, 2014; Cusi, 2012; Oh et al, 2008). The resulting FFA will be taken up by the liver where they are esterified into neutral triglycerides (TGs); however, an excess of FFAs overwhelms the capacity of the liver to esterify FFA, and induces lipotoxicity (Ibrahim, 2011). The increase in FFA can cause lipid peroxidation, which in turn can increase the production of pro-inflammatory cytokines; and can also exceed mitochondrial beta-oxidation further increasing the oxidative stress and inflammation (Schwenger and Allard, 2014).

The intermediary phospho-inositide-3-kinase (PI-3-kinase) is an important mediator of insulin action. When the insulin receptor is tyrosine-phosphorylated upon binding to insulin, it causes in turn tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. Upon phosphorylation, IRS-1 (the initiator in the pathway of glucose metabolism) induces stimulation of the PI-3-kinase-AKT/protein kinase B (PKB) pathway, resulting in recruitment of glucose transporters 4 (GLUT4). In turn, GLUT4 leads to increased glucose entry into cells for storage as glycogen or use for energy production (Schreuder et al, 2008). However, intracellular accumulation of FFAs in obesity leads to impairments in insulin signaling through the IRS1/PI-3-kinase/ GLUT4 pathway (Oh et al, 2008). IRS-1 tyrosine phosphorylation leads to serine phosphorylation, thereby interrupting the pathway for the transport of glucose via the GLUT transporters to the membrane. Inflammatory kinases that have been found to induce this inhibitory serine phosphorylation are, IKB-kinase-β (IKK-β), jun-kinase-1 (JNK-1) and suppressor-of-cytokine-signalling-3 (SOCS3) (Schreuder et al, 2008).

Moreover, the link between increased circulating FFAs and IR might involve accumulation of triglycerides and fatty acid-derived toxic lipid metabolites (diacylglycerol, fatty acyl-CoA and ceramides) in muscle and liver (Saltiel and Kahn, 2001), which activates intracellular inflammatory pathways within hepatocytes and Kupffer cells (the liver counterpart of adipose tissue macrophages) and other immune cells, in resemblance to defects within adipocytes (Cusi, 2012).

b. Adipose hepatocyte interactions

There is an increased body of evidence suggesting that the adipose tissue is a key player in hepatic steatosis (Ibrahim, 2011). Expansion of the adipose tissue during the development of obesity, results in an activation of the death receptor and mitochondrial pathways of apoptosis. An increase in adipocyte death results in the recruitment of macrophages to adipose tissue, with subsequent development of dysfunctional, insulin-resistant adipocytes that release excessive amounts of FFA and cause insulin resistance and lipoapoptosis in distant tissues including the liver, muscle, pancreatic beta cells, other (Feldstein, 2010).

The adipose tissue acts as an endocrine tissue by producing multiple proteins, collectively referred to as adipokines. Dysregulation of adipokines/cytokines (example: TNF-α, adiponectin and leptin) are strongly involved in the pathogenesis of MetS, the development of fatty liver and the progression from fatty liver to NASH (Polyzos et al, 2010). More specifically, adiponectin which is the most abundant and adipose-specific adipokine, has potent anti-inflammatory, antidiabetic, antiatherogenic and insulin-sensitizing properties by decreasing gluconeogenesis, de novo lipogenesis and FFAs influx in the liver and by increasing FFAs β-oxidation. Since the hormone is essential to the control of hepatic lipogenesis, decreased secretion of adiponectin in obesity alters lipid metabolism and insulin sensitivity in the liver leading to hepatic steatosis and VLDL dysregulation in NAFLD as well as necroinflammation in NASH (Polyzos et al, 2010). However, it should be mentioned that whether patients are obese or not, the adipose tissue insulin resistance is found in the majority of patients with NAFLD, with

the liver behaving as the metabolic sensor of the dysfunctional adipose tissue and a main target of this lipotoxic state (Cusi, 2012).

c. Liver de novo lipogenesis (DNL)

Hepatic DNL also contributes to the steatosis. Excess FFAs not only induces hepatic insulin resistance but also impairs insulin clearance in vitro (Svedberg et al, 1990) and in vivo (Wiesenthal et al, 1999) and in humans (Carpentier et al, 2000), which leads to the typical hyperinsulinemia of insulin-resistant states and of patients with NAFLD. Hyperinsulinemia in turn stimulates hepatic sterol regulatory element binding protein 1c (SREBP-1c) activity and is coupled with increased hepatic DNL increasing the production and storage of triglycerides (Cusi, 2012). However, even though DNL is increased in NAFLD, it contributes less to the total hepatic triglyceride pool (26%) in comparison to fatty acids derived from the adipose tissue that account for the majority (60%) of hepatic triglyceride accumulation in NAFLD (Donnely et al, 2005).

d. Lipid outflow:

Under normal physiological conditions, apolipoprotein (apo) B facilitates lipid export from the liver by incorporating TG and formation of VLDL by the ER (Briohny et al, 2011). Insulin is a strong promoter of ApoB degradation via the PI3K pathway and thus has an influence on the number of VLDL particles synthesized. However, in IR states, the PI3K pathway is eliminated to a certain extent (Schreuder et al, 2008). Therefore in IR, a hyper afflux of FFA to the liver, combined with a defective insulin affects in the inhibition of Apo B synthesis and secretion, resulting in hepatic lipid

accumulation and an exaggerated VLDL-Apo B secretion (Adiels et al., 2006). Actually, VLDL secretion rate increases linearly with increasing intrahepatic TG accumulation; eventually, increased hepatic VLDL secretion lowers HDL cholesterol levels and leads to small, dense, LDL cholesterol, the typical triad of NAFLD (Cusi, 2012).

On the other hand, hepatic VLDL-TG export in NAFLD is inadequate to normalize hepatic TG content (Cusi, 2012). In addition, it is likely that the production of VLDL/apoB might decrease as NAFLD progresses secondary to the impairment of hepato cellular function (Jiang et al., 2013). ApoB-100 synthesis, a rate-determining step in hepatic lipid export, is markedly attenuated in patients with NASH which is likely to contribute to the net retention of lipids in the liver predisposing to NASH (Charlton et al., 2002).

e. Oxidation

Most fatty acids are metabolized through β-oxidation mainly in the mitochondria, but also in peroxisomes and microsomal ω-oxidation by members of the cytochrome P450 in the endoplasmic reticulum. The latter 2 pathways become more important in conditions of increased FFA availability in the liver, including NAFLD (Musso et al, 2009). In IR states, the amount of FFA available for oxidation exceeds the mitochondrial capacity; consequently a bulk of acetyl CoA enters the citric acid cycle and results in the delivery of electrons to the respiratory chain, where they generate reactive oxygen species (ROS) (Schreuder et al., 2008).

Moreover, several lines of evidence suggest that mitochondrial function is impaired in human NASH, with different factors being involved in the pathogenesis of mitochondrial dysfunction, including NEFAs, lipid peroxidation products, and TNF-α. As the oxidative capacity of the mitochondria becomes impaired, cytosolic fatty acids accumulate; hence alternative pathways (peroxisomal β-oxidation and microsomal ω-oxidation) are activated, resulting in the formation of additional ROS (Musso et al, 2009).

***6. From hepatic steatosis to NASH***

Factors responsible for the progression from simple fatty liver to NASH are widely researched and include: TNF-α expression, lipid peroxidation and mitochondrial dysfunction (Schreuder et al, 2008). In addition, oxidative stress and increased ROS production through cytochrome P450 activation, further increase inflammatory cytokines; hepatic stellate cells activation leads to fibrosis and collagen deposition; dysregulation of lysosomal metabolism and endoplasmic reticulum stress lead to apoptosis. Moreover, increased ROS production decreases ATP and nicotinamide dinucleotide concentrations leading to DNA and protein damage resulting in impaired membrane structure and function (Tessari et al., 2009).

**E. Influence of nutrients on NAFLD**

Excessive energy intake leads to obesity, which in turn increases the risk for NAFLD; however the quality and not only the quantity of energy could play an important role for the development and progression of NAFLD (Schwenger and Allard,

2014). In fact, the effect of different dietary constituents on whole-body metabolism and its regulation through effects on hormones, transcription factors, and lipid metabolic pathways are considered to play a central role in the pathophysiology of NAFLD (Zivkovic et al., 2007).

The effects of each macronutrient on NAFLD are reviewed in details in the literature; however they are beyond the scope of this study. In general, saturated fat and fructose seem to stimulate hepatic lipid accumulation and progression into NASH, whereas unsaturated fat, choline, antioxidants, and high-protein diets rich in isoflavones seem to have a more preventive effect (De Wit et al., 2012).

The influence of high fat especially saturated fat diet will be discussed in details below.

## F. Lipids

### a. *Fat digestion and absorption*

About 97% of dietary lipids are in the form of triglycerides and the rest are in the form of phospholipids and cholesterol (Mahan and Escott-Stump, 2004).

