AMERICAN UNIVERSITY OF BEIRUT

ACUTE EFFECT OF PHOSPHORUS ON POSTPRANDIAL PARTITIONING OF ENERGY

by
YARA SAMIR BEYH

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Nutrition and Food Sciences
of the Faculty of Agriculture and Food Sciences
at the American University of Beirut

Beirut, Lebanon
April 2017

AMERICAN UNIVERSITY OF BEIRUT

ACUTE EFFECT OF PHOSPHORUS ON POSTPRANDIAL PARTITIONING OF ENERGY

by

YARA SAMIR BEYH

Approved by:

Dr. Omar Obeid, Professor
Nutrition and Food Sciences — Advisor

Dr. Imad Toufeili, Professor
Nutrition and Food Sciences — Member of Committee

Dr. Samer Kharroubi, Associate Professor
Nutrition and Food Sciences — Member of Committee

Dr. Hala Ghattas, Assistant Professor
Epidemiology and Population Health — Member of Committee

Date of thesis defense: April 19, 2017

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: BEYH (Last) YARA (First) SAMIR (Middle)

☑ Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☑ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes
after : **One ---- year from the date of submission of my thesis, dissertation, or project.**
Two ---- years from the date of submission of my thesis, dissertation, or project.
Three ---- years from the date of submission of my thesis, dissertation, or project.

Signature 24-04-2017
Date

ACKNOWLEDGMENTS

Throughout the work on my thesis project, I have had help and support from a number of people to whom I owe gratitude and respect for sharing their knowledge and time.

First of all, I owe my deepest gratitude to my advisor Dr. Omar Obeid, from whom I learnt a lot and who guided me throughout the experimental procedures and writing of my thesis.

I would also like to thank every member of my committee, Dr. Imad Toufeili, for being a father figure throughout the whole journey; Dr. Hala Ghattass, for being the light of hope; and finally Dr. Samer Kharroubi, for his unconditional moral and professional support. It has been a pleasure being guided by a group of leading figures in the world of research.

I owe a special and enormous gratitude to my colleagues and true friends Carla El Mallah and Marie-Ellizabeth Ragi for their constant help and support.

And finally, I would like to share my utmost gratitude to the wonderful parents I have, for their unconditional love, faith in me, and their support and encouragement at every turn point throughout the bitter and sweet times.

AN ABSTRACT OF THE THESIS OF

Yara Samir Beyh for Master of Science
Major: Nutrition

Title: Acute Effect of Phosphorus on Postprandial Partitioning of Energy

Lately, modernization and changes in dietary habits including the decreased phosphorus intake are increasing obesity rates. It is known that phosphorus provides ATP with its phosphate, making it vital for energy production, thus, the importance of its employment in carbohydrates metabolism for energy production. Previous studies have shown that hypophosphatemia reduces ATP production and in turn leads to a reduction in protein and glycogen synthesis and impaired fat oxidation. However, the acute effect of phosphorus manipulation of meal on *in vivo* postprandial glycogen has never been studied. The aim of this study is to investigate the acute effect of phosphorus on glycogen synthesis after the ingestion of a meal.

After receiving the approval of the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut, forty five male Sprague-Dawley rats, of around 200 g, were randomly divided into five groups and kept under monitoring for 2 weeks: control group (water), two groups sacrificed after 2 hours from food ingestion (with and without phosphorus) [(2-p) & (2+P)] and two groups sacrificed after 4 hours from food ingestion (with and without phosphorus) [(4-p) & (4+P)]. In vivo glycogen synthesis was measured using labeled $^{13}C_1$ glucose, by determining its distribution on the different carbons of the glucose molecules.

Total glycogen production changed according to time and on phosphorus intake, with the maximal production being in the group (4+P), and the most preferred route for glycogen synthesis was the direct pathway. However, these observations were influenced by other factors, mainly hormonal factors, including insulin, ghrelin and GLP-1.

In conclusion, phosphorus did show an influence on the amount of glycogen synthesized in the different experimental groups, as well as in the pathway preferred for the production, leaving us the possibility to further investigate the results and expand it to other fuels, such as protein and fat.

CONTENT

ILLUSTRATIONS

Figure

TABLES

ABBREVIATIONS

%	Per Cent
&	And
/	Per
<	Less Than
=	Equal To
>	Greater Than
±	Plus or Minus
X	Multiply
°C	Degrees Celsius
α	Alpha
2,3-DPG	2,3-diphosphoglycerate
2-P	2 hours without phosphorus
2+P	2 hours with phosphorus
3C	Three Carbons
4-P	4 hours without phosphorus
4+P	4 hours with phosphorus
ATP	Adenosine Tri-Phosphate
ADP	Adenosine Di-Phosphate
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate Protein Kinase
AUB	American University of Beirut
CAC	Citric Acid Cycle
cAMP	Cyclic Adenosine Monophosphate
CHO	Carbohydrates

CRSL	Central Research Science Laboratory
D2O	Deuterium Oxide
dL	Deciliter
E	Energy
EC	Extracellular
ECF	Extracellular Fluid
EDTA	Ethylenediaminetetraacetic Acid
FFA	Free Fatty Acids
FGF-7	Fibroblast Growth Factor-7
FGF-23	Fibroblast Growth Factor-23
g	Grams
G3P	Glyceraldehyde-3-Phosphate
G6P	Glucose-6-Phosphate
G6Pase	Glucose-6-Phosphatase
HDL	High Density Lipoprotein
HFCS	High Fructose Corn Syrup
Hr	Hour
IACUC	Institutional Animal Care and Use Committee
IC	Intracellular
IGT	Impaired Glucose Tolerance
IMP	Inosine Monophosphate
Kcal	Kilocalorie
Kg	Kilogram
KH2PO4	Monopotassium Phosphate
KOH	Potassium Hydroxide
L	Liter

M	Molar
MEPE	Matrix Extracellular Phosphoglycoprotein
Mg	Milligram
mL	Milliliter
mmol	Millimol
MPE	Molar Percent Excess
MW	Molecular Weight
N	Normal
NAD	Nicotinamide Adenine Dinucleotide
Nb	Number
ng	Nanogram
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OTC	Over the counter
P	Phosphorus
Pi	Inorganic Phosphate
PI	Phosphorus Intake
PCA	Perchloric Acid
PEP	Phosphoenolpyruvate
pM	Picomol
PTH	Parathyroid Hormone
RBC	Red Blood Cell
RDA	Recommended Dietary Allowance
ROS	Reactive Oxygen Species
SD	Standard Deviation
sFRP-4	Secreted Frizzled Related Protein-4

TG	Triglycerides
UL	Upper Limit
Wt	Weight

CHAPTER I
INTRODUCTION

Nowadays, with the increasing rise of obesity and non-communicable diseases including metabolic syndrome, diabetes, cardiovascular diseases, among others, there is an increased interest in understanding and developing new protective measures (Obeid et al., 2010; Obeid et al., 2014; Bassil & Obeid, 2016). One of the first steps in this milestone would be to understand energy partitioning after the ingestion of a meal. Energy or fuel portioning serves as the steady supply of energy yielding substrates to meet the needs of the body throughout the day (Friedman, 1998). Moreover, food intake is influenced by the source and the pathway of metabolizing and synthesizing the nutrients (Friedman, 1998).

One of the most important fuels that are utilized in the body, are carbohydrates. Their partitioning is not as clear as that of fat, since they can be stored as glycogen, stored as fat, or oxidized (Friedman, 1998). In addition to the type of carbohydrate ingested, such as fructose, complex carbohydrate, other types of simple carbohydrates, resistant starch, or other, since they differ in their metabolic process.

Moreover, there are hormones that influence the metabolism of carbohydrate along the way, including GLP-1, ghrelin and insulin. In fact, ghrelin plays a role in the initiation and termination of food intake (Kojima & Kangawa, 2005), while GLP-1 and insulin act in the process of glycogen production and utilization (Bouskila et al., 2008; Dent et al., 1990; Luque et al., 2002).

Furthermore, phosphorus has been shown to participate and influence the rate of food intake, activity of hormones in the processes, and weight gain (Obeid et al., 2014; Schmid et al., 2011; Bassil & Obeid, 2016; Fritsch et al., 2015).

Therefore, this study has been conducted to investigate the effect of phosphorus on hormonal activities and their implications on glycogen synthesis as part of the energy partitioning process after food ingestion.

CHAPTER II
LITTERATURE REVIEW

A- Phosphorus Overview

1. Distribution in the Body

Phosphorus (P) is an essential multivalent non-metal nutrient, mostly found in its bound phosphate form (PO_4) in nature, rather than in its free form (Kalantar et al., 2010). In the human body, P constitutes 1 to 1.4% of lean body mass and 0.65 and 1.1% of the total body weight, which amounts to 700 g in adults (Aloai et al. 1985; Institute of Medicine, 1997; Heany, 2012). The latter are not proportionally distributed since the majority (85%) are found bound to calcium in the skeleton (bones and teeth) as hydroxyapatite, while the remaining 15% are unevenly distributed between the soft tissues (14%) and the extracellular fluids (ECF) (1%) (Farrow & White, 2010; Gattineni & Baum, 2012; Obeid, 2013c).

The share of P found in the ECF appears in several states, mostly in the form of free ions such as $H2PO_4^-$, HPO_4^{2-} and PO_4^{3-} (Levi & Popovtzer, 1999), where those 85% are constantly regulated to the narrow range of 2.5-4.5 mg/dL (Shills et al., 2005; Amanzadeh & Reilly, 2006; Takeda et al., 2012). Whereas, about 10% are found bound to proteins such as albumin and the remaining 5% are chelated with other compounds namely calcium, magnesium and sodium (Yano & Sugimoto, 2009). Despite the small fraction of the inorganic form (P_i), it is of great importance to the human body and it reflects the status of

P stores (Takeda et al., 2012). However, in the intracellular (IC) space, P is a part of organic compounds such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine phosphate, and nicotinamide adenine dinucleotide (NAD), thus its involvement in energy (E) production and nutrients metabolism pathways (Takeda et al., 2012).

2. Functions in the Body

As mentioned earlier, P is a part of organic compounds involved in E production and metabolism in the human body, in addition to its circulation in the ECF. Therefore, P has vital roles both on the EC and IC levels. It is involved in the mineralization of bones and teeth, cell structure (i.e. phospholipids in cell membranes, nucleotides, phosphoproteins), urinary buffer through maintaining the acid-base balance (McDowell, 2003; Amanzadeh & Reilly, 2006; Gattineni & Baum, 2012), oxygen carrying and releasing capacity of hemoglobin since it is a part of the 2,3-diphosphoglycerate (2,3-DPG) (Gattineni & Baum, 2012), and functioning of muscles and nervous system through the phosphorylation of intermediates involved in cellular signaling (Kimutai et al., 2009). Additionally, P is involved in the E and metabolic pathways by being integrated in ATP, as well as being a major constituent of key enzymes implicated in glycogenesis, glycolysis, gluconeogenesis, and oxidative phosphorylation of major nutrients, mainly carbohydrates (CHO) (Ditzel & Lervang, 2009; Takeda et al., 2012).

3. Requirements & Intake

Nowadays, the average dietary intake of P is between 0.8 and 1.5 g/day (Amanzadeh & Reilly, 2006; Gattineni & Baum, 2012) compared to the daily intake of our ancestors, which used to reach 2.5 g/d (Food & Nutrition Board, 1997; Gattineni & Baum, 2012). This reduction, almost by half, is mainly caused by the change in the dietary habits, and the increased intake and availability of poor sources of P such as sugars, oils, high fructose corn syrup (HFCS), and sweeteners, in addition to the processed and refined grains, constituting about 50% of the daily food intake (Obeid, 2013). Nevertheless, based on the Food & Nutrition Board of the Institute of Medicine, the intake in both periods still fall between the recommended dietary allowance (RDA), which is 0.7 g/d for both females and males adults above the age of 19, and below the upper tolerable limit (UL), which is 4 g/d for adults between the ages of 19 and 70 years and 3 g/d for older adults (>70 years) (Food & Nutrition Board, 1997; Bergman et al., 2009; Takeda et al., 2014).

The normal intake of P is due to its wide availability from different sources, which could be divided in two main categories. The first one is the natural organic form found in protein rich foods like most animal products (milk, meat, fish, poultry…), which has a fair absorption level ranging between 40-60% (Kalantar et al., 2010). It is also found in cereals, grains and seeds like the unrefined whole grains (whole wheat flour, brown rice…); however, the absorption rate is very poor due to its availability in the phytate form, constituting 60-90% of total available P (Uribarri, 2007). The reason behind the poor absorption of P coming from phytate is caused by the lack of the enzyme phytase in the human body, which is required for the hydrolysis and absorption of P (Kumar et al., 2010;

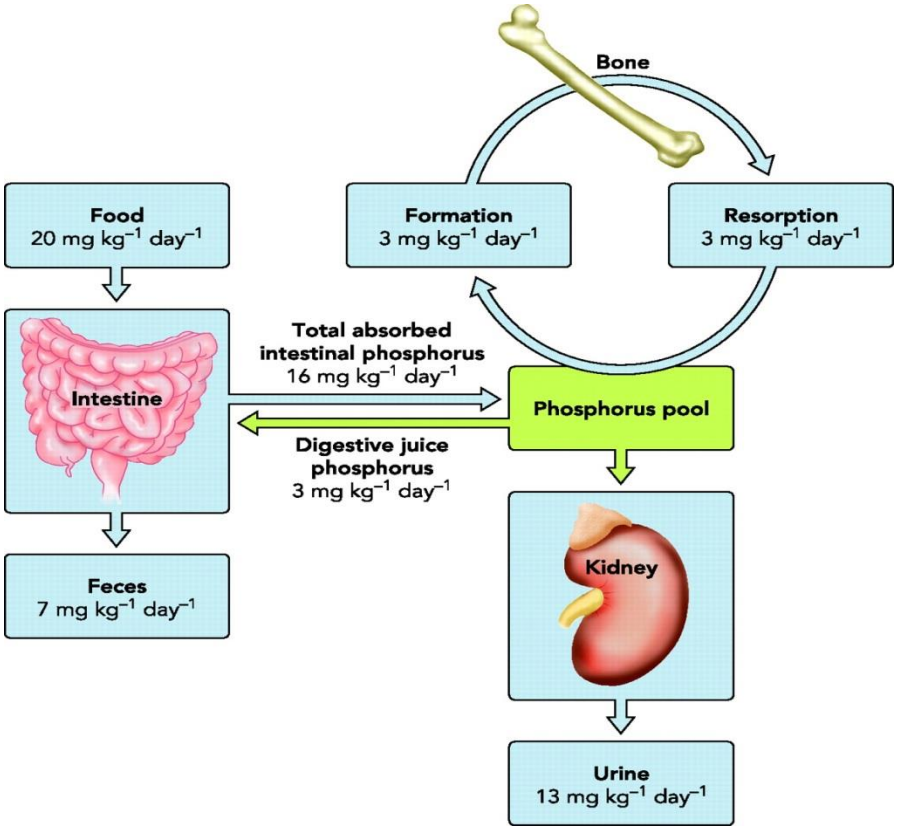
Heany, 2012). However, the bacteria and yeasts have the capacity of naturally producing some phytase, thus rendering the colonic bacteria to release some absorbable P (Heany, 2012). Refined grains and cereals have a lower phytate level than unrefined products, however, the process of milling, extraction and fermentation leads to losses in P up to 70%, as well as losses of other nutrients (calcium, magnesium, potassium) (Calvo & Uribarri, 2013; Obeid, 2013; Obeid & Hachem, 2014). The second source of P intake is in the inorganic form, mainly coming from supplements, some over the counter (OTC) drugs, water, enriched/fortified foods, and food additives (sodas, processed meats, juices, sports drinks…) (Murphy-Gutekunst, 2007; Calvo & Uribarri, 2013), which contribute to a significant and uncontrolled source of P, with high absorption rates (~90%) (Kalantar et al., 2010).