The digestion and absorption of fat by the small intestine requires mechanisms that solubilize the fat and its digestion products through the following stages:

- Minor digestion of triacylglycerols starts in the mouth and stomach by lingual and gastric (acid-stable) lipases respectively.
- Large fat globules leaving the stomach are emulsified in the small intestine by bile salts and phospholipids secreted by the liver.
- Pancreatic lipase digests fat at the surface of the emulsion droplets, forming fatty acids and monoglycerides.

- The water-insoluble products of lipase action combine with bile salts and form micelles which facilitate the passage of lipids to the mucosal surface. Bile salts then undergo enterohepatic recycling.
- Free fatty acids and monoglycerides are absorbed across the luminal membranes of epithelial cells, within which they are reesterified to form triglycerides.
- Export from intestinal cells to the lymphatic system by exocytosis as chylomicrons coated with Apo B48 and containing triacylglycerols, cholesterol esters and phospholipids.

***b. Hepatic metabolism of lipids***

After export from intestinal cells, chylomicrons which contain Apo B48, ApoA-1, and ApoCs undergo hydrolysis in the circulation to produce cholesterol-dense lipoprotein remnants. The chylomicron (CM) remnant particles, after utilization of lipids by peripheral tissues, are taken up by the hepatocytes (Jiang et al., 2013). The liver then reassembles fatty acids delivered by CM remnants in apoB-100 containing VLDL (another triglyceride rich lipoprotein). Mature VLDLs are secreted through vesicle-mediated exocytosis (Jiang et al., 2013). Liver cells are involved in many pathways of lipid metabolism: liposynthesis, de novo lipogenesis, fatty acid b-oxidation, lipolysis, and lipoprotein synthesis and export.

## G. Saturated Fat (SFA) and NAFLD

Several researchers have demonstrated a relationship between increased dietary fat consumption and NAFLD including Zelber-Sagi et al. (2007) and Sathiaraj

et al. (2011). More specifically, Toshimitsu et al (2007) found that the ratio of polyunsaturated/saturated fatty acid intake in NASH patients was lower than in the healthy population. Hepatic fat accumulation per se is not injurious according to the two-hit hypothesis, however, secondary insults (e.g. ROS, inflammatory cytokines) imposed upon the fatty liver are essential for progression to steatohepatitis. Therefore, it is proposed that saturated fatty acids represent an intrinsic second hit that hastens the development of NASH. Moreover, various experimental models suggest that lipid-induced cell toxicity and apoptosis is specific to or made more severe by SFAs (Gentile and Pagliassotti, 2008).

The mechanisms by which SFA induce apoptosis in many cells including hepatocytes are unclear. However, ceramides which have been linked to both insulin resistance and apoptosis tend to accumulate via enhanced de novo synthesis using palmitate or increased sphingomyelin breakdown. Moreover, inhibition of ceramide production prevents saturated fatty acid-induced apoptosis. Therefore, it has been hypothesized that ceramides play a role in SFA induced apoptosis (Gentile and Pagliassotti, 2008). Nevertheless, SFAs disrupt ER homeostasis inducing ER stress and apoptosis in liver cells via mechanisms that do not appear to involve ceramide accumulation (Wei et al., 2006). Indeed, ER stress and apoptosis are some of the factors responsible for the progression from simple fatty liver to NASH as mentioned earlier.

## H. Phosphorus and NAFLD

Although research over the last decade has greatly enhanced our understanding of the pathophysiology of NAFLD, nonetheless, numerous questions

remain unanswered. For example, what are the metabolic abnormalities that initiate the development of NAFLD, and what biochemical processes mediate the transition from simple steatosis to NASH?

Concern is raised on the relationship between dietary phosphorus and NAFLD since a recent study showed that dietary Pi restriction plays an important role in the development of fatty liver disease and hyperlipidemia induced by a high-cholesterol diet through the regulation of lipid metabolism–related gene expression in the liver (Tanaka S. et al, 2013). Moreover, hepatic steatosis was induced in Apolipoprotein E knock-out mice receiving a low phosphorus diet, and it was accompanied by greater liver weight and alanine transaminase (Ellam et al, 2011). Likewise, hypophosphatemia has been reported in studies of large numbers of individuals with metabolic syndrome as mentioned earlier.

At this point, interest is raised on the role of phosphorus in progression of NAFLD since it is required in ATP production. Theoretically, ATP depletion through increased adenylate kinase activity or inhibition of respiration raises the intracellular AMP/ATP ratio and should result in AMPK activation to recover ATP (Tessari et al, 2009). However, it has been shown in a human pilot study that unlike control subjects, NASH patients were unable to recover from a modest ATP-depleting challenge (Cortez-Pinto et al., 1999). Defects in oxidative phosphorylation, an impaired major respiratory chain activity and reduced ATP synthesis have been identified in the mitochondria of fatty liver. When mitochondrial oxidation is impaired, FFA

accumulates in the cytosol and is alternatively oxidized via the peroxisomes (β) and microsomes (ω-oxidation), resulting in further production of ROS (Tessari et al, 2009).

A study by Perez-Carreras et al. (2003) demonstrated a severe impairment in hepatic ATP recovery in NASH; in addition, body mass index was inversely correlated with ATP recovery even in the healthy lean control subjects. Although these findings are not precise but they do suggest that defects in energy conservation (i.e., mitochondrial dysfunction) may occur before fatty liver disease appears. Eventually this will increase the susceptibility for disease when the liver experiences other insults like increased ROS; liver fat infiltration may ultimately exacerbate mitochondrial dysfunction perpetuating the vicious cycle. Therefore, it becomes apparent that the reduced ATP synthesis in NAFLD may be the cause and not the consequence of hepatic lipid accumulation (Musso et al., 2009).

It should be mentioned that high fructose intake has been reported in a number of animal and human studies to have important hepatic consequences including the development of NAFLD (Yilmaz, 2012). Initial fructose metabolism in the liver involves the phosphorylation of fructose to fructose-1-phosphate by fructokinase which is specific for fructose and not rate limited. Thus the high activity of fructokinase can trap phosphorus and result in hepatic ATP depletion. As a consequence, high fructose consumption may contribute to NAFLD pathogenesis since fructose-induced ATP depletion promotes hepatic necroinflammation (Nseir et al., 2010).

Our hypothesis is that phosphorus seems to embody the missing link between the metabolic derangements that occur in insulin resistance/metabolic syndrome and NAFLD.

## I. Animal models of NAFLD/NASH

Table 2.2 shows the biochemical and pathological characteristics of animal models of NAFLD/NASH. There are many genetic models; however, our interest is in nutritional models of NAFLD. Since high fat diet has shown to induce obesity, IR, steatosis, steatohepatitis, and fibrosis, therefore we decided to use the high fat model to induce NAFLD.

**Table 1 Biochemical and pathological characteristics of animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis**

| Model | Obesity | Insulin resistance | Steatosis | Steatohepatitis | Fibrosis |
|---|---|---|---|---|---|
| SREBP-1c transgenic mice | No (decreased adiposity) | Yes | Yes | Yes | Yes |
| Ob/ob mice | Yes | Yes | Yes | No (does not develop spontaneously) | No (resistant to fibrosis) |
| Db/db mice | Yes | Yes | Yes | No (does not develop spontaneously) | No (does not develop spontaneously) |
| KK-$A^y$ mice | Yes | Yes | Yes | No (does not develop spontaneously) | No (does not develop spontaneously) |
| *PTEN* null mice | No | No | Yes | Yes | Yes |
| *PPAR-α* knockout mice | No | No | No (steatosis occurs in the starved state) | No | No |
| *AOX* null mice | No | No | Yes | Yes | No |
| *MAT1A* null mice | No | No | Yes | Yes | Yes |
| Methionine and choline deficiency | No (decreased weight and adiposity) | Hepatic insulin resistance | Yes | Yes (severe) | Yes |
| High fat | Yes | Yes | Yes | Yes (mild) | Yes |
| Cholesterol and cholate (atherogenic diet) | No (decreased weight) | Hepatic insulin resistance | Yes | Yes | Yes |
| Fructose | No | Yes | Yes | No/Yes | No |

Table 2.2: Biochemical and pathological characteristics of animal models of NAFLD/NASH. (Source: Takahashi et al (2012)).

## J. Objective:

A review article on animal models of NAFLD/NASH showed that a high fat diet can induce obesity, insulin resistance, steatosis, steatohepatitis, and fibrosis (Takahashi et al, 2012). In addition, Lieber et al conducted a study on Sprague-Dawley rats, fed a high-fat diet ad libitum for 3 weeks (71% of energy from fat, 11% from carbohydrates, 18% from proteins) and found that rats developed panlobular steatosis. By feeding rats a high-fat liquid diet, Lieber et al reproduced the typical hepatic lesions of NASH, inflammation, and early fibrosis (Lieber et al, 2004). Moreover, a recent study on Lewis and Sprague-Dawley rats of both genders fed standard or high-fat diet for three weeks showed that livers of both strains and genders fed the HF diet demonstrated evidence of steatosis and Sprague-Dawley rats presented macrovesicular steatosis accompanied by pronounced fibrosis (Stoppeler et al, 2013). Therefore, in the present study male Sprague-Dawley rats will be given a high fat diet to induce NAFLD; at the same time rats will be given varying concentrations of phosphorus supplementation to investigate the role of dietary phosphorus in the development and progression of NAFLD in rats.

# CHAPTER III

# MATERIALS AND METHODS

## A. Experimental Procedure

All procedures performed were in accordance with the guidelines for ethical care of experimental animals, and the experimental protocol was approved by the Institutional Animal Care and Use Committee of the American University of Beirut.

### *1. Animal Housing*

Six week-old male Sprague-Dawley rats weighing between 190-290 g were used for the experiment. Fifty rats were included in the study; however analysis was made only for 44 rats since six died during the experimental period. Rats were housed individually in wire mesh-bottomed cages to facilitate the collection of food being spilled by rats. Room temperature was maintained at 22±1°C with 12:12 dark-light cycles. The rats had free access to water and were fed ad libitum on an adaptation diet for one week to familiarize them to the environment and diet.

### *2.Experimental design:*

Following adaptation, rats were randomly allocated into 5 groups and had free access to water and fed ad libitum either a control diet or a high fat diet for 8 weeks. The diets were as follows:

- Group 1: Control
- Group 2: High Fat/ Free Phosphorus* (HF/P-Free)
- Group 3: High Fat/ Very Low Phosphorus (HF/Very Low P)

- Group 4: High Fat/ Low Phosphorus (HF/Low P)
- Group 5: High Fat/ Normal Phosphorus (HF/Normal P)

The high fat diets were prepared using the same ingredients and same proportions, with the exception of Potassium Phosphate (MERK, ProAnalysis) which was used in different proportions to manipulate phosphorus concentrations of the different groups. In addition, egg white (Appendix 4) was purchased from Dyets, inc. and used in all 5 groups instead of casein since it has a lower percentage of phosphorus (890 mg P/kg egg white) than casein which has 7500 mg P/kg. Table 3.1 shows the composition of the adaptation diet in addition to the experimental diets, and Table 3.2 shows the contribution of each of the macronutrients to the total diet in terms of energy and weight.

Table 3.1: Diet Composition

| **Ingredients (g/kg)** | **Adaptation diet** | Group 1 **Control** | Group 2 **HF/P-Free** | Group 3 **HF/Very Low P** | Group 4 **HF/Low P** | Group 5 **HF/Normal P** |
|---|---|---|---|---|---|---|
| Casein | 198 | 0 | 0 | 0 | 0 | 0 |
| Egg White [β] | 0 | 200 | 200 | 200 | 200 | 200 |
| DL-methionine | 2 | 0 | 0 | 0 | 0 | 0 |
| Corn Oil | 100 | 100 | 100 | 100 | 100 | 100 |
| Lard (Ghee) | 0 | 0 | 300 | 300 | 300 | 300 |
| Starch | 300 | 300 | 300 | 300 | 300 | 300 |
| Sucrose | 300 | 300 | 0 | 0 | 0 | 0 |
| Mineral mix | 35* | 35* | 35** | 35** | 35** | 35** |
| Vitamin mix*** | 10 | 10 | 10 | 10 | 10 | 10 |
| Cellulose | 55 | 49.202 | 55 | 52.5 | 49.202 | 42.62 |
| Potassium Phosphate | 0 | 5.798 | 0 | 2.5 | 5.798 | 12.38 |
| **% Phosphorus** | **0.3** | **0.3** | **0.0178** | **0.075** | **0.15** | **0.3** |
| mg Phosphorus/ Kcal | 0.7317 | 0.7317 | 0.0318 | 0.1339 | 0.2678 | 0.5357 |

*Appendix 1: Composition of the mineral mix.

**Appendix 2: Composition of the phosphorus-free mineral mix.

***Appendix 3: Composition of the vitamin mix.

Table 3.2: Contribution of each of the macronutrients to the total diet in terms of energy and weight.

| | **Control** Group 1 | **High Fat Groups** Groups 2-5 |
|---|---|---|
| **Macronutrients (% by Weight)** | | |
| Carbohydrates | 60 | 30 |
| Fat | 10 | 40 |
| Protein | 20 | 20 |
| **Macronutrients (% Energy)** | | |
| Carbohydrates | 58.53 | 21.43 |
| Fat | 21.95 | 64.3 |
| Protein | 19.5 | 14.3 |
| **Kcal/g** | 4.1 | 5.6 |

One day before sacrificing the rats, pots including the diets were removed from the cages and rats were fasted overnight. The next morning, rats were anesthetized using isoflurane and blood was drawn by venipuncture into tubes containing EDTA and placed directly in ice boxes. Next, livers, kidneys, and epididymal fat pads were directly excised and weighed. Directly after that, 2 portions of the liver were cut and placed in 2 different tubes for biopsy; and the remaining was placed in a nylon bag and frozen in liquid nitrogen, then stored at -20°C till analysis.

**B. Evaluation of animal and organ weights**

All animals were weighed once per week until the end of the experimental protocol. At the end of the 8 weeks on the diets, the animals were euthanized and the wet weight of the livers, epididymal fat pads and kidneys were measured.

**C. Food intake**

Rats were maintained on the experimental diet for 8 weeks and food intake was measured twice per week. Food intake was calculated by subtracting the weight of the pot from the previous weight taking into consideration food spilled by rats.

**D. In vivo Body Composition Analysis**

Fat mass and fat free mass were measured using VET BioImpedance Spectroscopy Analysis, (ImpediVetTM Vet BIS1) provided by ImpediMed Limited. This measurement was taken two days before sacrificing the rats. First, rats were anesthetized using isoflurane for the duration of the BIS measurements. Then, needles and electrodes were attached per manufacturer's instructions and measurement was performed five consecutive times for each rat and the average was calculated to compare between the different groups.

**E. Plasma Analysis**

At the end of the experiment, after collecting blood samples into EDTA tubes, these samples were centrifuged at 3500 rpm for 15 minutes to separate the plasma for the determination of glucose, triglyceride, total cholesterol, HDL-cholesterol, LDL-

cholesterol, total phosphate, Alanine aminotransferase (ALT), Aspartate amino-transferase (AST), and insulin. The plasma were aliquoted and stored at (−80°C) till the time of conducting the biochemical analyses.

### *1. Glucose, triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, and total phosphate.*

Plasma glucose, triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, and total phosphate were determined using the Vitros 350/250 AT Chemistry Systems (Ortho-Clinical Diagnostics, Johnson & Johnson, New York).

### *2. Alanine aminotransferase (ALT), Aspartate amino-transferase (AST)*

ALT and AST are run on Cobas 6000 instrument based on the following principle: The oxacids formed in the transaminase reaction are measured indirectly by enzymatic reduction to the corresponding hydroxyacids, and the accompanying change in NADH concentration is monitored spectrophotometrically.

### *3. Insulin*

Insulin was measured using an insulin kit provided by Dia Metra for the direct solid phase immunoenzymatic determination of insulin level. Results were read on Thermo Scientific Multiskan GO.

## F. Hepatic Fat Extraction

Hepatic total fat extraction was applied according to the Folch method (Folch et al, 1957). A portion of the liver (1g) designated for fat extraction was placed in a

previously weighed centrifuge tube. The centrifuge tubes with the liver portions were weighed again then freeze dried in a Labconco FreeZone 6L freeze dryer for 2 days. After freeze drying, tubes containing liver portions were weighed again and the liver was crushed in the tube using a glass rod till it became a powder. Then 20 ml of chloroform:methanol (2:1 by volume) were added to the tube, mixed with a vortex, and shaken in an orbital shaker for 15 minutes. The mixture was then centrifuged in an IEC Centa centrifuge (International Equipment Company- USA) for 15 minutes at 3500 rpm and the top layer containing the fat was discarded. Extraction with chloroform and methanol was done twice. Next, the tubes containing the non-fat layer were placed under the fume hood to dry for 2 days. Finally, the centrifuge tubes were weighed with the remaining residues.

## G. Liver Histology

Liver samples were collected for histology to study the progression of NAFLD.

### *1. Histopathologic Examination*

Rat liver tissue was processed into 3-4 μm thick formalin-fixed paraffin embedded tissue sections and stained with hematoxylin and eosin (H&E). Histopathologic examination consisted of assessing steatosis grade and distribution with a score=0 (<5%); score=1 (5%-33%); score=2 (>33%-66%) and score=3 (>66%). Location was defined as steatosis distribution with a score=0 (zone 3); score=1 (zone 1); score=2 (azonal); or score=3 (panacinar). Microvesicular steatosis was recorded as either score=0 (not present) or score=1 (present). Lobular inflammation was

semiquantified according to a score=1 (< 2 foci per 200x field); score=2 (2-4 foci per 200x field); or score=3 (>4 foci per 200x field).