B- Phosphorus Homeostasis

P anion is tightly controlled in the human body in order to keep its serum levels in the appropriate ranges, 2.5-4.5 mg/dL or 0.8-1.5 mmol/L (Shills et al., 2005; Amanzadeh & Reilly, 2006; Takeda et al., 2012). This process is accomplished through a juggling between several factors including organs and regulatory compounds, influencing its absorption, release and excretion (**Figure 1**). The two most important regulatory organs are the intestines and kidneys (Irving, 1973), where the intestines regulate the absorption at a rate of 16 mg/Kg/d, and the kidneys are responsible for the reabsorption of 80% of the absorbed P (13 mg/Kg/d) (Uribarri, 2007; Berndt & Kumar, 2009; Weinman & Lederer, 2012).

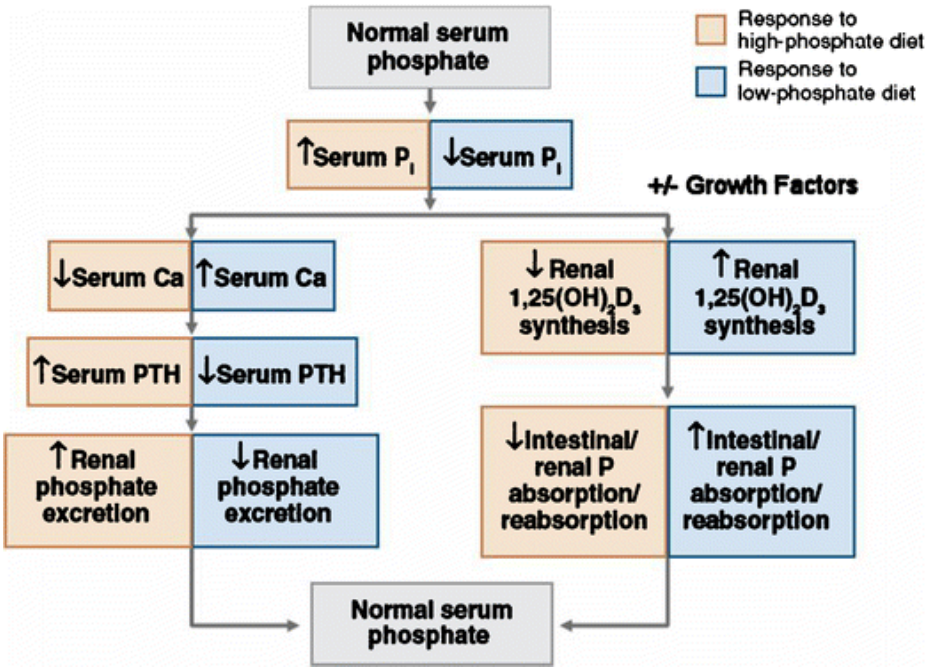
Additionally, the bones contribute to the regulation of 3 mg/Kg/d in the process of their resorption and formation (Berndt & Kumar, 2009). However, the regulation accomplished by these organs is supported by important regulatory compounds including the parathyroid hormone (PTH), calcitriol (Vitamin D), phosphate transporters, and phosphatonins (namely Fibroblast Growth Factor-23 (FGF-23), FGF-7, secreted frizzled related protein-4 (sFRP-4), and matrix extracellular phosphoglycoprotein (MEPE)) (Berndt & Kumar, 2009; Kuro-o, 2010; Goretti et al., 2012; Penido & Alon, 2012) (**Figure 2**). Each of these regulatory factors will be explained independently.

Figure 1: Phosphorus Homeostasis in the Human Body



Berndt, T., & Kumar, R. (2009). Novel mechanisms in the regulation of phosphorus homeostasis. *Physiology*, *24*(1), 17-25.

Figure 2: Mechanisms of Adaptation to Change in Phosphorus Homeostasis



Shaikh, A., Berndt, T., & Kumar, R. (2008). Regulation of phosphate homeostasis by the phosphatonins and other novel mediators. *Pediatric Nephrology*, *23*(8), 1203-1210.

1. Intestinal Absorption

The absorption of P occurs unevenly throughout the length of the small intestine, where 35% takes place in the duodenum, 25% in the jejunum and 40% in the ileum (Noori et al., 2010). This process may occur in two different pathways. The first is the active transcellular pathway, used for small concentrations of P, which necessitates the presence of the sodium dependent phosphate type IIb co-transporter, facilitated by 1,25-dihydroxyvitamin D (Amanzadeh & Reilly, 2006; Kalantar et al., 2010). The second pathway is the concentration-dependent passive diffusion, paracellular transport, used when

there is a high concentration of P in the intestinal lumen, thus higher amounts of P ingested are associated with increased absorption (Amanzadeh & Reilly, 2006; Uribarri, 2007). Nevertheless, intestinal absorption of P is influenced by several factors including dietary intake, bioavailability, gastric acidity, and interaction with other nutrients such as calcium, magnesium and sodium (Kalantar-Zadeh et al., 2010).

2. Renal Absorption

The kidneys are a crucial regulator for irreversible Pi homeostasis, since they are responsible of reabsorbing 80% of the absorbed P (Shils et al., 2006) in two major areas: the glomerulus and the proximal tubules. The process requires the type IIa (Forster et al., 2006) and type III (Murer et al., 2004) sodium-phosphate co-transporters in order to achieve the filtration at the level of the glomerulus and the reabsorption at the level of the proximal tubules (Urer et al., 2004; Forster et al., 2006). Nevertheless, this regulation is directly related to the P status, where it increases with hyperphosphatemia, and decreases with hypophosphatemia. Moreover, some hormones have a stimulatory effect on the process, such as insulin, growth hormone, FGF-23 and thyroid hormone (Murer et al., 2000; Farrow & White, 2010), while others rather exert an excretory effect such as calcitonin, glucagon and glucocorticoids (Murer et al., 2000; Uribari, 2007).

3. Bones

Bone remodeling is another regulator of Pi concentrations in the body, through the indirect actions of PTH and FGF-23, which influence the osteoblastic and osteoclastic activities. PTH stimulates osteoclasts in bones to release P into the EC space, while FGF-23 has protective hypophosphatemic effects on bones against the elevated P levels that could negatively influence the osteoblastic activities (Tiosano & Hochberg, 2009).

4. Hormones

a. Parathyroid Hormone (PTH)

The primary role of PTH is to maintain normal serum calcium levels (Penindo & Alon, 2012) while keeping a balanced serum P concentration (Goretti et al., 2012). In fact, elevated levels of P will stimulate PTH synthesis by the parathyroid gland, which will act on several organs, mainly the kidneys, to inhibit P reabsorption at the proximal tubule level (Forster et al., 2006).

b. Calcitriol ($1,25\text{-}(OH)_2D_3$)

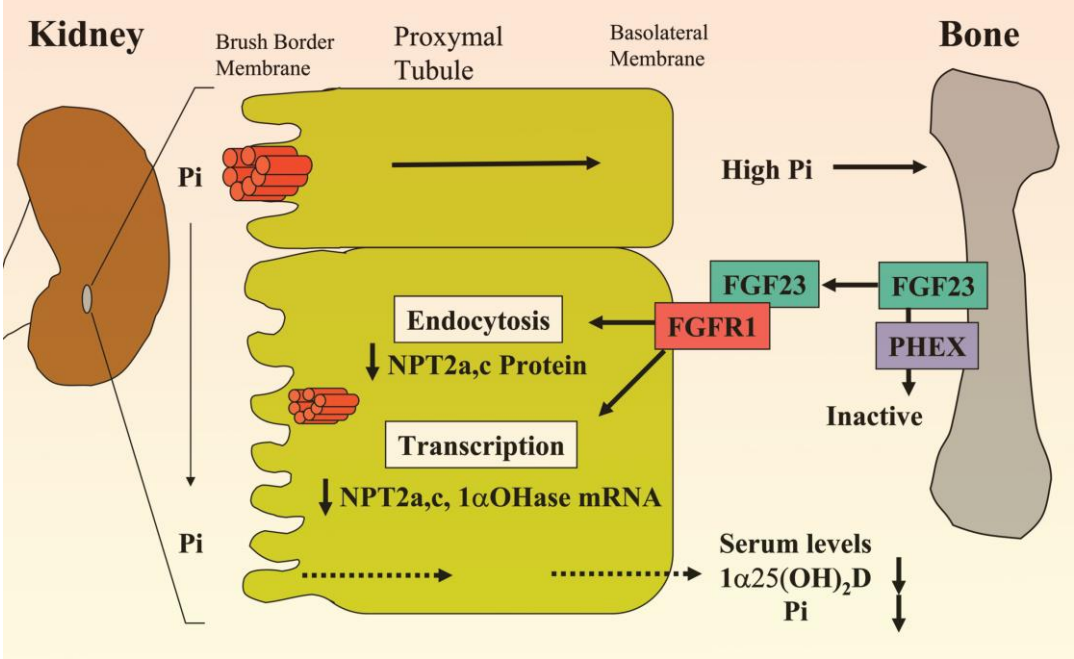
Vitamin D production is induced by PTH since the latter stimulates the production of the enzyme responsible for the 1-α-hydroxylation (Bouillon, 2006). However, the influence of PTH is suppressed by the elevated levels of calcitriol, which provides the activated form of Vitamin D a bi-directional role in P homeostasis (Bergwitz & Juppner,

2010; Goretti et al., 2012). It could either have a direct stimulatory effect on P absorption in the enterocytes at the intestinal level (Bouillon, 2006); or an indirect effect on renal reabsorption through the suppressing effect on PTH (Penindo & Alon, 2012). Nevertheless, calcitriol may still stimulate the release of P into the circulation via the activation of osteoclastic resorption (Penindo & Alon, 2012; Goretti et al., 2012).

c. Phosphatonins

Phosphatonins are a recently discovered family of peptides involved in P regulation, namely: FGF-23, FGF-7, MEPE, and sFRP-4 (Penindo & Alon, 2012). FGF-23 is secreted by bones and it exerts a hypophosphatemic effect through decreasing renal P reabsorption at the level of the proximal tubules by downregulating the expression of sodium-phosphate co-transporters and increasing its excretion (Penindo & Alon, 2012). Moreover, it has an indirect regulatory effect on serum P through reducing its intestinal absorption. The latter is accomplished through enhancing the enzyme 24-α-hydroxylase instead of 1-α-hydroxylase, thus reducing the production of calcitriol or active form of the vitamin D (Berndt et al., 2007; Kuro-o, 2010; Goretti et al., 2012; Penindo & Alon, 2012) (**Figure 3**).

Figure 3: Role of FGF-23 in the Regulation of Phosphorus Homeostasis



Takeda, E., Yamamoto, H., Nashiki, K., Sato, T., Arai, H., & Taketani, Y. (2004). Inorganic phosphate homeostasis and the role of dietary phosphorus. *Journal of cellular and molecular medicine*, *8*(2), 191-200.

C- Health Implications

Numerous studies to date have shown that imbalances in P homeostasis, hypophosphatemia or hyperphosphatemia, lead to systemic disturbances associated with increased risk of metabolic diseases (Tanaka et al., 2013; Calvo & Uribarri, 2013). For instance, high P levels may have detrimental effects on bone health through disrupting the hormonal balance (Henry et al., 1979; Hazim et al., 2014; Obeid et al., 2014), in addition to the development of cardiovascular diseases and endothelial dysfunction through the increased production of reactive oxygen species (ROS) and the inactivation of the endothelial nitric oxides (NO) synthase (Ketteler et al., 2005; Takeda et al., 2012; Calvo &

Uribarri, 2013; Takeda et al., 2014); while hypophosphatemia induces an increased intake of food, especially fatty foods which would increase the risk of developing fatty liver disease, hypertension (Elliott et al., 2008; Ditzel & Levrang, 2010) and obesity (Obeid et al., 2010; Obeid, 2013; Tanaka et al., 2013), further to inducing insulin resistance and impaired glucose tolerance (IGT) which creates a vicious cycle of hyperinsulinemia followed by a further drop in P levels (Kalaitzidis et al., 2005; Khattab et al., 2011), besides its involvement in glycemic control and its indirect correlation with the lipid profile (elevated LDL and TG) (Lippi et al., 2009; Obeid, 2013).

One of the many possible mechanisms that could explain the interrelationship between P and the above mentioned metabolic disturbances may be associated to the production of adenosine triphosphate (ATP), which is dependent upon P availability (Solomon & Kirkby, 1990; Haglin, 2001), and it's implicated in multiple metabolic processes (Levi & Popovtzer, 1999; Takeda et al., 2012).

D- Phosphorus and Energy Production (ATP)

P constitutes an important part of ATP, where the production of the latter depends tremendously on the former; therefore, any impaired P metabolism will lead to an impaired ATP metabolism (Tetens, 2009; Schmid et al., 2011; Li et al., 2015). In fact, Fritsch et al. showed in their study (2015) that ATP production in obese has been stimulated through an increased postprandial Pi increasing the ATP synthase in the liver. Furthermore, ATP production, especially hepatic ATP, depends upon two factors further to adequate P levels.

First, the amounts of free P stored within cells is limited, thus the tissues depend on the ECF P as their metabolic P source, and therefore, if ECF P is low, it will lead to a reduced cellular function. Second, fractional P absorption is constant across a broad level of intake, thus inferring a lack of adaptive response to increase P absorption at low intakes as it is the case with other micronutrients (Obeid, 2013). However, P availability might be compromised by some dietary factors, including high glucose and high fructose foods, which are poor sources of P (Schmid et al., 2011; Obeid et al., 2010; Obeid, 2013). For instance, fructose is known for its "phosphate-sequestering" capacity, leading to the accumulation fructose-1-phosphate (F1P) in the liver due to the absence of a phosphorylation feedback mechanism to halt this process, thus making P unavailable for other essential metabolic reactions including ATP production (Obeid, 2013). Additionally, IC P uptake is influenced by the level of insulin release, where peripheral P uptake has been shown to be inversely related to insulin level by means of a sodium dependent transporter (Schmid et al., 2011; Obeid, 2013). Furthermore, a study by Bassil & Obeid (2016) showed that a state of insulin resistance (IR) may reduce ATP production due to reducing the uptake of substrates required for the process, mainly P and glucose, which could be corrected through supplying P in a meal, hence, stimulating substrates uptake by liver, thus the ulterior increase in ATP production and thermogenesis (Bassil & Obeid, 2016). Therefore, under a high CHO-low P meal, plasma Pi status would decrease due to its stimulated peripheral uptake mediated by the increased insulin release in order to employ it in the phosphorylation of different metabolites uptake by extrahepatic tissues, hence creating a competitive state between ATP production and phosphorylation of other compounds (Obeid et al., 2010; Obeid, 2013).

E- Phosphate and Carbohydrate Metabolism

Throughout several studies conducted over decades, low serum P has been associated with disturbances in nutrient metabolism, namely that of CHO (Bansal, 1990; Haglin, 2001; Baker et al., 2002; Park et al., 2009; Celik & Andiran, 2011; Takeda et al., 2012). To the fact, hypophosphatemia has been initially linked to IGT, and then it was associated with disturbed glucose utilization, to be followed by decreased insulin sensitivity and hyperinsulinemia (Marshall et al., 1978; Haap et al., 2006; Gudmundsdottir et al., 2008).