### *2. Oil Red O (ORO) Examination*

ORO was performed according to the described protocol (Melhem et al. Nature protocols-2013). Briefly fresh frozen rat liver tissue was embedded into cryomolds and sectioned into 5 µm sections on a cryostat (Leica). Sections were then stained in ORO and semiquantified using image j software (http://rsbweb.nih.gov/ij). Tissue sections were imaged at five high-power (400x) fields and converted to 8-bit grayscale images; this was followed by an image threshold predefined according to a rat liver section negative for steatosis and microvesicular steatosis on H&E and ORO staining. Subsequently image analysis for ORO surface area staining was determined.

## H. Statistical Analysis

The data were analyzed using the statistical package Mini-Tab 13.1 for Windows, and statistical treatment of the data included one way analysis of variance (ANOVA). Specific comparisons were made using Fisher's pairwise comparisons between groups for continuous variables and Tukey Pairwise Comparisons among levels of group for categorical variables. All values are expressed as mean ± SD. A two-sided *P* value <0.05 was considered to be statistically significant.

# CHAPTER IV

# RESULTS

Rats fed on the experimental diets appeared healthy during the feeding period, with no pathological signs or abnormalities.

## A. Body Weight and Weight Gain

Rats were randomly allocated into groups and there was no significant difference in body weight between the different groups at the beginning of the experiment as shown by the initial body weight in Table 4.1. Results for final body weight showed that HF/P-Free and HF/Very Low P diets had significantly lower final body weight than all other groups (P-value<0.001). In addition, the HF/Low P and HF/Normal P had almost the same final body weight and weight gain as the group receiving the control diet. Comparing high-fat groups among each other, we can see that rats fed on HF/P-Free and HF/Very Low P diets had a significantly lower final body weight than the other groups, with the HF/P Free group having the least weight gain among groups and it was significantly different than all other groups (P<0.001). Moreover, as P concentration increases, there was a trend towards increasing final body weight and total weight gain over the experimental period between the high fat groups.

## B. Food Intake and food efficiency

Food intake was measured twice per week and the average food intake per group is presented in Table 4.1. Comparing food intake among all groups; we can see that it was significantly different (P<0.001), with the HF/Normal P group having

significantly the highest food intake among all other groups. It should be mentioned that even though HF/Normal P and Control groups had the same phosphorus concentrations in the diet, however HF/Normal P had significantly higher caloric intake than control group. Moreover, there was a significant difference between all high fat groups ($P<0.001$) with a trend towards increasing food intake over the experimental period between the high fat groups as P concentration increases in the diet.

Food efficiency presented as weight gain (g)/100 calorie was significantly different between all groups. Specifically, it was significantly lower in phosphate restricted groups (HF/ P Free and HF/ very Low P) compared to the 3 other groups (P-value for all groups$<0.001$). Comparing high fat groups alone showed similar results which is significantly lower food efficiency in phosphate restricted groups (HF/ P Free and HF/ very Low P) compared to the 2 other groups (P-value for High fat groups$<0.001$).

**C. Fat mass and fat free mass**

Percentage of fat mass and fat free mass in all groups of rats receiving the experimental diets were similar as shown in Table 4.1. Although HF/P Free group had a lower percentage of fat mass than all other groups, however it did not reach statistical significance.

Table 4.1: Body weight, weight gain and average food intake of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high fat diet with different concentrations of phosphorus (Mean ±SD).

| | Control (n=9) | HF/P Free (n=8) | HF/Very Low P (n=9) | HF/Low P (n=9) | HF/Normal P (n=9) | ANOVA P-value (All groups) | ANOVA P-value (High-fat groups) |
|---|---|---|---|---|---|---|---|
| Initial Body Weight (g) | 241.3±33.6 | 243.2±33.6 | 241.3 ± 29.1 | 249.2 ± 23.9 | 247.9 ±26.8 | 0.966 | 0.927 |
| Final Body Weight (g) | 532.1±72.8 $^{a}$ | 418.6 ±58.5 $^{b(\beta)}$ | 466.9 ± 63.6 $^{b(\beta)}$ | 559.8 ± 85.7 $^{a(\alpha)}$ | 562.9 ± 47.9 $^{a(\alpha)}$ | 0.000 | 0.000 |
| Weight gain (g) | 290.8 ± 48.6 $^{a}$ | 175.4 ± 26.1 $^{c(\omega)}$ | 225.5 ± 44.2 $^{b(\beta)}$ | 310.6 ± 67.1 $^{a(\alpha)}$ | 315.0 ± 35.5 $^{a(\alpha)}$ | 0.000 | 0.000 |
| Food Intake (Calorie/day) | 90.8 ± 8.1 $^{b}$ | 77.9 ± 7.6 $^{c(\omega)}$ | 89.7 ± 8.5 $^{b(\beta)}$ | 95.8 ± 9.6 $^{a,b(\alpha,\beta)}$ | 98.9 ± 8.3 $^{a(\alpha)}$ | 0.000 | 0.000 |
| Weight gain (g) /100 calorie | 5.1 ± 0.6 $^{a}$ | 3.6 ± 0.3 $^{b(\beta)}$ | 3.9 ± 0.5 $^{b(\beta)}$ | 5.1 ± 0.6 $^{a(\alpha)}$ | 5.1 ± 0.3 $^{a(\alpha)}$ | 0.000 | 0.000 |
| Fat mass (%) | 24.5 ± 3.2 | 22.0 ± 5.7 | 24.7 ± 5.6 | 24.6 ± 4.3 | 26.0 ± 2.9 | 0.482 | 0.387 |
| Fat Free Mass (%) | 75.5 ± 3.2 | 78.0 ± 5.7 | 75.3 ± 5.6 | 75.4 ± 4.3 | 74.0 ± 2.9 | 0.482 | 0.387 |

*ANOVA One Way Analysis of Variance (ANOVA) using Fisher Pair Wise comparison*

$^{a,b,c}$ *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P for all groups<0.05).*

$^{\alpha,\beta,\omega}$ *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P for High-fat groups<0.05).*

**D. Kidneys and Epididymal Fat Pads (EDF) weights**

Table 4.2 summarizes kidneys and EDF weights of the different groups.

Kidneys weights did not show any significant difference between the groups. However when kidney weight was calculated as percentage of total body weight, HF/P-Free and HF/Very Low P groups showed significantly higher percentage than other groups (P-value for High fat groups: 0.002, and P-value for all groups: 0.001).

Regarding EDF, rats fed on HF/P-Free and HF/Very Low P diets had a significantly lower EDF weight than the other groups, with the HF/P Free group having the least EDF weight and it was significantly different than all other groups (P-value for all groups: 0.008). Among high fat groups, EDF weight is showing the same trend as final body weight and total weight gain which is as P concentration increases among the high fat groups, EDF weight increases. Moreover, the percentage of EDF from total body weight showed same results as total EDF weights. It should be noted that, even though total weight gain was not significantly different between groups receiving control or HF/normal P diets, EDF weight and EDF (g/100g Body weight) of HF/Normal P group was significantly higher than the control group knowing that both groups received the same concentration of phosphorus.

Table 4.2: Kidneys and Epididymal Fat Pads (EDF) weights of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high fat diet with different concentrations of phosphorus (Mean ±SD).

| | Control (n=9) | HF/P Free (n=8) | HF/Very Low P (n=9) | HF/Low P (n=9) | HF/Normal P (n=9) | ANOVA P-value (All groups) | ANOVA P-value (High-fat groups) |
|---|---|---|---|---|---|---|---|
| Kidney Weight (g) | 3.6 ± 0.5 | 3.3 ± 0.9 | 3.3 ± 0.5 | 3.2 ± 0.4 | 3.4 ± 0.3 | 0.432 | 0.790 |
| Kidney (g/100g body weight) | $0.7 \pm 0.1^{a,b}$ | $0.8 \pm 0.2^{a(\alpha)}$ | $0.8 \pm 0.1^{a(\alpha)}$ | $0.6 \pm 0.1^{c(\beta)}$ | $0.6 \pm 0.02^{b,c(\beta)}$ | 0.001 | 0.002 |
| E.D.F. Weight (g) | $9.7 \pm 3.3^{b,c}$ | $6.5 \pm 2.8^{c(\omega)}$ | $9.3 \pm 4.3^{b,c(\beta,\omega)}$ | $11.1 \pm 4.6$ $^{a,b(\alpha,\beta)}$ | $13.7 \pm 3.9^{a(\alpha)}$ | 0.008 | 0.006 |
| E.D.F. (g/100g Body weight) | $1.9 \pm 0.5^{b,c}$ | $1.5 \pm 0.5^{c(\beta)}$ | $1.99 \pm 0.6$ $^{a,b,c(\alpha,\beta)}$ | $2.1 \pm 0.6$ $^{a,b(\alpha,\beta)}$ | $2.5 \pm 0.5^{a(\alpha)}$ | 0.013 | 0.01 |

*ANOVA One Way Analysis of Variance (ANOVA) using Fisher Pair Wise comparison*

$^{a,b,c}$ *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P( for all groups)<0.05).*

$^{\alpha,\beta,\omega}$ *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P for High-fat groups<0.05)*

**E. Liver weight and % Fat in dry weight of liver**

Although there was a trend towards increasing liver weight between the high fat groups as phosphorus concentration increases, however it did not reach statistical significance. Likewise, liver weight as percentage of total body weight did not show any difference between all the groups when comparing all groups together and high fat groups alone (Table 4.3).

Liver fat accumulation was assessed and results showed a statistically significant difference between all groups (P-value for all groups: 0.001). Percentage of fat in the liver in group receiving the control diet was significantly lower than all high fat groups.

In addition, % Fat in dry weight of liver showed the same trend as final body weight and EDF weight, which is as P concentration increases among the high fat groups, % fat in dry weight of liver increases, however it did not reach statistical significance (P-value for high fat groups: 0.083).

Table 4.3: Liver weight, percentage of dry weight, and percentage of fat in dry weight of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high fat diet with different concentrations of phosphorus (Mean ±SD).

| | Control (n=9) | HF/P Free (n=8) | HF/Very Low P (n=9) | HF/Low P (n=9) | HF/Normal P (n=9) | ANOVA P-value (All groups) | ANOVA P-value (High-fat groups) |
|---|---|---|---|---|---|---|---|
| Liver Weight (g) | 15.9 ± 2.4 | 14.0 ± 2.8 | 15.7 ± 2.7 | 17.9 ± 6.1 | 18.9 ± 4.6 | 0.113 | 0.107 |
| Liver (g/100g body weight) | 3.1 ± 0.2 | 3.4 ± 0.3 | 3.5 ± 0.3 | 3.4 ± 0.7 | 3.5 ± 0.5 | 0.467 | 0.945 |
| % Dry Weight of liver | 30.6 ± 2.1 [c] | 31.6 ± 2.1 [b,c] | 35.3 ± 5.1 [a,b] | 35.6 ± 6.2 [a] | 36.4 ± 2.3[a] | 0.011 | 0.129 |
| % Fat in dry weight of liver | 4.9 ± 3.2 [c] | 10.1 ± 7.5 [b,c] | 19.4 ± 10.7 [a,b] | 19.1 ± 16.1 [a,b] | 25.0 ± 9.2 [a] | 0.001 | 0.083 |

*ANOVA One Way Analysis of Variance (ANOVA) using Fisher Pair Wise comparison*

[a,b,c] *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P( for all groups)<0.05).*

[α,β,ω] *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P for High-fat groups<0.05)*

## F. Plasma analysis

Table 4.4 summarizes average plasma analysis of the different groups.

HDL cholesterol decreased significantly in the high-fat groups compared to the control group. Moreover, between the high fat groups, there was a statistical significance in HDL level between the group receiving normal phosphorus and P-Free diet which had the lowest value for HDL. Figure 4.1 shows plasma HDL concentrations of the different groups.

Total cholesterol was significantly higher in the control group than the high fat groups; though it was not different between the high fat groups. Additionally, LDL cholesterol, triglycerides, and glucose did not show any difference between groups.

Even though plasma insulin concentration did not reach any significant difference, but it did show a trend toward increasing as P concentration increases in the high fat groups.

Regarding plasma phosphorus concentration, the HF/P-Free group showed a significantly lower level than the HF/Very Low P and HF/Low P groups; however, it was similar to the HF/Normal P group.

Table 4.4: Plasma analysis of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high fat diet (Mean±SD).

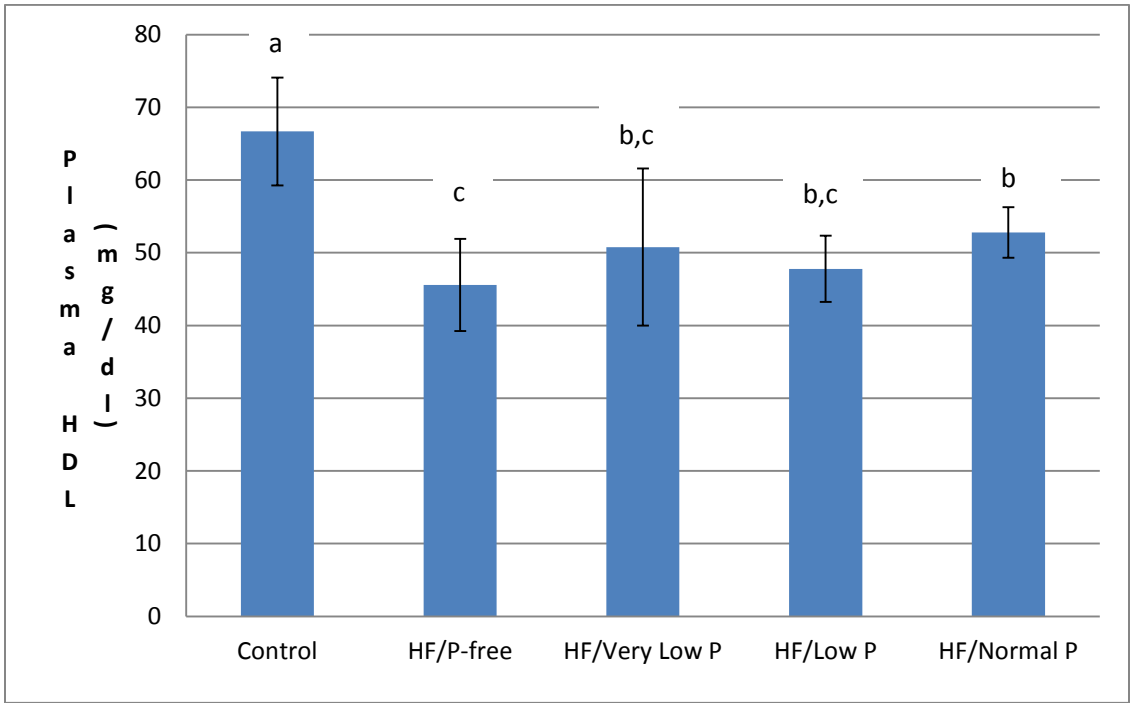
| | Control (n=9) | HF/P Free (n=8) | HF/Very Low P (n=9) | HF/Low P (n=9) | HF/Normal P (n=9) | ANOVA P-value (All groups) | ANOVA P-value (High-fat groups) |
|---|---|---|---|---|---|---|---|
| HDL (mg/dl) | 66.7 ± 7.4 [a] | 45.6 ± 6.4 [c] | 50.8 ± 10.8 [b,c] | 47.8 ± 4.6 [b,c] | 52.8 ± 3.5 [b] | 0.000 | 0.186 |
| LDL (mg/dl) | 40.6 ±15.3 | 33.6 ± 6.2 | 32.0 ± 9.8 | 28.8 ± 8.5 | 31.6 ± 17.9 | 0.359 | 0.864 |
| Triglycerides (mg/dl) | 49.2 ± 18.8 | 40.3 ± 7.7 | 40.4 ± 8.9 | 39.0 ± 9.8 | 60.4 ± 33.1 | 0.104 | 0.066 |
| Total cholesterol (mg/dl) | 117.2 ± 20.5 [a] | 87.3 ± 7.8 [b] | 90.8 ± 14.0 [b] | 84.3 ± 9.8 [b] | 96.6 ± 19.7 [b] | 0.001 | 0.305 |
| Glucose (mg/dl) | 299.3 ± 94.3 | 246.0 ± 107.1 | 254.9 ± 92.9 | 293.1 ± 107.3 | 342.4 ± 91.6 | 0.289 | 0.199 |
| Insulin (µIU/ml) | 5.3 ± 2.4 | 3.8 ± 1.0 | 5.0 ± 1.7 | 5.5 ± 2.6 | 6.7 ± 3.0 | 0.160 | 0.089 |
| Phosphorus (mg/dl) | 7.4 ± 1.3[b,c] | 6.5 ± 0.9 [c (β)] | 7.9 ± 1.2 [a,b(α)] | 8.8 ± 1.2 [a(α)] | 6.6 ± 1.0 [c(β)] | 0.001 | 0.000 |

*ANOVA One Way Analysis of Variance (ANOVA) using Fisher Pair Wise comparison*

[a,b,c] *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P( for all groups)<0.05).*

[α,β,ω] *Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P for High-fat groups<0.05)*

Figure 4.1: HDL concentrations of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high fat diet with different concentrations of phosphorus (Mean ±SD).