The relationship between P and glucose metabolism has been linked through several mechanisms, mainly the involvement of P in the phosphorylation step of CHO intermediates in glycolysis and glycogenesis (Jaedig et al., 1993). In fact, Pi is known to stimulate the activity of hexokinase and phosphofructokinase, and serves as a substrate for glyceraldehyde-3-phosphate (G3P) dehydrogenase (Ditzel & Levrang, 2010), in addition to the essentiality of ATP at the level of G3P dehydrogenase and 3 phosphoglycerate kinase in the glycolytic and gluconeogenic pathways (Schmid et al., 2011). Hence, an impaired P level would limit this process (Xie et al., 2000; Haglin, 2001; Kalaitzidis et al., 2005). Furthermore, the chronic hypophosphatemia state has been associated with insulin resistance and consequently secondary hyperinsulinemia (Marshall et al., 1978; Haglin, 2001; Haap et al., 2006). The latter may be explained by the altered cell membrane structure since the phospholipids constituting it are affected in low serum P levels, thus hindering the adherence of insulin to its cell receptors (Davis et al., 1979; Anderson & Moore, 2004), or that a decreased level of P imposes the decrease of ATP production, thus

the decreased insulin level that would lead to IGT (Obeid, 2013). Moreover, a decreased Pi level increases glucose production from gluconeogenesis and glycogenolysis possibly through an upregulation of the glucose-6-phosphatase (G6Pase) gene expression triggered by increased cyclic adenosine monophosphate (cAMP) levels (Xie et al., 2000; Celik & Andiran, 2011).

It has been mentioned earlier that P is involved in glycogenesis, which is the production of glycogen from the available glucose molecules in the post-absorptive state. The latter is solely dependent upon the level of available glucose-6-phosphate (G6P), which in turn depends upon the concentration of glucose in the blood (Wehmeyer et al., 1993). The glycogenic pathway in the body is influenced by the metabolic state, and it could follow either a direct or an indirect pathway (Kunnecke & Seelig, 1991; Wehmeyer et al., 1993). The direct pathway emerges at high blood glucose levels (> 10 mmoL/L), where there is a higher phosphorylation rate of glucose leading to the production of G6P by the increased activity of glucokinase in the liver (Wehmeyer et al., 1993). However, under reduced blood glucose (< 10 mmoL/L), the latter is considered a poor substrate for glycogen production, known as the "glucose paradox" (Kunnecke & Seelig, 1991; Wehmeyer et al., 1993). Hence, an alternative pathway involving a three-carbon (3-C) intermediate will take the lead in glycogen production. This process involves cleavage of glucose to either lactate or pyruvate. In such case, if glucose enters the peripheral tissues or the red blood cells (RBCs), it will be converted to lactate, which will then serve as a gluconeogenic substrate in the liver for glycogen production through its intermediates, oxaloacetate and fumarate, that will generate phosphoenolpyruvate (PEP) that could serve

as a 3-C substrate for indirect glycogen production. Otherwise, pyruvate entering the citric acid cycle (CAC) will generate oxaloacetate, which will be used in gluconeogenesis for glycogen production (Kunnecke & Seelig, 1991; Wehmeyer et al., 1993).

CHAPTER III
MATERIALS AND METHODS

A. Experimental procedure

1. Animal housing

Forty, six week old, male Sprague-Dawley rats weighing between 200-250 grams were used. The rats were housed individually in transparent Plexiglas cages installed in a temperature controlled room at 22±2°C, with the appropriate 12/12 hours of light/dark cycle. The rats had free access to water and they were served an ad libitum control diet for a period of one week, which was considered the adaptation phase.

2. Experimental diet

All rats were fed the same experimental iso-caloric control diet over two weeks. The control diet (**Table 1**) served as the base for the diet provided by gavage (**Table 2**) to the rats on the day of the sacrifice. However, P was excluded from the diet on the experimental day, except for two groups out of the five. The dietary ingredients included egg white powder, corn oil, cornstarch, sucrose, cellulose, P free mineral mix, vitamin mix, and potassium phosphate. Egg white powder was used as the source of protein since it is known to have negligible amounts of P; hence, it provides the essential amino acids without affecting the level of P in the diet (Taylor et al., 2011).

Table 1: Control diet provided to animals over two week period

Ingredients	Diet %(Kcal)	Diet (g/100 g Food)
Egg white (Protein)	20 (80)	22.5
Corn oil (Fat)	10 (90)	5
Corn starch (CHO)	30 (120)	30.75
Sucrose (CHO)	30 (120)	30.75
Cellulose (%)	5.5	-
Mineral mix (%) **	3.5	-
Vitamin mix (%)	1	-
KH2PO4 (P added) (%)	0.27	1.18
Total calories (Kcal)	410	-

*1.5 mg P/ 1 g egg white

**Phosphorus free

MW KH2PO4 = 136 g of which P = 31 g

Table 2: Diet provided to animals on day of sacrifice by gavage

Ingredients	Groups 2 & 4	Groups 3 & 5
Diet % (Kcal)		
Egg white (Protein)	20 (80)	20 (80)
Corn oil (Fat)	10 (90)	10 (90)
Corn starch (CHO)	30 (120)	30 (120)
Sucrose (CHO)	30 (120)	30 (120)
Cellulose	5.5%	5.5%
Mineral mix**	3.5%	3.5%
Vitamin mix	1%	1%
Total calories (Kcal)	410	410
Diet (g/100 g Food)		
Egg white* Of which % P	22.5 0.03%	22.5 0.03
Corn oil	5	5
Corn starch	30.75	30.75
Sucrose	30.75	30.75
KH2PO4 (P added) %	-	0.27
Total P %	-	0.3

*1.5 mg P/ 1 g egg white

**Phosphorus free

3. Experimental Design

This study has been approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (AUB). All forty rats were placed on a one week adaptation period in order to get used to their environment. Then, they were provided with the same diet for an additional week, which was part of the experimental procedure. Following that, the rats were randomly allocated to five experimental groups (**Table 3**), with 8 rats per group, for the day of the sacrifice.

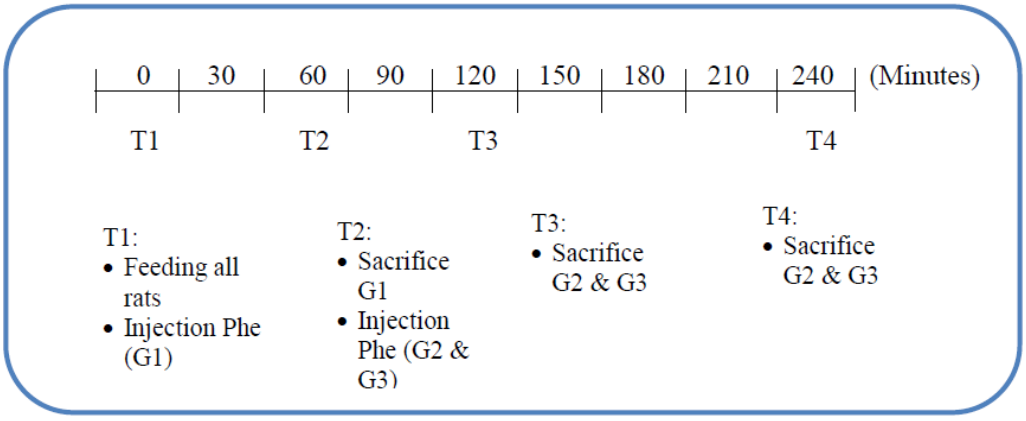
Table 3: The distribution of the five experimental groups

	Group 1 (control)	Group 2 (2-P)	Group 3 (2+P)	Group 4 (4-P)	Group 5 (4+P)
Food (gavage)	3 mL water	1 g	1 g	1 g	1 g
Phosphorus	NO	NO	YES	NO	YES
^{13}C-enriched glucose	NO	100 mg added to the food			
L-[ring-2H5]Phenylalanine	1 mL 40% enrichment given as an intraperitoneal injection 60 minutes after gavage, except Group 1 (immediately after gavage)				
Sacrifice time	1 hour post gavage	2 hours post gavage		4 hours post gavage	

The rats were kept ad libitum on the control diet for two weeks, where their weights were recorded at the beginning of the study, after one week, and the night of the sacrifice.

At the end of the two weeks, the rats were fasted overnight. The following day, they were provided their appropriate diets through gavage, and an additional 1 mL of water was used to clean the feeding tube in order to make sure all the food has been ingested. The ^{13}C-enriched glucose was added to the gavage, and after one hour, the animals were given 1 ml of L-[ring-2H5] Phenylalanine 40% molecular enrichment via an intraperitoneal injection, except for Group 1, whose rats were given the injection immediately after gavage. Following that, the animals were sacrificed by decapitation according to the specified timetable. Blood samples were collected in EDTA tubes that were placed immediately on ice. Then, the sacrificed animals were dissected, and their organs (liver, intestines, leg adipose tissue, leg muscle) were collected, rinsed thoroughly with ice-cold saline (154 mmol NaCl/L at pH 7.4), snap-frozen in liquid nitrogen, and stored at -80°C pending analysis. Blood samples were then centrifuged at 3500 (g) for 15 minutes at 3°C and plasma aliquots were collected and stored at -80°C pending analysis. **Figure 4** shows in details the development of the experimental procedure on the day of the sacrifice.

Figure 4: Summary of the Experimental Time Frame (the tasks executed from T1 to T4 are simplified, where in the experimental phase it was a full day)



B. Analytical Procedure

1. Blood Analysis

a. Plasma Glucose, Lipid Profile, and Phosphorus Analysis

Plasma glucose, triglycerides (TG), total cholesterol (TC), HDL, total phosphorus were determined using an enzymatic colorimetric method on the Vitros 350/250 Chemistry System (Ortho Clinical Diagnostics, Johnson and Johnson, 50-100 Holmers Farm Way, High Wycombe, Buckighamshire, HP 12 4DP, United Kingdom).

b. Plasma Insulin, Total Ghrelin, and Total GLP-1

Plasma insulin, total ghrelin, and total GLP-1 concentrations were determined through the enzyme immunoassay on the Multiskan GO (ELISA, Thermo Fisher Scientific, Waltham, MA, USA). Plasma insulin was determined using the rat/mouse ELISA kit (EZRMI-13K), the rat/mouse (total) ghrelin ELISA Kit (EZRGRT-91K) was used to determine total plasma ghrelin, and the rat/mouse (total) GLP-1 ELISA Kit (EZGLP1T-36K) was used to determine total plasma GLP-1. All kits were provided by EMD Millipore Corporation (Billerica, MA, USA). Total concentrations were then determined from their appropriate standard curves.

All three tests were performed using a microwell plate by adapting two highly specific antibodies to bind to the analytes in focus. Then, the coloring agent tetra methyl benzidine (TMB) was added, followed by a stop solution to halt the reaction. The intensity of the color that appeared is proportional to the amount of the analyte in the sample. Afterwards, the absorbance was measured using a spectrophotometer, and a standard curve was plotted. An equation was extracted from the latter and it was used to calculate the concentrations of the targeted analytes.

2. Glycogen Synthesis

The distributions of the ^{13}C-enriched glucose in the rats' livers were measured by Nuclear Magnetic Resonance (NMR) spectroscopy, using the BRUKER Avance III HD NMR Spectrometer (Billerica, MA, USA) at the Central Research Science Laboratory (CRSL) at AUB.

In order to run the analysis, the samples were prepared following specific steps. First of all, the weights of the livers were recorded; then, a weight ranging between 3-5 g was taken from each liver and dissolved in 15 mL 30% KOH (prepared by dissolving 30 g KOH in 100 mL deionized water) in a water bath for two hours. Then, volume was made up to 25 mL using 30% KOH. From each sample, a 10 mL aliquot was taken, to which 20 mL ice cold ethanol was added, mixed using a vortex and refrigerated overnight at 4°C. The next day, samples were centrifuged at 3000 (g) for 10 minutes at 4°C. The supernatant was discarded and the pellets were dissolved in 5 mL 10% PCA (prepared by dissolving 20 mL 60% PCA in 100 mL deionized water) in a water bath (50°C) for 15 minutes, then re-precipitated in 10 mL ice cold ethanol for two hours at 4°C. The same process was repeated a second time, while using 5 mL deionized water to re-dissolve the pellets. A final centrifugation was done and the pellets were kept to dry at room temperature for two days. Following that, pellets were re-constituted over a 15 minutes incubation period at 55°C with 5 mL 0.04M Sodium Acetate Buffer pH 4.5 (prepared by dissolving 0.33 g Na acetate in 100 mL deionized water and adding 0.6 mL of acetic acid to reach the desired pH), and 100 µL amyloglucosidase (dissolving 0.054 g amyloglucosidase in 2 mL deionized water) for a 15 minutes incubation. The samples were mixed and then stored at -20°C overnight, and

then freeze dried overnight. The resulting samples were re-dissolved with 5 mL deionized water and filtered to remove any residues and impurities using a filter flask and a pump. Then, they were stored at -20°C overnight and then freeze dried overnight. At this point forward, each sample was dissolved with 0.8 mL 99% D2O and analyzed on the NMR spectrometer. Those readings were used to determine the percentage contribution of direct and indirect glycogen synthesis pathways in the liver.

3. Total Glycogen

The total amount of glycogen produced in the liver was generated after running an analysis on Vitros for a fraction of the liver samples prepared for the NMR readings based on the colorimetric procedure, and then the approximate amount of glycogen was calculated according to the total weight of the liver.

C. Statistical Analysis

Data are expressed as means ± SD of all values. Data analysis was done using the statistical software package IBM SPSS Statistics 24. Results were analyzed by a general linear model, with time and P as the factors, in addition to the interaction representing time x phosphorus. Significance was established at a $P\text{-}value < 0.05$, and comparisons were made between groups using Fisher's pairwise comparison.

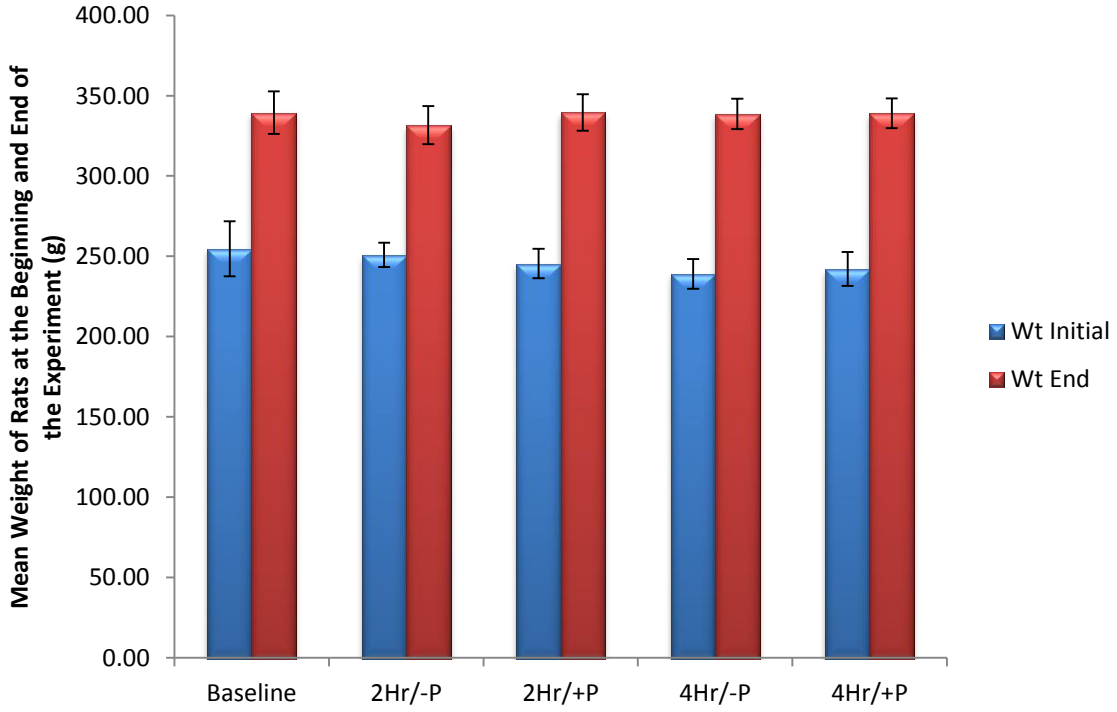
CHAPTER IV
RESULTS

A. Body and liver weights

All values are expressed as mean ±SD (standard deviation). The weights of the rats were recorded twice over the two weeks period, the first time at the beginning of the experimental phase, and the second time on the night of the sacrifice. The total weight gain has been computed, and the livers' weights were recorded before using them in the experiment.