*ANOVA One Way Analysis of Variance (ANOVA) using Fisher Pair Wise comparison*
*[a,b,c] Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P( for all groups)<0.05).*

## G. Liver function tests

Liver enzymes Alanine aminotransferase (ALT) and Aspartate Aminotransferase (AST) showed no statistical difference between all groups receiving experimental diets as shown in Table 4.5.

## H. Liver Histology

### *3. Oil Red O (ORO) Examination*

Comparing all groups, Oil-Red-O analysis did not show any significant difference between the groups. Even though HF/P Free group showed lower fat accumulation, however it did not reach statistical significance.

### *4. Histopathologic Examination*

Upon staining liver samples with hematoxylin and eosin for histopathological examination, microvesicular steatosis was present in all groups receiving the high-fat diet; being significantly higher in the HF/Normal P group. In fact, all rats in the HF/Normal P group were presented with microvesicular steatosis, in contrast to all other groups. Macrovesicular steatosis presented as steatosis grade was also present in all groups receiving the high fat diet. However, although the P Free and Very Low P groups had a higher steatosis grade but it did not reach statistical significance. In addition, number of rats presented with steatosis grade of more than 1 were approximately the same in the high fat groups.

Lobular inflammation was also present in all groups; however, there was no statistical difference among them; with the highest number of rats presented with lobular inflammation grade of more than 1 in the HF/normal P group.

Fibrosis, Portal inflammation, Mallorys hyaline, and glycogenated nuclei were not present in all groups of rats subjected to the experimental diets.

Table 4.5: Liver enzymes ALT and AST levels of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high fat diet with different concentrations of phosphorus (Mean ±SD).

| | Control (n=9) | HF/P Free (n=8) | HF/Very Low P (n=9) | HF/Low P (n=9) | HF/Normal P (n=9) | ANOVA P-value |
|---|---|---|---|---|---|---|
| AST (IU/L) | 91.5±54.7 | 89.0 ± 57.8 | 75.0 ± 14.2 | 88.1 ± 34.5 | 95.7 ± 26.9 | 0.869 |
| ALT (IU/L) | 41.0±31.4 | 39.4 ±17.2 | 35.5 ±12.0 | 43.1 ±21.5 | 46.6±18.1 | 0.861 |

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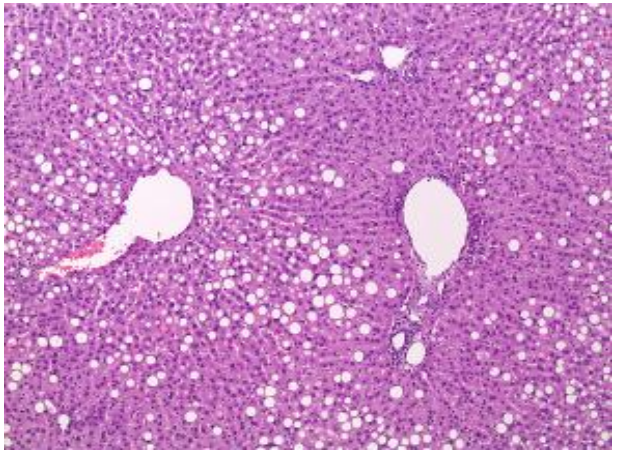
Table 4.6: Liver biopsy results of male Sprague-Dawley rats maintained for 8 weeks either on a control or a high-fat diet with different concentrations of phosphorus (Mean ± SD).

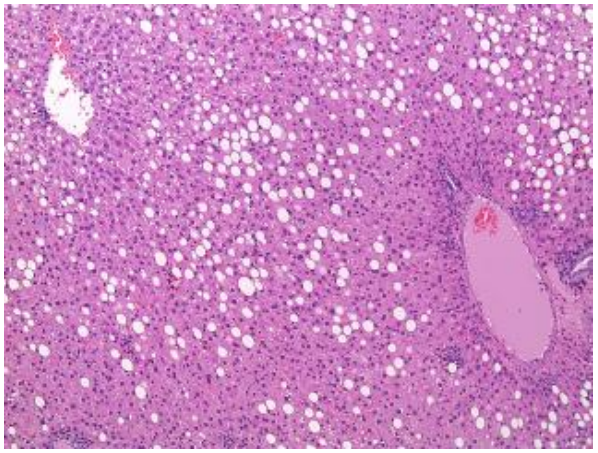
| | Control (n=9) | HF/P Free (n=8) | HF/Very Low P (n=9) | HF/Low P (n=9) | HF/Normal P (n=9) | ANOVA P-value (All groups) | ANOVA P-value (High-fat groups) |
|---|---|---|---|---|---|---|---|
| Steatosis Grade | 0 [b] | 0.88 ± 1.25 [a,b] | 1.56 ± 1.51 [a] | 0.67 ± 1.00 [a,b] | 0.67 ± 0.87 [a,b] | 0.056 | 0.345 |
| Steatosis Grade (1-3) | 0/9 | 3/8 | 5/9 | 4/9 | 4/9 | | |
| Location | 0.11 ± 0.33 | 1.25 ±1.49 | 1.67 ±1.58 | 1.11 ± 1.27 | 1.11 ±1.45 | 0.163 | 0.829 |
| Microvesicular steatosis | 0.22 ±0.44 [b] | 0.63 ±0.52 [a,b] | 0.67 ± 0.5 [a,b] | 0.78 ± 0.44 [a,b] | 1.00 ± 0.0 [a] | 0.008 | 0.259 |
| Microvesicular steatosis (present) | 2/9 | 5/8 | 6/9 | 7/9 | 9/9 | | |
| Lobular inflammation | 0.33 ± 0.50 | 0.75 ± 0.89 | 0.67 ± 0.71 | 0.67 ± 0.87 | 0.89 ± 0.60 | 0.586 | 0.918 |
| Lobular inflammation (1-2) | 3/9 | 4/8 | 5/9 | 4/9 | 7/9 | | |
| Oil-Red-O | 3128 ± 3479 | 1697 ± 793 | 3543 ± 2886 | 4781 ± 3072 | 2753± 2378 | 0.258 | 0.246 |

*ANOVA One Way Analysis of Variance (ANOVA) using Tukey Pair Wise comparison*
*[a,b,c] Values in the same row with different superscripts are significantly different based on Tukey's pair wise comparison (P( for all groups)<0.05).*

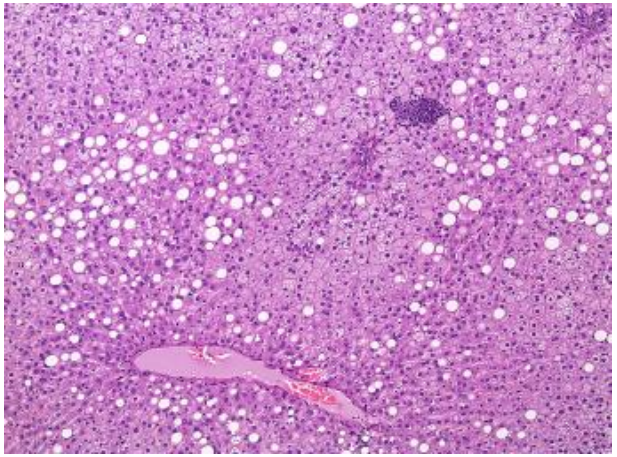
Figure 4.2: Histological examination of the liver with hematoxylin and eosin (H & E) staining in rats maintained for 8 weeks on high-fat diet with different concentrations of phosphorus.



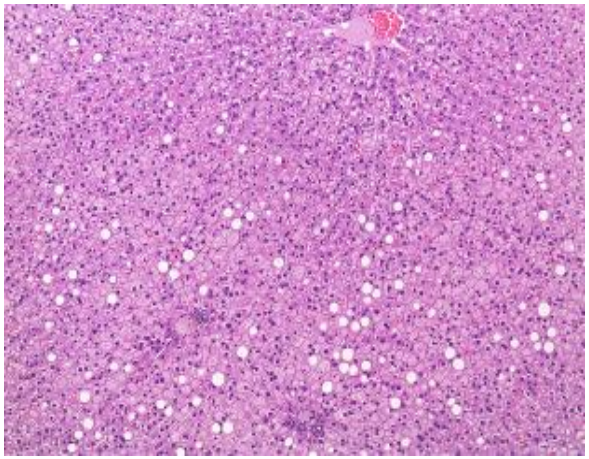
(A) High Fat/ P Free group



(B) High Fat/ Very Low P group



(C) High Fat/ Low P group



(D) High Fat/ Normal P group

# CHAPTER V

# DISCUSSION

The term non-alcoholic fatty liver disease (NAFLD) refers to the range of diseases characterized by fatty infiltration of the liver ranging from benign steatosis, to the more severe steatohepatitis (NASH), or cirrhosis; in the absence of significant ethanol consumption or viral infection (Guturu et al, 2012). Owing to its high prevalence worldwide and potentially serious sequelae, NAFLD is a major public health concern and research efforts are focusing on understanding the underlying pathophysiology of hepatic steatosis with the expectation to identify novel therapeutic targets.

Concern is raised on the relationship between dietary phosphorus and NAFLD in 2 studies mentioned earlier which found that dietary Pi restriction plays an important role in the development of fatty liver disease (Tanaka et al, 2013; and Ellam et al, 2011). We hypothesized that P seems to embody the missing link between metabolic derangements that occur in insulin resistance/metabolic syndrome and NAFLD. Therefore, our objective was to investigate the role of dietary phosphorus on the development and progression of high fat diet induced NAFLD in rats by manipulating the phosphorus concentration of the different groups. To identify the effects of phosphate restriction, egg white was used as the source of protein since it has a very low phosphorus concentration (890 mg P/kg).

In the present study, we mimicked the nutritional rat model developed by Lieber et al (2004) to study the progression of fatty liver disease, since they showed that Sprague-Dawley rats given a high fat diet (71% of energy from fat) reproduces the key features of human NASH.

Landsman et al. (2005) reported that 0.02–0.04% low phosphorus diets decreased food intake and body weight in 5-weeks old male Sprague-Dawley rats, and then phosphorus repletion led to an immediate increment in body weight associated with a similarly rapid effect on food intake. Moreover, Ohnishi et al. (2007) showed that male rats fed either a low phosphorus (LPD: 0.02%) or a normal phosphorus diet (NPD: 0.6%) for 15 days, weight gain in NPD was as twice higher than rats fed a LPD through its direct effect on increasing appetite and thus food intake. Our results were similar to these two studies since rats fed on HF/P-free and HF/very low P diets had a significantly lower food intake and total weight gain than the other groups. In addition, the group receiving the HF/normal P diet had total body weight gain almost as twice higher than the group receiving the HF/P free group.

In addition, EDF which represents whole body adipose tissue is showing the same trend as final body weight and total weight gain which is as P concentration increases among the high fat groups, EDF weight and EDF as percentage of total body weight increases significantly.

Looking at food efficiency clarifies the image, food efficiency (weight gain/100 calories) was significantly lower in phosphate restricted groups (HF/ P Free and HF/ very Low P) compared to the three other groups. Therefore, it could be noticed from these results that upon feeding male Sprague-Dawley rats phosphate restricted

diets (0.0178% and 0.075% P), rats showed significant weight loss, low intake, and low energy efficiency than rats fed a P sufficient diet. There could be more than one reason for these observations. Phosphorus is needed in ATP production, nucleic acid synthesis, enzyme activity, phosphorylation of many proteins (Berndt and Kumar, 2008); therefore, P restriction might lead to abnormal functions in multiple organ sysytems. In fact, chronic hypophosphatemia has been linked to abnormal growth in children (Felsenfeld and Levine, 2012).

Accumulation of fat in the liver, which is the first hit in the development of NAFLD, measured by hepatic fat extraction and presented as percentage of fat in dry weight of liver are in parallel with results about total weight gain and EDF. In the present study, fat accumulation followed a tendency that is more parallel to the amount of phosphorus in the diet; the higher the phosphorus level, the more percentage of fat in the liver, being significantly highest in the HF/ Normal P group. There could be more than one reason for this observation. As we mentioned earlier, hepatic lipid accumulation results from an imbalance between lipid availability (from circulating lipid uptake or de novo lipogenesis) and lipid disposal (via free fatty acid oxidation or triglyceride-rich lipoprotein secretion) and eventually triggers lipoperoxidative stress and hepatic injury (Musso et al, 2009).

Lipogenesis is activated by high ATP concentration in the cell (Champe, 1994). However, hypophosphatemia results in adenosine triphosphate (ATP) depletion (Felsenfeld and Levine, 2012). Therefore, as P concentration increases in the diet, availability of P for ATP generation increases and hence lipogenesis increases secondary to high fat intake. Indeed, lipogenesis is a highly costly process for the cells.

ATP which is the energy carrier for all cells is one of the main determinant and controller of several biological reactions, including lipogenesis. Fatty acid synthesis starts with Acetyl-CoA being transported from the mitochondrion to the cytosol as citrate, and then transformed again to cytosolic Acetyl-CoA by citrate lyase which requires ATP. Subsequent reactions also involve ATP and other energy carrier compounds such as NADPH (Champe, 1994). Moreover, it has been proven that as the level of phosphorus in the diet increases, rates of lipogenesis increases as well (Foufelle et al, 1992).

Another explanation for the increased accumulation of fat in the liver in the high fat groups is due to decreased hepatic VLDL-TG export since its known that hepatic VLDL-TG export in NAFLD is inadequate to normalize hepatic TG content (Cusi, 2012). Indeed, ApoB-100 synthesis, a rate-determining step in hepatic lipid export, is markedly attenuated in patients with NAFLD which is likely to contribute to the net retention of lipids in the liver predisposing to NASH (Charlton et al., 2002).

Plasma analysis showed that upon feeding rats a high fat diet for 8 weeks, HDL cholesterol significantly decreased compared to the control group. Premature HDL is produced by the liver, becomes mature HDL in the circulation, and serves a central role in reverse cholesterol transport (Jiang et al., 2013). In fact, it has been proven that Low HDL is associated with insulin resistance, and NAFLD (Park et al., 2004). Moreover, the group receiving the P-Free diet had the lowest value for HDL between the high fat groups even though it did not reach statistical significance.

Therefore these results indicate that group receiving the P-Free diet should show a more advanced stage of NAFLD.

The characteristic lipid profile of subjects with NAFLD and NASH includes high triacylglycerol, low HDL, and increased small, dense LDL (Koruk et al., 2003). As we mentioned, HDL levels decreased in the high fat groups; however triglycerides, total cholesterol, and LDL cholesterol didn't decrease or show any significant difference between the groups. This could be explained by the fact that when VLDL secretion is inhibited into serum in NAFLD, accumulation of TG is inhibited and thus lead to a non significance increase (Foufelle et al, 2002). De Castro et al. (2013) which had similar findings, showed that a high-fat diet was not effective in increasing serum triglycerides in both young and adult Sprague-Dawley rats. The authors defended their results by saying that the synthesis of VLDL could be reduced in hepatocytes, since excess TG consumed can be directed to an oxidative route to form ketones and carbon dioxide. Moreover, another possible explanation is that the high-fat diet has provided low amount of carbohydrates for synthesis of triglycerides and/or the high concentration of saturated fat in the high fat diet could have reduced the hepatic secretion of VLDLs (De Castro et al., 2013).

Regarding plasma phosphorus concentration, the HF/P-Free group showed a significantly lower level than the HF/Very Low P and HF/Low P groups. These results are in accordance with results from Jara et al which showed that growing rats given a low-phosphate diet (Phosphate less than 0.05%), even though all other dietary

constituents remain normal, rapidly produces hypophosphatemia, and urine virtually free of phosphorus (Jara et al, 1999). Upon subjecting animals on a low Pi diet, serum Pi concentrations decrease followed by increase in circulating calcium concentrations, resulting in inhibited PTH release and reduced excretion of Pi by the kidney. An increase in the serum concentrations of 1,25- dihydroxyvitamin D3 (which occurs despite a reduction in PTH) increases the absorption of Pi in the intestine, and also increases renal Pi reabsorption in the proximal tubule (Sommer et al, 2007). This might explain the higher levels of P in the plasma in the HF/Very Low P and HF/Low P groups compared to the normal P group. In the normal P group there is no need for increased P reabsorption to compensate for the deficient dietary intake; therefore since phosphorus is acutely affected by the fed state and plasma analysis was performed while rats were fasting, this might be the reason behind lower P level in the normal P group.

Even though plasma insulin concentrations did not reach any significant difference between the high fat groups (P-value=0.089), but it did show a trend toward decreasing as P concentration decreases in the high fat groups. These results indicate that phosphate restriction has an effect on insulin secretion. Our results are in accordance with results from Castillo et al. (1982) and Zhou et al. (1991) who found impaired insulin secretion during phosphate depletion.

A deficiency in inorganic phosphate can cause severe bone abnormalities and stunted growth since it is known that Pi homeostasis is crucial to bone mineralization and muscle growth (Czarnogorski et al., 2004). Moreover, longstanding hypophosphatemia causes osteomalacia in adults (Tsuboi et al., 2000). Therefore in

vivo body composition analysis was done to determine the effects of different P concentrations on FFM and FM. However, no significant difference was found between the groups maybe due to the increased reabsorption of phosphorus in the P restricted groups.