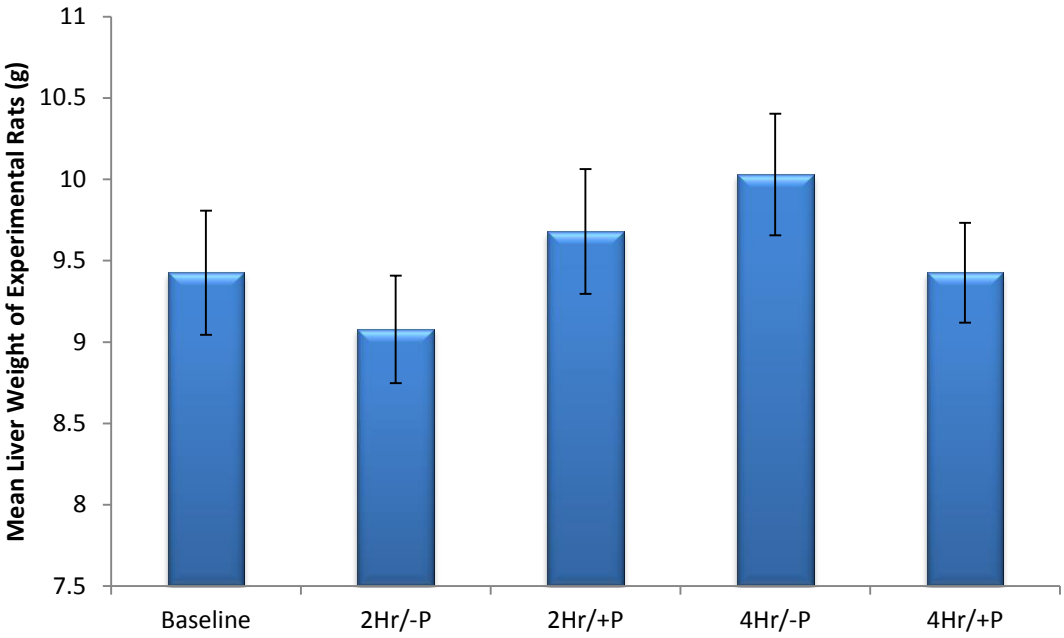
The average weight of the rats was 246.33 g (± 30.77) at the beginning of the experiment, with the highest weight scored by the control group, 254.66 g (± 48.68), and the lowest by the (4-P) group, 239.09 g (± 26.10). The average weight at the end of the two weeks was 337.83 g (± 29.53), the lowest being that of the (2-P) group, 331.73 (± 31.26), while the others had quite similar weights. As for the livers of the rats, they had an average weight of 9.54 g (± 0.99). As it is shown in **Table 4**, time, phosphorus, or their interaction had no significant effect on the weight gain pattern of the rats. The weights of the rats at the beginning of the experimental study and at the end are shown in **Figure 5**. Additionally, the bar graph in **Figure 6** shows the mean weight of livers for different groups.

Figure 5: Weight of rats at the beginning and at the end of the two weeks experimental period for the five different groups



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Figure 6: Mean Liver weights for the five experimental groups



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SD (standard deviation). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Table 4: Body Weight (g), Weight gain (g), and Liver Weight (g) of the five experimental groups of rats

						GLM *P-value*		
	Control	2-P	2+P	4-P	4+P	Time	PI	Interaction
Initial Body Wt (g)	254.66 ± 48.68	250.81 ± 20.06	245.46 ± 25.96	239.09 ± 26.10	242.19 ± 29.78	0.700	0.945	0.602
Final Body Wt (g)	339.40 ± 37.58	331.73 ± 31.26	339.56 ± 31.95	338.66 ± 26.86	339.04 ± 26.37	0.831	0.545	0.932
Total Wt Gain (g)	84.74 ± 35.96	80.91 ± 15.57	94.10 ± 24.37	99.58 ± 22.80	96.84 ± 17.90	0.398	0.511	0.439
Liver Wt (g)	9.43 ± 1.07	9.08 ± 0.87	9.68 ± 1.08	10.03 ± 1.06	9.43 ± 0.87	0.456	0.803	0.167

Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SD (standard deviation). A GLM was performed, with P and time as the two factors and their interaction (Time x P). Significance is found at *P-value* < 0.05.

(GLM: General Linear Model; PI: Phosphorus Intake; P: Phosphorus; Wt: Weight).

B. Blood Analysis

1. Glucose, Total Phosphorus, and Lipid Profile

Mean glucose level in the blood was not affected by neither time or PI, nor their interaction (Time x PI). The highest mean was observed for the group (2+P) and the lowest was observed for the control group, with respective values of 152.63 mg/dL (± 24.74) and 130.13 mg/dL (± 10.58). However, significant differences were seen between the groups, where all four groups (2-P, 2+P, 4-P, and 4+P) had significantly higher mean glucose levels than the control groups. This statistically significant difference was observed for the difference in times, with *P-values* of 0.22 and 0.015 for the 2Hr sacrifice time and 4Hr sacrifice time respectively, compared to the control. No statistically significant differences were seen for the PI (**Table 5**).

Mean total P was significantly affected by the time factor, with the highest values observed for groups sacrificed after 4 hours, with and without P, with respective values of 7.68 mg/dL (± 0.91) and 7.79 mg/dL (± 0.40). Moreover, the mean total P level was statistically significantly different between groups in terms of the time. In fact, groups sacrificed after 2 hours had a significantly different P level than the control group with a *P-value* of 0.021, and the groups sacrificed after 4 hours had a significantly different mean total P level than the 2Hr groups with *P-value* of 0.005 (**Table 5**).

As for the lipid profile, TG and HDL-Chol concentrations did not have any statistically significant changes in regards of time, PI or their interaction (Time x PI). However, mean total cholesterol concentration was significantly affected by the interaction (Time x PI) with a *P-value* of 0.045. In fact, the group (4+P) had a lower cholesterol level

than the group (2+P) with respective values 56.25 mg/dL (± 11.72) and 65.63 mg/dL (± 16.09), in which case the level of group (4+P) was lower than the control group (**Table 5**).

Table 5: Blood Profile- Glucose, Total Phosphorus, and Lipid Profile of the five experimental groups of rats

						GLM *P-value*		
	Control	2-P	2+P	4-P	4+P	Time	PI	Interaction
Glucose (mg/dL)	130.13 ± 10.58[a]	148.87 ± 20.17[b]	152.63 ± 24.74[b]	148.13 ± 19.76[b]	150.38 ± 26.27[b]	0.117	0.380	0.747
Total P (mg/dL)	7.74 ± 0.51[a]	6.84 ± 0.98[b]	7.18 ± 0.44[b]	7.79 ± 0.40[ac]	7.68 ± 0.91[ac]	**0.009**	0.571	0.459
Cholesterol (mg/dL)	63.63 ± 8.38	56.86 ± 13.28	65.63 ± 16.09	63.13 ± 11.79	56.25 ± 11.72	0.629	0.917	**0.045**
HDL-Cholesterol (mg/dL)	54.38 ± 4.03	49.57 ± 7.65	54.63 ± 11.21	52.88 ± 5.59	49.50 ± 4.69	0.520	0.886	0.081
TG (mg/dL)	54.16 ± 17.29	60.42 ± 18.51	53.75 ± 15.60	52.00 ± 15.09	53.00 ± 21.53	0.790	0.708	0.528

Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SD (standard deviation). A GLM was performed, with P and time as the two factors and their interaction (Time x P). Significance is found at *P-value* < 0.05, different subscripts indicate significant difference using Fisher's test.

(GLM: General Linear Model; PI: Phosphorus Intake; P: Phosphorus; TG: Triglyceride).

2. Mean Plasma Insulin, Ghrelin and GLP-1

Mean ghrelin concentration was significantly affected by time and PI but not by their interaction (Time x PI), with respective *P-values* of 0.027, 0.046 and 0.418. Additionally, time had significant influence on ghrelin level for the groups sacrificed after 2 hours, which has a *P-value* of 0.034. In fact, ghrelin level at 2Hr without P was lower than the 2Hr with P with levels 1.10 ng/dL (± 0.35) and 1.28 ng/dL (± 0.46), both lower than the control group, 1.57 ng/dL (± 0.42). However, no significant differences were seen between the control group and those sacrificed after 4 hours, or between the 2Hr and 4Hr groups (**Table 6**).

There was no statistically significant effect on both insulin and GLP-1 levels (**Table 6**).

Bar graphs in **Figures 7, 8 and 9** show the mean and standard errors of insulin, ghrelin and GLP-1 respectively.

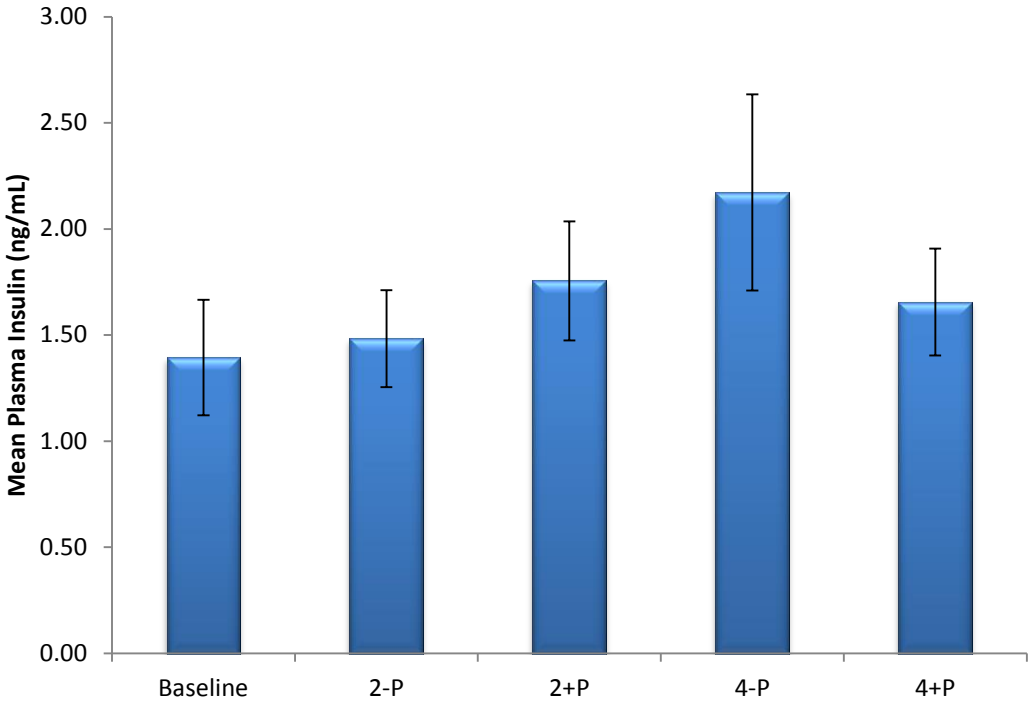
Table 6: Mean Plasma Insulin, GLP-1, and Ghrelin of the five experimental groups of rats

						GLM *P-value*		
	Control	2-P	2+P	4-P	4+P	Time	PI	Interaction
Insulin (ng/dL)	1.39 ± 0.77	1.48 ± 0.61	1.75 ± 0.79	2.17 ± 1.31	1.66 ± 0.67	0.348	0.705	0.228
Ghrelin (ng/dL)	1.57 ± 0.42[a]	1.10 ± 0.35[b]	1.28 ± 0.46 [b]	1.09 ± 0.27 [ab]	1.51 ± 0.42 [ab]	**0.027**	**0.046**	0.418
GLP-1 (pM)	33.89 ± 9.86	46.41 ± 15.20	37.31 ± 14.00	47.20 ± 23.95	45.41 ± 21.09	0.200	0.402	0.572

Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SD (standard deviation). A GLM was performed, with P and time as the two factors and their interaction (Time x P). Significance is found at *P-value* < 0.05, different subscripts indicate significant difference using Fisher's test.

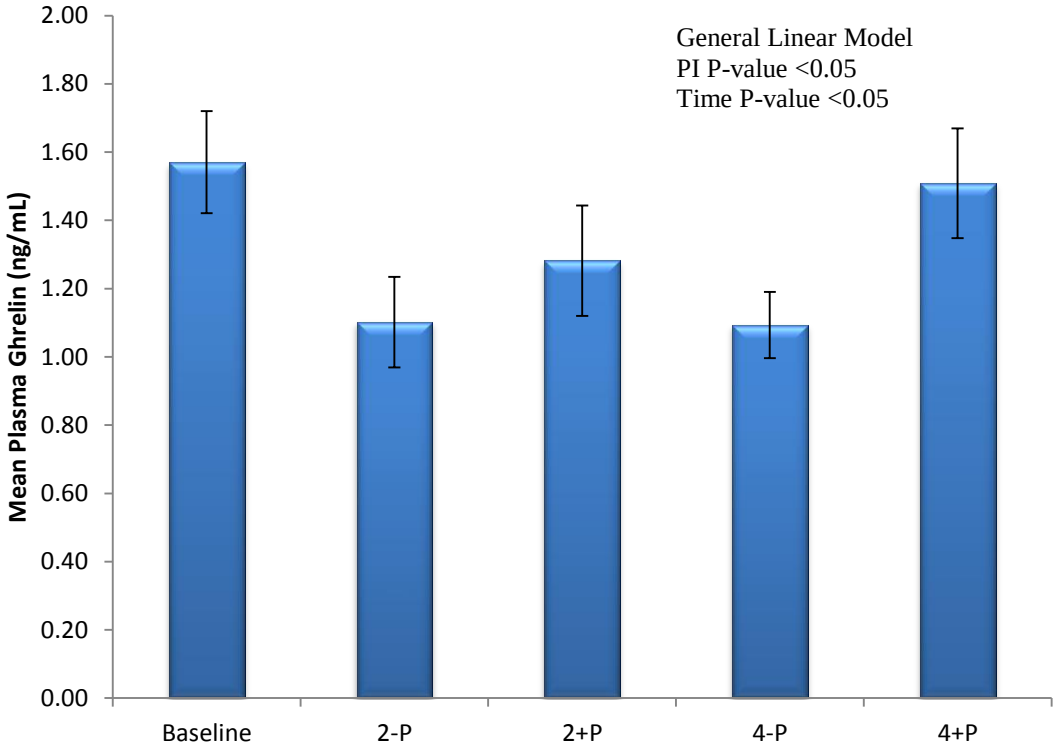
(GLM: General Linear Model; PI: Phosphorus Intake; P: Phosphorus).