Our objective was to induce NAFLD in rats by providing them with high fat diet (64.3% by energy from fat). Liver biopsy which is known as the "Gold standard" for diagnosis of hepatic steatosis and steatohepatitis (Oh et al, 2008) was done. Morphologically, depending on the size of the lipid vacuoles, hepatic steatosis can be classified as macrovesicular or microvesicular. Different hypothesis have been discussed for developing micro- or macrovesicular steatosis; it is more likely that microvesicular steatosis is related to mitochondrial dysfunction; however, the macrovesicular type may be due to disturbed protein synthesis (Stoppler et al., 2013).

Results showed that upon staining liver samples with hematoxylin and eosin for histopathological examination, microvesicular steatosis, macrovesicular steatosis presented as steatosis grade, and lobular inflammation were present in all groups receiving the high-fat diet.

Even though NAFLD was present, however contrary to the observation of NASH after 3 weeks of feeding Sprague-Dawley rats a high fat diet in Lieber et al (2013) rat model, our results showed only microvesicular hepatosteatosis with low grade macrosteatosis and lobular inflammation not reaching a score of 1after 8 weeks in rats fed a high fat diet. In addition, fibrosis, portal inflammation, mallorys hyaline, and

glycogenated nuclei were not present in all groups of rats subjected to the experimental diets. Therefore, we were unable to show any significant difference between fat groups, and hence effect of phosphate manipulation on NAFLD could not be made. Umbreen et al (2009) who also mimicked the nutritional model developed by Lieber et al to study the progression of fatty liver disease, found that extending the feeding period of the high fat diet for 5 weeks placed their rats with Type I to II NAFLD compared to the more advanced Type III state previously obtained after 3 weeks feeding by Lieber et al. They defended their results by saying that the milder condition obtained from their study was due to genetic modifiers found in their rats that resist disease progression. Our results do not contradict with the last study mentioned, therefore we can suppose that our rats might also have adapted to the high fat diet and resisted NAFLD progression.

De Castro et al. (2013) gave rats a high fat diet containing 370g/kg lard which is even more than we gave our experimental rats which (300g/kg lard). In young rats, the high fat diet induced NAFLD after 3 weeks. However, micro and macrosteatosis were not present in all rats and none developed tissue inflammation or fibrosis which does not contradict our results.

Another possible explanation is that although liver biopsy is known as the "Gold standard" for diagnosis of NAFLD, nevertheless, there are limitations to scoring systems based on liver biopsy. Paired biopsies have been evaluated in several published studies which concluded that there is a significant sampling variability and that the histological lesions of NASH are unevenly distributed throughout the liver parenchyma, hence leading to substantial misdiagnosis and staging inaccuracies (Oh et al., 2008).

# CHAPTER VI

# CONCLUSIONS AND RECOMMENDATIONS

Our main objective of this study was to investigate the role of dietary phosphorus on the development and progression of NAFLD in rats. Male Sprague-Dawley rats were given a high fat diet to induce NAFLD; at the same time rats were given varying concentrations of phosphorus to manipulate P concentrations.

Our results showed that upon feeding male Sprague-Dawley rats phosphate restricted diets (0.0178% and 0.075% P); rats had significant weight loss, low intake, and low energy efficiency than rats fed a P sufficient diet. In addition, plasma analysis showed that upon feeding rats a high fat diet for 8 weeks, HDL cholesterol significantly decreased compared to the control group.

Based on our results, we can conclude that male Sprague-Dawley rats upon administration of high fat diet tend to induce microvesicular steatosis, and mild macrovesicular steatosis and lobular inflammation. However, effect of P manipulation could not be detected.

A limitation of our study was that Lieber et al. (2013) gave rats a liquid diet with 71% of energy coming from fat. However, our experimental diet was not liquid and it consisted of 64.3% fat. In addition, we can predict that Sprague-Dawley rats might also have adapted to the high fat diet and resisted NAFLD progression.

In the future, more investigations in this area of research might be able to draw more on the relation between phosphorus and NAFLD especially that dietary Pi

restriction with a high cholesterol diet was found to play an important role in the development of fatty liver disease (Tanaka et al, 2013). Therefore, we recommend in the future giving rats a high cholesterol diet with P manipulation to induce NAFLD.

# APPENDIX 1

# MINERAL MIX

The mineral mix used is the AIN-93G mixture purchased from Dyets inc. Experimental diets & Ingredients for Laboratory Animals used at 35 g/kg diet. The mixture is composed of the following:

| Ingredients | g/kg mixture |
|---|---|
| Calcium Carbonate | 357 |
| Potassium Phosphate (monobasic) | 196 |
| Potassium citrate $H_2O$ | 70.78 |
| Sodium Chloride | 74 |
| Potassium Sulphate | 46.6 |
| Magnesium Oxide | 24 |
| Ferric Citrate, U.S.P | 6.06 |
| Zinc Carbonate | 1.65 |
| Manganous Carbonate | 0.63 |
| Cupric Carbonate | 0.3 |
| Potassium Iodate | 0.01 |
| Sodium Selenate | 0.01025 |
| Ammonium Paramolybdate 4 $H_2O$ | 0.00795 |
| Sodium Metasilicate 9 $H_2O$ | 1.45 |
| Chromium Potassium Sulfate 12 $H_2O$ | 0.275 |

# APPENDIX 2

## P-FREE MINERAL MIX

Phosphorous free mineral mix used is Phosphorous Deficient AIN-76 Salt Mix (Used at 35 g/Kg diet) from Dyets. Inc. Experimental Diets & Ingredients for Laboratory Animals.
The mixture is composed of the following:

| Ingredients | g/kg mixture |
|---|---|
| Calcium Carbonate | 368 |
| Sodium Chloride | 74 |
| Potassium Citrate, monohydrate | 220 |
| Potassium Sulfate | 52 |
| Magnesium Oxide | 24 |
| Manganous Carbonate | 3.5 |
| Ferric Citrate | 6 |
| Zinc Carbonate | 1.6 |
| Cupric Carbonate | 0.3 |
| Potassium Iodate | 0.01 |
| Sodium Selenite | 0.01 |
| Chromium Potassium Sulfate | 0.55 |
| Sucrose, finely powdered | 250 |

# APPENDIX 3

# VITAMIN MIX

The vitamin mix used is the AIN-76A mixture purchased from Dyets inc. Experimental diets & Ingredients for Laboratory Animals.
The mixture is composed of the following

| Vitamin | g/kg mixture |
|---|---|
| Thiamin HCl | 0.60 |
| Riboflavin | 0.60 |
| Pyridoxine HCl | 0.70 |
| Niacin | 3.00 |
| Calcium Pantothenate | 1.60 |
| Folic acid | 0.20 |
| Biotin | 0.02 |
| Vitamin $B_{12}$ (0.1%) | 1.00 |
| Vitamin A palmitate (500,000 IU/g) | 0.80 |
| vitamin $D_3$ (400,000 IU/g) | 0.25 |
| Vitamin E acetate (500 IU/g) | 10.00 |
| Menadione Sodium Bisulfite | 0.08 |
| Sucrose finely powdered | 981.1. |

# APPENDIX 4

# SPRAY DRIED EGG WHITE

Composition of the spray-dried egg white.

Typical Inspection Data

GENERAL*

| | | | |
|---|---|---|---|
| Protein | 82.4% | Moisture | 8.54% |
| Fat | 0.0% | Carbohydrates | 4.50% |
| Ash | 4.6% | Cholesterol | 0.00% |
| Food Energy | kcal/gram | 3.76 | |
| | kJ/gram | 15.73 | |

AMINO ACID COMPOSITION(Grams/100 grams Protein)*

| | | | |
|---|---|---|---|
| Arginine | 5.7 | Alanine | 6.2 |
| Aspartic Acid | 8.6 | Cystine | 2.4 |
| Glutamic Acid | 13.5 | Glycine | 3.6 |
| Histidine | 2.2 | Isoleucine | 5.9 |
| Leucine | 8.4 | Lysine | 6.0 |
| Methionine | 3.8 | Proline | 3.6 |
| Phenylalanine | 6.1 | Serine | 7.1 |
| Threonine | 4.3 | Tyrosine | 3.9 |
| Tryptophan | 1.5 | Valine | 7.2 |

VITAMIN CONTENT(mg./Kg.)

| | | | |
|---|---|---|---|
| Niacin | 7.2* | Pantothenic Acid | 19.60* |
| Vitamin B-6 | 0.2* | Riboflavin | 23.20* |
| Thiamin | 0.4* | B-12 | 0.005* |
| Vitamin D | 0.0** | Vitamin A | 0.0* |
| Biotin | 0.57** | Vitamin C | 0.0* |

MINERAL CONTENT(mg./Kg.)

| | | | |
|---|---|---|---|
| Calcium | 890* | Zinc | 1.6* |
| Phosphorus | 890* | Iron | 2.4* |
| Sodium | 12380* | Copper | 3.2** |
| Potassium | 11160* | Iodine | 0.05** |
| Sulfur | 17020* | Flourine | 1.6** |
| Chlorine | 10570** | Magnesium | 720* |

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