Figure 7: Mean Insulin Levels of the Five Experimental Groups of Rats



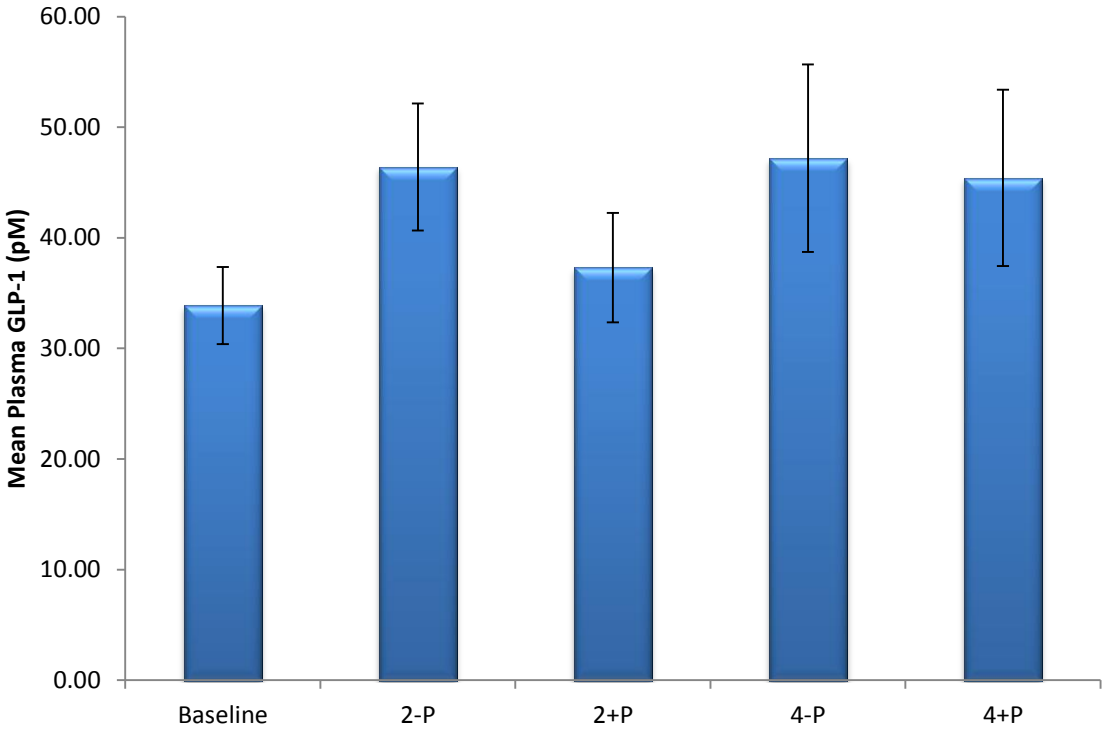
Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Figure 8: Mean Ghrelin Level for the Five Experimental Groups of Rats



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Figure 9: Mean GLP-1 Levels for the Five Experimental Groups of Rats



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

C. Glycogen Production

1. Total Glycogen Production and Glycogen per Gram Liver

The time factor showed statistically significant influence on total glycogen production with a *P-value* of 0.011, however, PI and the interaction (Time x PI) had no significant effect on glycogen production (**Table 7**). Moreover, between groups significant differences were observed for time, mainly between the control group and the 4Hr groups (*P-value* = 0.003), and to a lower extent between groups 2Hr and 4Hr (*P-value* = 0.047), but not between the control group and the 2Hr groups. In fact, total glycogen production showed higher results for groups sacrificed after 2 hours {2-P [114.34 mg (± 48.89)]; 2+P [98.44 mg (± 61.08)]}, and a further production for groups sacrificed after 4 hours {4-P [183.75 mg (± 133.14)]; 4+P [167.75 mg (± 149.66)]}. However, it is clearly seen that the production was lower for groups given P (2+P and 4+P) (**Table 7**).

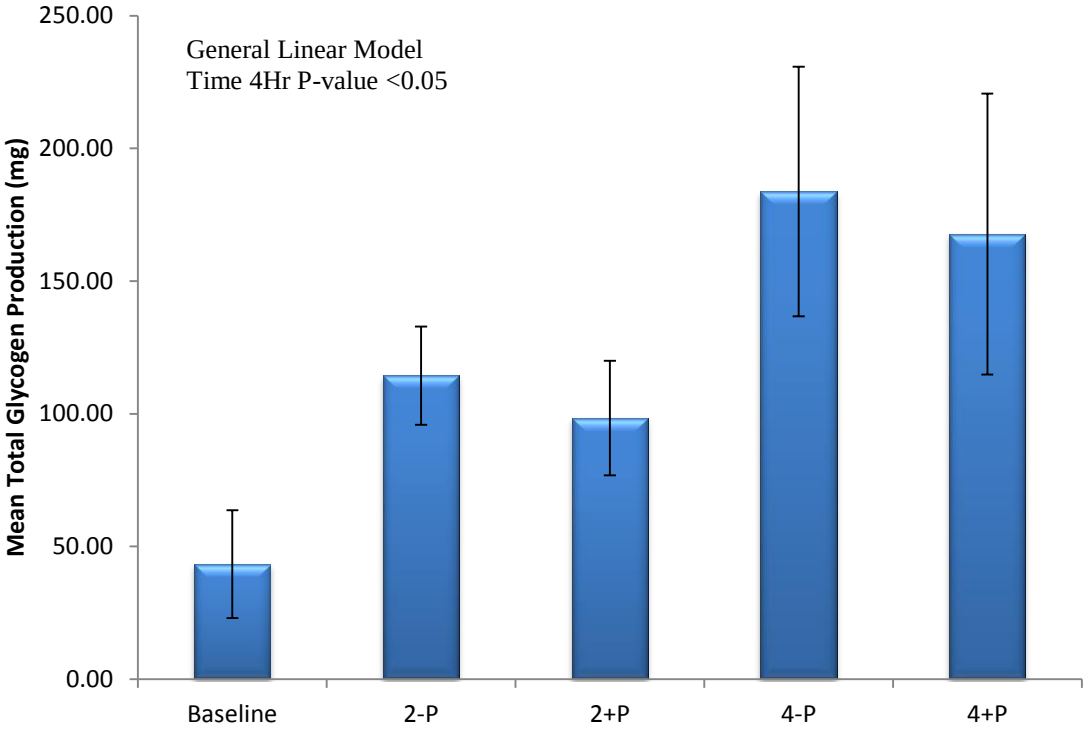
Similar results were observed for the glycogen production per gram liver, with the time factor showing significant influence (*P-value* = 0.014). However, the between groups significance was only observed between the control and the groups sacrificed after 4 hours (*P-value* = 0.004). Similarly, glycogen production per gram liver showed higher results for groups sacrificed after 2 hours {2-P [12.97 mg/g (± 6.48)]; 2+P [10.29 mg/g (± 6.52)]}, and a further production for groups sacrificed after 4 hours {4-P [18.49 mg/g (± 13.83)]; 4+P [17.00 mg/g (± 13.76)]}. However, it is clearly seen that the production was lower for groups given P (2+P and 4+P) (**Table 7**).

Despite the significant effect observed in terms of the time, it is important to highlight the existence of a large variation within the groups, which could have affected the final results of the significance in terms of PI and interaction (Time x PI). These variations

could have been caused by experimental errors such as glucose losses during the extraction phase.

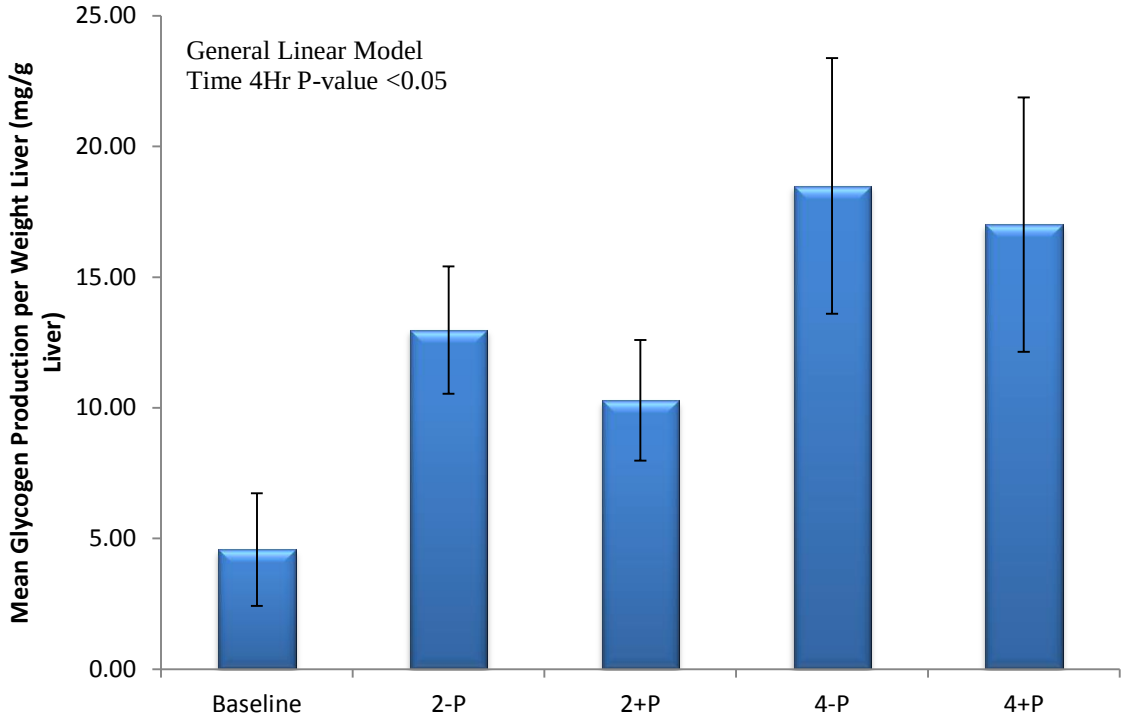
The trend in total glycogen production and glycogen production per gram liver are shown in the bar graphs in **Figures 10 and 11** respectively**.**

Figure 10: Mean Total Glycogen Production in the Five Experimental Groups of Rats



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Figure 11: Mean Glycogen Production per Gram Liver in the Five Experimental Groups of Rats



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Table 7: Total Glycogen Production and Glycogen Production per Gram Liver in the Five Experimental Groups of Rats

						GLM *P-value*		
	Control	2-P	2+P	4-P	4+P	Time	PI	Interaction
Total Gly Production (mg)	43.41 ± 57.47^{b}	114.34 ± 48.89^{bc}	98.44 ± 61.08^{bc}	183.75 ± 133.14^{a}	167.75 ± 149.66^{a}	**0.011**	0.804	0.857
Gly production per unit (mg/ g Liver)	4.57 ± 6.09^{b}	12.97 ± 6.48^{ab}	10.29 ± 6.52^{ab}	18.49 ± 13.83^{a}	17.00 ± 13.76^{a}	**0.014**	0.686	0.754

Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SD (standard deviation). A GLM was performed, with PI and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05, different subscripts indicate significant difference using Fisher's test.

(GLM: General Linear Model; PI: Phosphorus Intake; P: Phosphorus; Gly: Glycogen).

2. Direct Glycogen Production

Direct glycogen production obtained from the distribution of the $13C_1$ over the 12 carbons of glucose α and β in the NMR reading, a sample of the readings is shown in **Figure12**, showed a significant increase with time, with the highest level being at 4Hr sacrifice time when P was added, 45.94% (± 12.89), while the control group had almost no direct glycogen production with a mean of 2.24% (± 3.16). The statistically significant effect was seen with time only (*P-value* < 0.001), while PI and interaction (Time x PI) showed no significant effect (**Table 8**). Effectively, the between group differences were significant between the control group and simultaneously with the 2Hr and 4Hr sacrifice times, with a *P-value* < 0.001 for both. However, no differences were observed between the 2Hr and 4Hr sacrifice times (**Table 8**). Despite the absence of significant effect of PI on the overall results, there was a statistically significant difference between the groups who were given P and those who were not given P in the same time frame, thus between 2-P and 2+P on one hand and between 4-P and 4+P on the other hand, both differences have *P-values* <0.001 (**Table 8**).

The results are shown in the bar graph in **Figure 13**.

Figure 12: Sample of NMR Reading for the Distribution of $^{13}C_1$ Dsitribution on the 12 Carbons of theTwo Anomers (α & β) of the Glucose Molecule

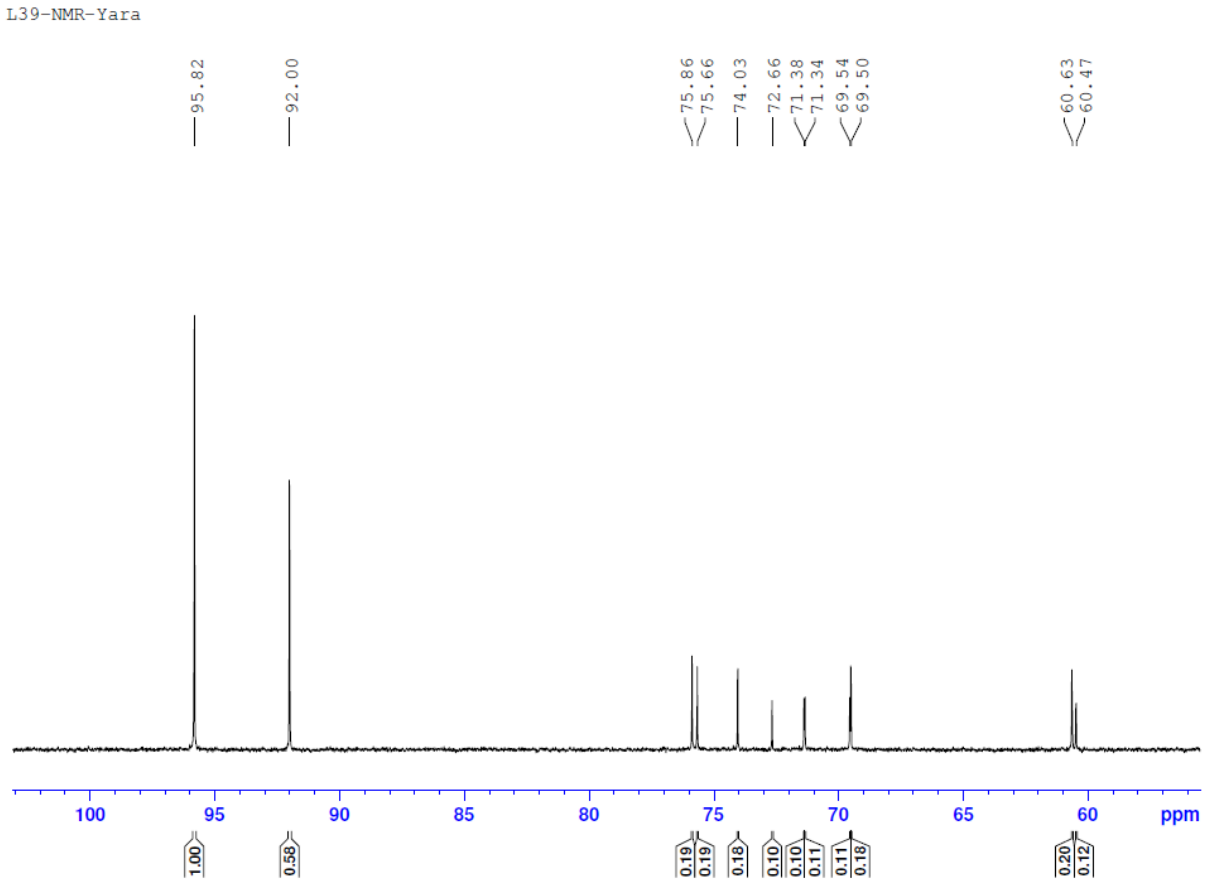
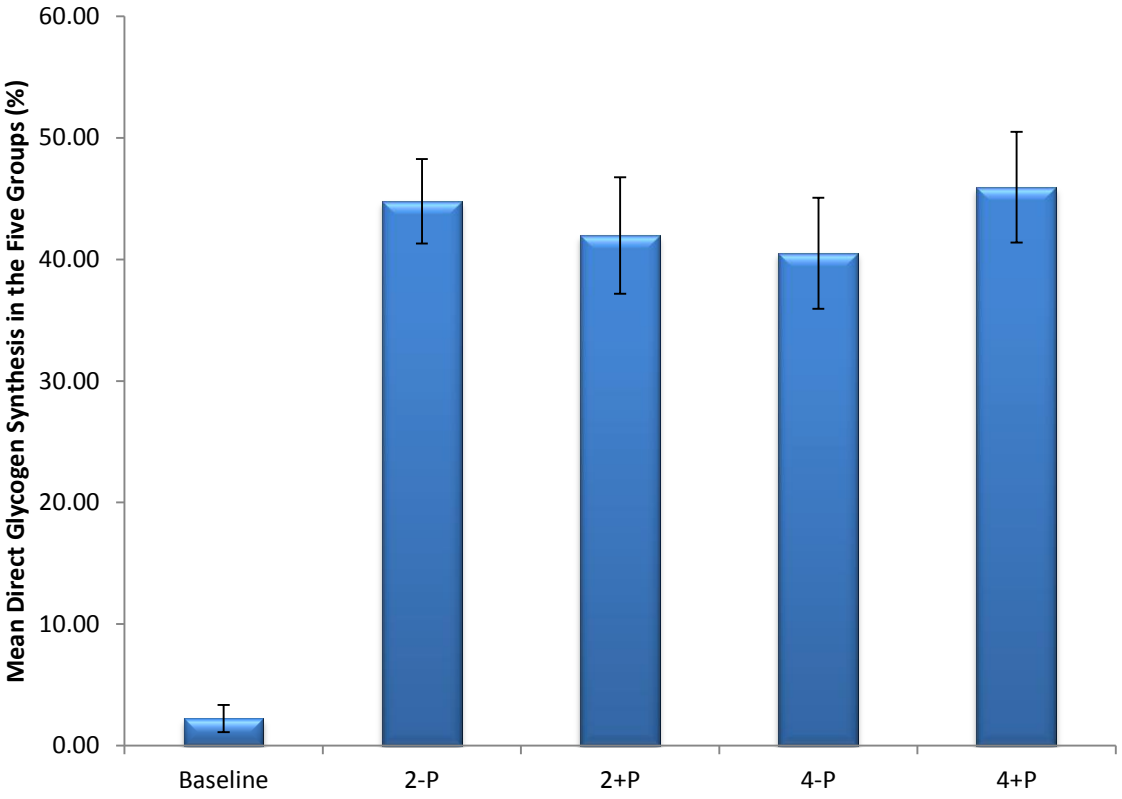


Figure 13: Mean Direct Glycogen Synthesis for the Five Experimental Groups of Rats



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

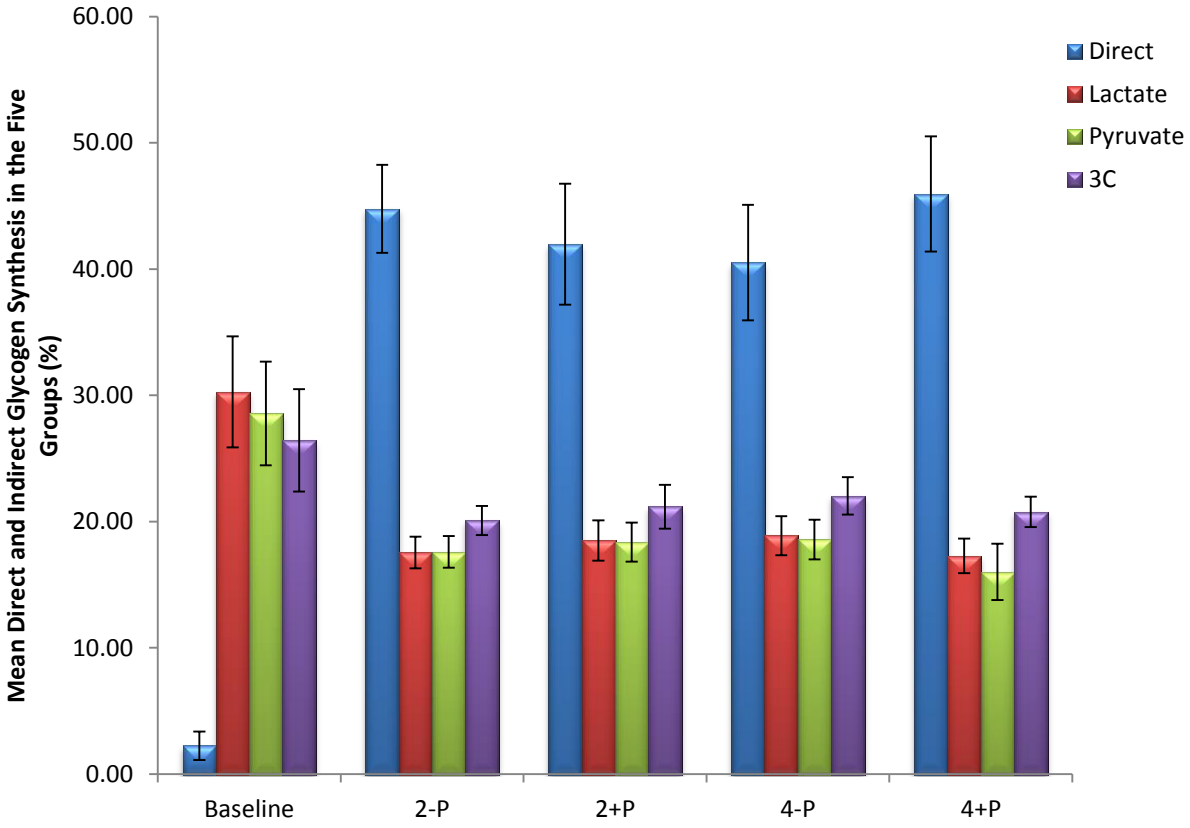
3. Indirect Glycogen Production

Indirect glycogen production is shown by glycogen production following three different pathways: lactate, pyruvate, and 3C. In this analysis, time has shown statistically significant effects on glycogen production through pyruvate (*P-value* = 0.005) and lactate (*P-value* = 0.001) (**Table 8**).

As for pyruvate, there was a statistically significant difference between groups in terms of time between control group and 2Hr and 4Hr sacrifice time simultaneously, with *P-values* of 0.001 for both differences. Similar results were observed for lactate, with *P-values* < 0.001 for both differences (**Table 8**). However, for the 3C pathway, a modest significant difference was observed only between the control group and the 2Hr sacrifice time (*P-value* = 0.047). These significant differences are seen since the indirect glycogen production in the control group is higher than that of the other groups, due to the fact that the others had a higher direct glycogen synthesis (**Table 8**).

A bar graph in **Figure 14** shows the different pathways of glycogen synthesis, including direct and indirect, in all five groups.

Figure 14: Mean Percentage Glycogen Production through Different Pathways for the Five Experimental Groups



Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SE (standard error). A GLM was performed, with PI (phosphorus intake) and Time as the two factors and their interaction (Time x PI). Significance is found at *P-value* < 0.05.

Table 8: Mean Percentages of Direct and Indirect Glycogen Synthesis for the Five Experimental Groups of Rats

						GLM *P-value*		
	Control	2-P	2+P	4-P	4+P	Time	PI	Interaction
Direct Gly (%)	2.24 ± 3.16^{a}	44.77 ± 9.22^{bc}	41.96 ± 13.53^{bd}	40.51 ± 12.93^{be}	45.94 ± 12.89^{bf}	**<0.001**	0.798	0.354
Pyruvate (%)	28.56 ± 11.59^{a}	17.60 ± 3.33^{b}	18.37 ± 4.36^{b}	18.57 ± 4.44^{b}	16.01 ± 6.31^{b}	**0.005**	0.740	0.526
Lactate (%)	30.27 ± 12.44^{a}	17.54 ± 3.16^{b}	18.49 ± 4.51^{b}	18.89 ± 4.36^{b}	17.28 ± 3.88^{b}	**0.001**	0.925	0.637
3C (%)	26.43 ± 11.47^{b}	20.08 ± 3.05^{a}	21.18 ± 4.93^{a}	22.04 ± 4.22^{ab}	20.77 ± 3.42	**0.168**	0.945	0.649

Control group was fed only water on the day of the sacrifice; Group 2-P was not given P and sacrificed 2 hours after gavage; Group 2+P was given P and sacrificed 2 hours after gavage; Group 4+P was given P and sacrificed 4 hours after gavage; Group 4-P was not given P and sacrificed 4 hours after gavage. Values for each group are expressed as mean ± SD (standard deviation). A GLM was performed, with PI and Time as the two factors and their interaction (Time x PI). Significance is found at P-value < 0.05, different subscripts indicate significant difference using Fisher's test.

(GLM: General Linear Model; PI: Phosphorus Intake; P: Phosphorus; Gly: Glycogen; 3C: 3 carbon).

CHAPTER V
DISCUSSION

Ingestion of low phosphorus containing protein, such as egg white, was shown to be associated with decreased glycogen concentration almost by half in the livers of rats (Xie *et al.*, 2000). Phosphate depletion of rats was also reported to impair myocardial glycogen and lipid metabolism including the reduction in long-chain and short-chain fatty acids oxidation (Brautbar *et al.*, 1984).

Dietary phosphorus deprivation in rats was reported to decrease myocardial glycogen, which was accompanied by inhibition of the enzymes of glycogen synthesis (Hörl *et al.*, 1983). The changes observed are analogous to those found in heart failure (Hörl *et al.*, 1980).

Furthermore, postprandial phosphorus availability seems to depend on phosphorus content of the meal, especially 60-90 minutes after meal ingestion. Thus, the postprandial metabolic need for phosphorus was found to be met by available limited free phosphorus (intra and extra-cellular), which seems to be sufficient for about 90 min. Thereafter, the postprandial metabolism would be dependent on phosphorus availability from ingested food. Therefore, it is expected that glycogen metabolism would be altered 1 ½ hour after meal ingestion. Such alteration would be expected to affect food intake and growth on the long term.

Furthermore, these alterations are expected to affect glycogen production in the liver, thus an increased level of production would be expected with time. Moreover, the availability of P plays a vital role in this process, and hence, when P is available, the synthesis rate is expected to increase. This been said, these syntheses in the study are reflected first in terms of total glycogen production in the liver (mg), or expressed as glycogen produced per gram liver (mg/g liver) from glucose uptake of from the circulation and its storage in the liver in the form of glycogen. In our experimental procedure, we measured total glycogen production in the liver and then computed the amount of glycogen produced per gram liver based on the weight of every liver, which was weighed before the start of the chemical analysis. Glucose was extracted from rats' livers using KOH and ethanol, similarly to the extraction procedure applied by Carroll et al. (1955) and Van der Vies (1953). Our results showed a mean total glycogen production of 121.73 mg (±108.33), with the lowest production observed in the control group [43.41 mg (±57.47)] and the highest level observed after 4 hours from feeding [183.75 mg (±133.14)]. These values are expressed as total glycogen per liver; however they are equivalent to 4.57 mg/g liver (±6.09) and 18.49 mg/g liver (±13.83) respectively. In their study, Carroll (1955) found an average of 44.40 mg glycogen per gram liver in the fed state, which is by far higher than the amounts we observed. However, at a fasted state, they obtained an average of 3.26 mg glycogen per gram liver, which is very close to our findings. Nevertheless, our results showed a large within group variation in the amount of glycogen produced, thus, hadn't it been for these variations, our findings could have been in line with theirs. One possibility to our variations is the experimental errors that might occur in the course of any study, such as loss of glucose during the extraction phase, or maybe from the animals responding to stress

during feeding. For a fact, the extraction of glucose requires repeated washing phases, where the supernatant is discarded after every wash. Therefore, some of the glucose might have stayed in the supernatant and did not get incorporated in the pellet at the bottom of the tube, thus leading to glucose losses in the process of this extraction step. Albeit, the maximal rate of glycogen produced was observed for group (4+P), 490.44 mg of total glycogen, which is higher than the maximal rate for group (4-P), 428.07 mg total glycogen. Similarly, the maximal rate for group (2+P) is 193.07 mg total glycogen, which is higher than the maximal rate for group (2-P), 163.12 mg total glycogen. These differences, even though not significant, they highlight the role of P in glycogen production alongside the effect of time, which could have been reflected by the mean production rate, hasn't been for the large within group variations. Additionally, direct glycogen production requires 4 ATP molecules, which depends upon the level of available P for the restoration of those ATPs. Therefore, the groups who were given P might have had a higher availability of ATP for the synthesis of glycogen.

After having determined the total amount of glycogen produced, we were able to determine the preferred pathway for the synthesis. In fact, both possible pathways, direct and indirect, were observed in all groups and they are expressed by the percentage of contribution of the specific pathway. These contributions are deduced from the labeled $^{13}C_1$ distribution on the 12 different peaks on the NMR graphical reading. In the NMR reading, each peak represents a carbon atom of the glucose molecule in its two anomers, α & β, each having 6 carbons. Thus, the higher the amount of labeled $^{13}C_1$ remaining on C_1, reflects the higher the percentage of the direct synthesis of glycogen. However, glycogen production

can be accomplished through indirect pathways, notably through lactate, pyruvate and 3C. Indirect glycogen synthesis could be accomplished through pyruvate when the $^{13}C_1$ is found on C_3 and C_4. This pathway is followed when pyruvate enters the citric acid cycle as either acetyl CoA or oxaloacetate. An ulterior pathway would be accomplished through lactate when the labelled carbon is detected on C_2 and C_5. Lactate would then contribute to glycogen production through its intermediate, oxaloacetate, or other symmetrical molecules like succinate and fumarate. Lastly, the 3C molecule is responsible for the glycogen synthesis when the labelled $^{13}C_1$ is distributed to C_6 (Wehmeyer et al., 1994). This method for detecting direct and indirect glycogen production has been adopted by several researchers under different conditions, including Wehmeyer et al. in 1994, Morikawa et al. in 1996, and Kunnecke & Seelig in 1991. In our study, glycogen production from the direct pathway ranged between -1.08% and 69.00%, which is higher than the results obtained in the study conducted by Wehmeyer in 1994, who found on average 29% glycogen production emerging from the direct pathway, measured after 2 hours of continuous infusion with glucose. If we compare their results with our results at 2 hours where our rats were given food by gavage and left to rest for the following 2 hours, we still had higher levels, including the group who was given P. However, in a different study by Kunnecke & Seelig (1991), their results come in agreement with our results, where they found around 67% of glycogen production coming from the direct pathway, although they did not detect the participation of the indirect pathway. Yet, it is important to note the fact that in neither of the studies, the rats were provided with P, which is the opposite situation in our case. In fact, the lowest levels- in which the direct pathway was quasi non-existent- were observed for the control group that was fasted overnight and was not given any food on the day of the

sacrifice, while the higher rates were observed for the groups who were fed by gavage a very small quantity of food, thus a small amount of carbohydrates that would allow the much higher production of direct glycogen compared to the results of Wehmeyer (1994). Additionally, the highest level of production, with significant difference from other groups, was observed for group (4+P) with an average of 45.95% ± (12.89), thus implying that P must have played a role in glycogen production. Furthermore, Morikawa showed in their study the importance of ATP for the production of glycogen, mainly through its participation in glycogenesis and gluconeogenesis in the liver, thus highlighting the crucial role of P in the process, reflected in the highest direct glycogen production rate observed in the group who was given P. For a fact, a study conducted by Bassil & Obeid (2016) showed that providing a meal with supply of P affects hepatic ATP production and thus leads to a faster and higher rate of glucose and P uptake by the liver. Additionally, direct glycogen synthesis depends on blood glucose concentration, which is proportional to the activity of glucokinase for the phosphorylation step of glucose to G6P in the liver, requiring an elevated blood glucose level (>180 mg/dL) (Wehmeyer et al., 1994; Morikawa et al., 1996). Nevertheless, in our study, the mean glucose level was 145.90 mg/dL, and it ranged between 105.00 mg/dL and 209.00 mg/dL. Therefore, it is most likely that our rats had a reasonably elevated blood glucose level to activate glucokinase in the liver. Albeit, this was not the case of the control group who had a mean blood glucose level of 130.13 mg/dL (± 10.58), thus glucokinase couldn't have been activated, and the glycogen production would be directed towards the indirect pathways, hence G6 P would be produced via gluconeogenetic pathways including lactate, pyruvate and 3C (Wehmeyer et al., 1994). In fact, time showed a significant influence on the indirect glycogen production, mainly

through lactate and pyruvate, which were the preferred substrate for the control group with an average of 30.27% (± 12.44) and 28.56% (± 11.59) respectively, which was in agreement with the Wehmeyer study (1994), where they found that lactate and pyruvate are more preferred than 3C. However, for the fed groups, the preferred substrate for glycogen production after the direct pathway was the 3C. in addition to the level of blood glucose for directing glycogen synthesis, the baseline hepatic glycogen will dictate as well the amount of glycogen to be produced, where a lower baseline level increases the rate of glycogen synthesis and thus indirectly ATP synthesis (Musso et al., 2010).

However, the synthesis of total glycogen, whether through a direct or indirect pathway, couldn't be achieved without the support of some hormones that are usually implicated in food intake and glucose homeostasis, namely insulin, GLP-1, and ghrelin. In addition, P is implicated in the pathways and performance of these hormones.

Our results showed no significance when it comes to the blood level of GLP-1, not even between group variations. However, GLP-1 is an incretin hormone secreted in the small intestine as a response to food ingestion (Hansen et al., 1999; Luque et al., 2002; Ben-Shlomo et al., 2011). Additionally, it stimulates insulin secretion via specific receptors on islets β cells of the pancreas, providing it with the incretin effects character, simultaneously inhibiting the release of glucagon (Hansen et al., 1999; Luque et al., 2002; Ben-Shlomo et al., 2011). These functions allow GLP-1 to regulate plasma glucose levels in several ways. For instance, Luque et al. (2002) mentioned in their study that GLP-1 stimulates parameters involved in glucose metabolism in the liver and muscles, such as the stimulation of glycogen synthesis and glycogen synthase α activity. The latter constitutes the direct roles

of GLP-1, whereas the indirect role is most probably accomplished through cAMP, which stimulates GLP-1 secretion (Hansen et al., 1999; Luque et al., 2002; Ben-Shlomo et al., 2011). Additionally, P is a crucial part of cAMP, thus the influence of P on both GLP-1 levels and glycogen synthesis rate. For a fact, a vicious cycle is created between GLP-1, cAMP and P, where when the available levels of the latter increase, they increase the production of cAMP which further stimulate GLP-1 secretion, and thus glycogen production would increase. However, our results showed a lower level of GLP-1 in groups (2+P) and (4+P) in comparison to (2-P) and (4-P). Thus, in order to obtain results in line with other studies, the experimental procedure could be necessitating either a larger sample size, a higher level of P intake, or possibly measuring the active GLP-1 instead of only measuring total GLP-1.

As for insulin, studies have showed up to this date that P homeostasis and insulin levels have a mutual influence on one another. In fact, insulin plays a major role in Pi uptake to the intracellular space by the Na dependent transporter (Schmid et al., 2011), where they have an inversely related association (Obeid, 2013). Moreover, insulin has a leading role in the production of ATP (Bassil & Obeid, 2016) through promoting the uptake of the substrates needed for the production, glucose and P, in addition to influencing the turnover of the two labile groups of P in the ATP molecule (Sacks, 1945). Reciprocally, when there is a reduced level of P available, glucose won't be cleared from the circulation, thus leading to glucose intolerance and hence insulin resistance (Bassil & Obeid, 2016). This relationship is explained by the necessity of insulin to activate the oxidation process of glucose by activating the enzyme glycogen synthase by phosphorylation, thus the need for

P in the process (Sacks, 1945; Bouskila et al., 2008). The phosphorylation step is required in order to transform glucose into G6P, which is the form under which glucose can enter the cell and undergo the glycogenetic process (Bouskila et al., 2008), and the rate of glycogen synthesis depends on the blood concentration of G6P (Wehmeyer et al., 1993). Additionally, P is required for the secondary phosphorylation step through cAMP that will also stimulate glycogen synthesis. Therefore, P in cAMP is required for direct glycogen synthesis and for synthesis through increasing GLP-1 levels (Dent et al., 1990). Our results show elevated insulin and total P levels in groups who were given P, which is in accordance with the existent studies. For instance, the highest percentage of direct glycogen synthesis has been scored for the (4+P) group, showing the importance of time in glycogen production, in addition to the availability of P which is crucial for the phosphorylation step. Furthermore, P is an important part of ATP, which is also needed for the production of glycogen, whether from the direct or the indirect pathways. Further to this point, total glycogen production increased with time, and the maximal production rate has been scored for the groups (2+P) and (4+P) compared to (2-P) and (4-P), even if this is not reflected in the mean total glycogen production due to the large within group variation. Additionally, group (4+P) scored the highest direct glycogen production rate (45.94%) with the lowest mean insulin level (1.66 ng/dL), indicating that this group was able to phosphorylate its glucose and take it to the direct glycogen production pathway, thus it has been cleared from the circulation and insulin wasn't needed to remove it.

Ghrelin is an acylated peptide that stimulates the release of growth hormone, secreted from the anterior pituitary gland, and plays an important role in food intake and

nutrients metabolism, thus its involvement in glycogen production (Nakazato et al., 2001; Lee et al., 2008; Kojima & Kangawa, 2005). Under usual conditions, ghrelin levels are expected to decrease after the ingestion of a meal (Wren et al., 2011; Nakazato et al., 2001), which is reflected in our results, where all four groups [(2-P), (2+P), (4-P) and (4+P)] had a lower ghrelin level than the control group who was given only water. Furthermore, it ghrelin levels would increase few hours after food intake in order to start initiating hunger (Wren et al., 2011; Nakazato et al., 2001), which is also reflected in our results, since the ghrelin levels for groups sacrificed after 4 hours were higher than both control groups and groups sacrificed after 2 hours. However, until today, findings about the relationship between ghrelin and insulin are still controversial, since some studies deduce an inverse relationship, while others deduce a direct relationship (Lee et al., 2008; Kojima & Kangawa, 2005). Our results showed both observations. For instance, ghrelin level for groups (2-P) was 1.10 ng/dL (±0.35) with an insulin level of 1.48 ng/dL (± 0.61), while when insulin levels increased for group (4-P) to 2.17 ng/dL (± 1.31), ghrelin levels remained quite the same, 1.09 ng/dL (± 0.27). However, when P was given to rats in their meals, insulin increased at 2 hours (2+P) compared to (2-P) to have a mean of 1.75 ng/dL (±0.79), which was accompanied by an increased ghrelin level to 1.28 ng/dL (±0.46). Albeit, at 4 hours, the group provided with P had a decreased insulin level with a mean of 1.66 ng/dL (±0.67), accompanied by an increased ghrelin level with a mean of 1.51 ng/dL (±0.42). These results were conforming to the findings in the literature about the relationship between insulin and ghrelin. Additionally, the decreased insulin levels at 4 hours for the group (4+P) compared to (4-P) is quite normal, since P would require insulin in order to phosphorylate glucose and start the glycogen synthesis pathway. Furthermore,

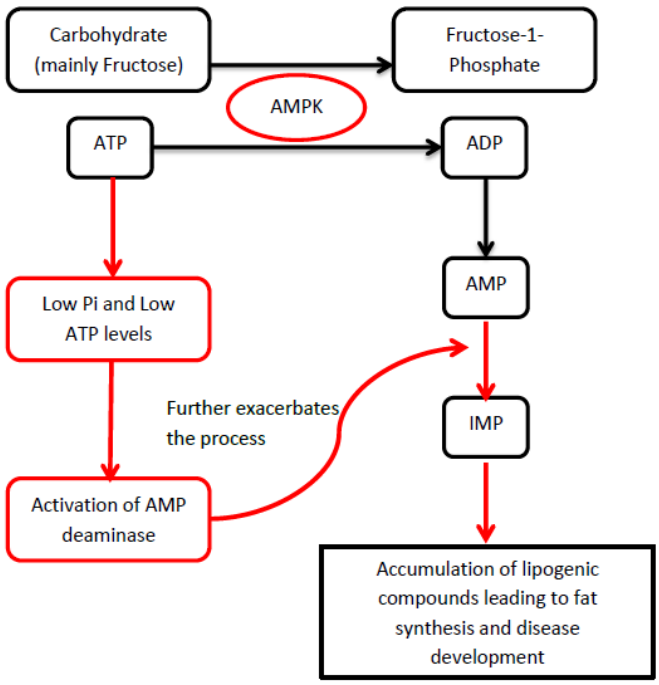
the presence of P has been shown to decrease ad libitum food intake due to the improved utilization of many metabolites, and hence the production of ATP (Obeid et al., 2010; Obeid et al., 2013), which would lead to a decreased ghrelin level caused by a decreased desire to eat. This effect is seen when comparing the control group to groups (2+P) and (4+P) who were given P.

Regardless the pathway of glycogen synthesis, or the hormonal regulation imposed on it, several studies have showed to this date the tremendous effect of carbohydrates intake, notably fructose, on the production and depletion of IC ATP and Pi, and how its level may affect the synthesis of glycogen, lipid and protein in the body (Musso et al., 2010; Johnson et al., 2010; Bawden et al., 2015). It has been well established that the metabolism of any type of carbohydrates requires phosphorylation, which is an energy expensive step, imposing the use of ATP and P. However, glucose is more tightly regulated than fructose, which lacks the phosphorylation feedback (Johnson et al., 2010; Bawden et al., 2015). The process is guided mainly by AMP-activated protein kinase (AMPK), which after being activated by ATP, binds glycogen and controls its synthesis. The latter would indirectly regulate lipid and protein syntheses (Musso et al., 2010; Johnson et al., 2010; Bawden et al., 2015). For a matter of fact, when a source of carbohydrate is ingested, ATP is sent for phosphorylation, which reduces the IC concentration of ATP and P, leading to the accumulation of adenosine monophosphate (AMP), which is broken down by AMP deaminase, executing the opposite functions of AMPK. Therefore, this process, if uncontrolled, would lead to a higher level of lipogenesis, accumulating in the hepatocytes

since HMG-CoA reductase is one of the enzymes activated by this process. **Figure 15** below would better explain the process through which this cycle occurs.

It is worth noting that this study being conducted on animal models has been controlled as much as possible. However, similarly to any other experimental procedure, there is always a window for the inability to control the food intake of the animals over the two weeks adaptation phase, the physiological responses occurring during the sacrifice phase or how every animal responded to the treatment.

Figure 15: Pathway for Carbohydrate metabolism into lipids



ADP: adenosine triphosphate; AMP: adenosine monophosphate; IMP: inosine monophosphate.

Under regular conditions, elevated levels of AMP or IMP would inhibit the phosphorylation process in order to avoid the activation of AMP deaminase, which is not the case under fructose intake, leading to the unbalance production of lipids and their accumulation in the hepatocytes.

CHAPTER VI
CONCLUSION

Understanding energy partitioning after a meal ingestion containing P is important in order to understand the mechanism behind the different processes in products syntheses in the human body. For this reason, this study consisted of an animal model which has been built to study the synthesis of glycogen, as the first part in energy partitioning.

Our results showed that when P has been added to the meal ingested before sacrifice, there has been an increased production of total glycogen, which was reflected by the distribution of production over the groups. Furthermore, the preferred pathway for this production was the direct pathway. One of the many reasons that led to this pathway is the adequate presence of P that is of high essentiality to start the process of glycogen synthesis in the liver, which is the phosphorylation of glucose to G-6-P, in addition to the improved utilization of insulin and its improved sensitivity. Further to these important factors, remain the participation of other hormones in the process, including GLP-1 and ghrelin, which are influenced by the level of available P in addition to the time factor.

This been said, it is important to continue working on this platform to better understand how the process is occurring in the cells, which could be done by the enrichment of the labeled $^{13}C_1$ using the GC-MS approach. Additionally, it is important to direct the analyses towards proteins and fat syntheses in order to get a better picture of how

energy is being portioned in the body following a meal ingestion including P, which will further elucidate the hypotheses relating P to weight management and disease development.

BIBLIOGRAPHY

Aloia, J. F., Cohn, S. H., Vaswani, A., Yeh, J. K., Yuen, K., & Ellis, K. (1985). Risk factors for postmenopausal osteoporosis. *The American journal of medicine*, 78(1), 95-100.

Amanzadeh, J., & Reilly, R. F. (2006). Hypophosphatemia: an evidence-based approach to its clinical consequences and management. *Nature Clinical Practice Nephrology*, 2(3), 136-148.

Anderson, G. & Moore, S. (2004). Dietary Proteins in the Regulation of Food Intake and Body Weight in Humans. *Journal of Nutrition*, 134: 974S–979S.

Bansal, V.K. (1990). Serum Inorganic Phosphorus. In: Walker HK, Hall WD, Hurst JW, editors. Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition. Boston: Butterworths. Chapter 198. Available from: https://www.ncbi.nlm.nih.gov/books/NBK310/

Bassil, M. S. & Obeid, O. A. (2016). Phosphorus Supplementation Recovers the Blunted Diet Induced Thermogenesis of Overweight and Obese Adults: A Pilot Study. *Nutrients*, 8(12), 801.

Bawden, S. J., Stephenson, M. C., Ciampi, E., Hunter, K., Marciani, L., MacDonald, I. A., ... & Gowland, P. A. (2016). Investigating the effects of an oral fructose challenge on hepatic ATP reserves in healthy volunteers: A 31 P MRS study. *Clinical Nutrition*, 35(3), 645-649.

Ben-Shlomo, S., Zvibel, I., Shnell, M., Shlomai, A., Chepurko, E., Halpern, Z., ... & Fishman, S. (2011). Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase. *Journal of Hepatology*, 54(6), 1214-1223.

Bergman, C., Gray-Scott, D., Chen, J. & Meacham, S. (2009). What is next for the Dietary Reference Intakes for Bone Metabolism Related Nutrients beyond Calcium: Phosphorus, Magnesium, Vitamin D, and Fluoride? *Critical Reviews in Food Science and Nutrition*, 49:2, 136-144.

Bergwitz, C. & Jüppner, H. (2010). Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. *Annual Review of Medicine*, 61, 91-104.

Berndt, T. & Kumar, R. (2009).Novel mechanisms in the regulation of phosphorus homeostasis. *Physiology*, 24(1), 17-25.

Berndt, T., Thomas, L. F., Craig, T. A., Sommer, S., Li, X., Bergstralh, E. J. & Kumar, R. (2007). Evidence for a signaling axis by which intestinal phosphate rapidly modulates renal phosphate reabsorption. *Proceedings of the National Academy of Sciences*, 104(26), 11085-11090.

Boullion, R. (2006). Vitamin D: From photosynthesis, metabolism, and action to clinical application. See Ref. 100, pp. 1435–1463.

Bouskila, M., Hirshman, M. F., Jensen, J., Goodyear, L. J., & Sakamoto, K. (2008). Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, 294(1), E28-E35.

Brautbar, N., Tabernero-Romo, J., Coats, J., & Massry, S. (1984). Impaired myocardial lipid metabolism in phosphate depletion. *Kidney International*, 18-23.

Calvo, M.S. & Uribarri, J. (2013). Contributions to Total Phosphorus Intake: All Sources Considered. *Dialysis*, 26(1): 54–61.

Carroll, N. V., Longley, R. W., & Roe, J. H. (1956). The determination of glycogen in liver and muscle by use of anthrone reagent. *Journal of Biological Chemistry*, 220, 583-593.

Çelik, N. & Andıran, N. (2011). The relationship between serum phosphate levels with childhood obesity and insulin resistance. *Journal of Pediatric Endocrinology and Metabolism*, 24(1-2), 81-83.

Davis, J. L., Lewis, S. B., Schultz, T. A., Kaplan, R. A. & Wallin, J. D. (1979). Acute and chronic phosphate depletion as a modulator of glucose uptake in rat skeletal muscle. *Life Sciences*, 24:629–632.

Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., & Cohen, P. (1990). The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature*, 348(6299), 302.

Ditzel, J. & Lervang, H. (2009). Disturbance of inorganic phosphate metabolism in diabetes mellitus: temporary therapeutic intervention trials. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 2 173–177.

Ditzel, J. & Lervang, H. (2010). Lifestyle diseases and cardiovascular risk factors are interrelated to deficiencies of major substrates in ATP synthesis. *Vascular Health and Risk Management*, 6:829–836.

Elliott, P., Kesteloot, H., Appel, L.J., Dyer, A.R., Ueshima, H., Chan, Q., Brown, I.J., Zhao, L. & Stamler, J. (2008). INTERMAP Cooperative Research Group. Dietary phosphorus and blood pressure: international study of macro- and micro-nutrients and blood pressure. *Hypertension*, 51(3):669-75.

Farrow, E. G., & White, K. E. (2010). Recent advances in renal phosphate handling. *Nature Reviews Nephrology*, 6(4), 207-217.

Food and Nutrition Board, Institute of Medicine. (1997). Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride.

Forster, I. C., Virkki, L., Bossi, E., Murer, H. & Biber, J. (2006). Electrogenic kinetics of a mammalian intestinal type IIb Na+/Pi cotransporter. *The Journal of Membrane Biology*, 212(3), 177-190.

Fritsch, M., Koliaki, C., Livingstone, R., Phielix, E., Bierwagen, A., Meisinger, M., ... & Wolff, C. (2015). Time course of postprandial hepatic phosphorus metabolites in lean, obese, and type 2 diabetes patients. *The American journal of clinical nutrition*, 102(5), 1051-1058.

Gattineni, J., & Baum, M. (2012). Genetic disorders of phosphate regulation. *Pediatric Nephrology*, 27(9):1477-87.

Goretti, M., Penido, G. & Alon, U. Phosphate homeostasis and its role in bone health. *Pediatric Nephrology*, (2012) 27:2039–2048.

Gudmundsdottir, H., Starnd, A.H., Kjeldsen, S.E., Hoieggen, A., & Os, I (2008). Serum phosphate, blood pressure, and the metabolic syndrome-20-year follow up of middle-aged men. *The Journal of Clinical Hypertension*, 10(11):814-821.

Haap, M., Heller, E., Thamer, C., Tschritter, O., Stefan, N. & Fritsche, A. (2006). Association of serum phosphate levels with glucose tolerance, insulin sensitivity and insulin secretion in non-diabetic subjects. *European journal of clinical nutrition*, 60(6), 734-739.

Håglin, L. (2001). Hypophosphatemia: cause of the disturbed metabolism in the metabolic syndrome. *Medical Hypotheses*, 56(6), 657-663.

Hansen, L., Deacon, C. F., Ørskov, C., & Holst, J. J. (1999). Glucagon-Like Peptide-1-(7–36) Amide Is Transformed to Glucagon-Like Peptide-1-(9–36) Amide by Dipeptidyl Peptidase IV in the Capillaries Supplying the L Cells of the Porcine Intestine 1. *Endocrinology*, 140(11), 5356-5363.

Hazim, J., Hlais, S., Ghattas, H., Shatila, D., Bassil, M. & Obeid O. (2014). Phosphorus supplement alters postprandial lipemia of healthy male subjects: a pilot cross-over trial. *Lipids In Health and Disease*, 13:109.

Heaney, R. P. (2012). Phosphorus. Present knowledge in nutrition (pp. 447-458) Wiley-Blackwell.

Henry, Y., Gueguen, L. & Rerat A. (1979). Influence of the level of dietary phosphorus on the voluntary intake of energy and metabolic utilization of nutrients in the growing rat. *British Journal of Nutrition*, 42(1): 127-137.

Hörl, W., Kreusser, W., Heidland, A., & Ritz, E. (1980). Abnormalities of Glycogen Metabolism in Cardiomyopathy of Phosphorous Depletion.Advances in Experimental Medicine and Biology Phosphate and Minerals in Health and Disease, 343-350.

Hörl, W., Kreusser, W., Rambausek, M., Heidland, A., & Ritz, E. (1983). Glycogen metabolism in phosphorus-depleted rats. *PubMed*, 9(3), 113-8.

Institute of Medicine, Food and Nutrition Board. (1997). Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. National Academy Press. Washington, DC.

Irving, J. T. (1973). Chapter 7 – Blood Calcium and Phosphorus. In J. T. IRVING (Ed.), Calcium and phosphorus metabolism (pp. 53-63) Academic Press.

Jaedig, S., Lindgärde, F. & Arborelius, M. (1993). Increased postprandial energy expenditure in obese women after peroral K-and Mg-phosphate. *Mineral and Electrolyte Metabolism*, 20(3), 147-152.

Johnson, R. J., Sanchez-Lozada, L. G., & Nakagawa, T. (2010). The effect of fructose on renal biology and disease. *Journal of the American Society of Nephrology*, 21(12), 2036-2039.

Kalaitzidis, R., Tsimihodimos, V., Bairaktari, E., Siamopoulos, K. C. & Elisaf, M. (2005). Disturbances of phosphate metabolism: another feature of metabolic syndrome. *American Journal of Kidney Diseases*, 45(5), 851-858.

Kalantar-Zadeh, K., Gutekunst, L., Mehrotra, R., Kovesdy, C. P., Bross, R., Shinaberger, C. S., ... & Kopple, J. D. (2010). Understanding sources of dietary phosphorus in the treatment of patients with chronic kidney disease. *Clinical Journal of the American Society of Nephrology*, 5(3), 519-530.

Kalantar-Zadeh, K., Gutekunst, L., Mehrotra, R., Kovesdy, C. P., Bross, R., Shinaberger, C. S., ... & Kopple, J. D. (2010). Understanding sources of dietary phosphorus in the treatment of patients with chronic kidney disease. *Clinical Journal of the American Society of Nephrology*, 5(3), 519-530.

Ketteler, M., Gross, M.L. & Ritz, E. (2005). Calcification and cardiovascular problems in renal failure. *Kidney International Suppl*., (94):S120-7.

Khattab, M., Azar, S., Mattar, M. & Obeid, O. (2011). Effect of phosphorus on the oral glucose tolerance test. Proceedings of the Nutrition Society, 70(OCE3), E60.

Kimutai, D., Maleche-Obimbo, E., Kamenwa, R. & Murila, F. (2009). Hypophosphatemia in children under five years with Kwashiorkor and marasmic Kwashiorkor. *East African medical journal*, 86(7).

Kojima, M., & Kangawa, K. (2005). Ghrelin: structure and function. *Physiological reviews*, 85(2), 495-522.

Kumar, V., Sinha, A.K., Makkar, H.P. & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry*, 120(4): 945-959.

Künnecke, B., & Seelig, J. (1991). Glycogen metabolism as detected by in vivo and in vitro13C NMR spectroscopy using [1, 2-13C2] glucose as substrate. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1095(2), 103-113.

Kuro-o, M. (2010). A potential link between phosphate and aging—lessons from klotho-deficient mice. *Mechanisms of Ageing and Development*, 131(4), 270-275.

Lee, C. C., Lee, R. P., Subeq, Y. M., Wang, C. H., Fang, T. C., & Hsu, B. G. (2008). Fasting serum total ghrelin level inversely correlates with metabolic syndrome in hemodialysis patients. *Archives of medical research*, 39(8), 785-790.

Levi, M., & Popovtzer, M. (1999). Disorders of phosphate balance. Chapter 7 in Atlases of Diseases of the Kidney, 1, 7-1.

Li, M., Li, L., Shi, X., Lin, L. & Lin, S. (2015). Effects of phosphorus deficiency and adenosine 5′-triphosphate (ATP) on growth and cell cycle of the dinoflagellate Prorocentrum donghaiense. *Harmful Algae*, 47, 35-41.

Lippi, G., Montagnana, M., Salvagno, G. L., Targher, G. & Guidi, G. C. (2009). Relationship between serum phosphate and cardiovascular risk factors in a large cohort of adult outpatients. *Diabetes Research and Clinical Practice*, 84(1), e3-e5.

Luque, M. A., Gonzalez, N., Marquez, L., Acitores, A., Redondo, A., Morales, M., ... & Villanueva-Penacarrillo, M. L. (2002). Glucagon-like peptide-1 (GLP-1) and glucose metabolism in human myocytes. *Journal of Endocrinology*, 173(3), 465-473.

Marshall, W. P., Banasiak, M. F. & Kalkhoff, R. K. (1978). Effects of phosphate deprivation on carbohydrate metabolism. *Hormone and Metabolic Research*, 10(05), 369-373.

McDowell, L. R. (2003). Chapter 2 - calcium and phosphorus. In L. R. McDowell (Ed.), Minerals in animal and human nutrition (second edition) (pp. 33-100). Amsterdam: Elsevier.

Morikawa, S., Inubushi, T., Takahashi, K., Terada, Y., Iwata, S., & Ozawa, K. (1996). Glucose and energy metabolism in rat liver after ischemic damage assessed by13C and31P NMR spectroscopy. *Journal of Surgical Research*, 63(2), 393-399.

Murer, H., Forster, I. & Biber, J. (2004). The sodium phosphate cotransporter family SLC34. *Pflügers Archives*, 447(5), 763-767.

Murphy-Gutekunst, L. (2007). Hidden phosphorus: where do we go from here? *Journal of Renal Nutrition*, 17(4), e31-e36.

Musso, G., Gambino, R., & Cassader, M. (2010). Emerging molecular targets for the treatment of nonalcoholic fatty liver disease. *Annual review of medicine*, 61, 375-392.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., & Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature*, 409(6817), 194-198.

Noori, N., Sims, J., Kopple, J., Shah, A., Colman, S., Shinaberger, C., Bross, R., Mehrotra, R., Kovesdy, C. & Kalantar-Zadeh, K. (2010). Organic and Inorganic Dietary Phosphorus and Its Management in Chronic Kidney Disease. *Iranian Journal of Kidney Diseases*, 4(2).

Obeid, O. (2013c). Phosphorus. Advanced Nutrition Minerals Course. Lecture conducted by the American University of Beirut, Lebanon.

Obeid, O. A. (2013). Low phosphorus status might contribute to the onset of obesity. *Obesity Reviews*, 14(8), 659-664.

Obeid, O., Dimachkie, S. & Hlais, S. (2010). Increased phosphorus content of preload suppresses ad libitum energy intake at subsequent meal. *International Journal of Obesity*, 34, 1446–1448.

Obeid, O.A., Hachem, D.H. & Ayoub, J.J. (2014). Refeeding and metabolic syndromes: two sides of the same coin. *Nutrition and Diabetes*, 4(6): e120.

Park, W., Kim, B.S., Lee, J.E., Huh, J.K., Kim, B.J., Sung, K.C., ... & Kim S.W. (2009). Serum phosphate levels and the risk of cardiovascular disease and metabolic syndrome: a double-edged sword. *Diabetes Research and Clinical Practice*, 83(1): 119-125.

Penido, M. G. M. & Alon, U. S. (2012). Phosphate homeostasis and its role in bone health. *Pediatric Nephrology*, 27(11), 2039-2048.

Sacks, J. (1945). The effect of insulin on phosphorus turnover in muscle. *American Journal of Physiology--Legacy Content*, 143(2), 157-162.

Schmid, A. I., Szendroedi, J., Chmelik, M., Krššák, M., Moser, E. & Roden, M. (2011). Liver ATP synthesis is lower and relates to insulin sensitivity in patients with type 2 diabetes. *Diabetes care*, 34(2), 448-453.

Shaikh, A., Berndt, T. & Kumar, R. (2008). Regulation of phosphate homeostasis by the phosphatonins and other novel mediators. *Pediatric Nephrology*, 23(8), 1203-1210.

Shills, M.E., Olson, J.A., Shike, M. & Ross, A.C. (2005). Modern Nutrition in Health and Disease (10th edition), Lippincott Williams and Wilkins. Chapter 10.

Shils, M. E. & Shike, M. (Eds.). (2006). Modern nutrition in health and disease. Lippincott Williams & Wilkins.

Solomon, S. M. & Kirby, D. F. (1990). The refeeding syndrome: a review. *Journal of Parenteral and Enteral Nutrition*, 14(1), 90-97.

Takeda, E., Yamamoto, H., Nashiki, K., Sato, T., Arai, H. & Taketani, Y. (2004). Inorganic phosphate homeostasis and the role of dietary phosphorus. *Journal of cellular and molecular medicine*, 8(2), 191-200.

Takeda, E., Yamamoto, H., Yamanaka-Okumura, H. & Taketani Y. (2012). Dietary phosphorus in bone health and quality of life. *Nutrition Reviews*, Vol. 70(6):311–321.

Takeda, E., Yamamoto, H., Yamanaka-Okumura, H. & Taketani, Y. (2014). Increasing Dietary Phosphorus Intake from Food Additives: Potential for Negative Impact on Bone Health. *Advanced Nutrition*, 5(1): 92–97.

Tanaka, S., Yamamoto, H., Nakahashi, O., Kagawa, T., Ishiguro, M., Masuda, M., ... & Takeda, E. (2013). Dietary phosphate restriction induces hepatic lipid accumulation through dysregulation of cholesterol metabolism in mice. *Nutrition Research*, 33(7), 586-593.

Taylor, L. M., Kalantar-Zadeh, K., Markewich, T., Colman, S., Benner, D., Sim, J. J., & Kovesdy, C. P. (2011). Dietary egg whites for phosphorus control in maintenance haemodialysis patients: a pilot study. *Journal of renal care*, 37(1), 16-24.

Tetens, I. (2009). Scientific Opinion on the substantiation of health claims related to phosphorus and function of cell membranes (ID 328), energy-yielding metabolism (ID 329, 373) and maintenance of bone and teeth (ID 324, 327) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006.

Tiosano, D. & Hochberg, Z. (2009). Hypophosphatemia: The common denominator of all rickets. *Journal of Bone and Mineral Metabolism*, 27(4), 392-401.

Uribarri, J. (2007). Phosphorus homeostasis in normal health and in chronic kidney disease patients with special emphasis on dietary phosphorus intake. *Seminars in Dialysis*, 20(4), 295-301.

Van der Vies, J. (1954). Two methods for the determination of glycogen in liver. *Biochemical Journal*, 57(3), 410.

Wehmeyer, N., Gunderson, H., Nauman, J., Savage, S. & Hartzell, C. (1994). Determination of the glycogen synthesis pathway by 13C nuclear magnetic resonance analysis. *Metabolism*, 43(1), 38-43.

Weinman, E. J. & Lederer, E. D. (2012). PTH-mediated inhibition of the renal transport of phosphate. *Experimental Cell Research*, 318(9), 1027-1032.

Wren, A. M., Seal, L. J., Cohen, M. A., Brynes, A. E., Frost, G. S., Murphy, K. G., ... & Bloom, S. R. (2001). Ghrelin enhances appetite and increases food intake in humans. *Journal of Clinical Endocrinology and Metabolism*, 86(12), 5992.

Xie, W., Tran, T., Finegood, D., & Werve, G. (2000). Dietary Pi deprivation in rats affects liver cAMP, glycogen, key steps of gluconeogenesis and glucose production. *Biochemical Journal*, 227-232.

Xie, W., Tran, T.L., Finegood, D.T. & van de W.G. (2000). Dietary P(i) deprivation in rats affects liver cAMP, glycogen, key steps of gluconeogenesis and glucose production. *Biochemistry Journal*, 352 (Part 1), 227–232.

Yano, S. & Sugimoto, T. (2009). Clinical aspect of recent progress in phosphate metabolism. Distribution of phosphorus and its physiological roles in the body: the form, distribution, and physiological function. *Clinical Calcium*, 19(6):771-6.