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E-NPP1 as a Potential Link Between Insulin Resistance and Bone Health in Type 2 Diabetes

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Received: August 22, 2016; **Published:** September 08, 2016

Abstract

Due to the rise of the obese population and the lack of physical activity such as sedentary lifestyles, the number of people with type 2 diabetes is anticipated to rise even higher in the near future. Patients with type 2 diabetes will develop complications such as retinopathy and osteoporosis in the future. In order to prevent and properly treat it, understanding the underlying molecular mechanisms leading to the onset and development of type 2 diabetes and its complications becomes imperative. The advancement of modern biomedical sciences allows the identification of many genetic and environmental factors contributing to the development of metabolic diseases. Ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP1) or plasma cell membrane glycoprotein 1 (PC-1) is a transmembrane glycoprotein that has been suggested to play a role in interfering with insulin signal transduction and affecting bone mineralization, a potential link between insulin resistance and osteoporosis. Evidence suggests that this enzyme, E-NPP1 may have a negative effect on insulin signal transduction, a potential cause of insulin resistance. On the other hand, it may also be an essential contributor for bone mineralization. Thus, this review was aimed to summarize the current understanding of E-NPP1's role in insulin resistance and bone health. We also offered some future perspectives for better understanding of the development of type 2 diabetes complications.

Keywords: *E-NPP1; K121Q polymorphism; Type 2 diabetes; Insulin resistance; Insulin signal transduction; Bone mineralization; Pyrophosphate; Phosphate; Osteoblast differentiation; Bone health; Obesity*

Introduction

Obesity has become a major public health issue in the United States and the world. It is estimated that one out of every three adults is obese in the U.S and that 2.1 billion people are overweight or obese worldwide [1,2]. In addition to the health problems, obese individuals will encounter social, economic, physiological, and behavior issues [3]. While there has been no significant change in the prevalence of obesity from 2004 to 2012, the number still remains high [1]. With more than 34.9% of the U.S. population being affected by obesity, the number of obesity related conditions is also on the rise. Obesity increases the risk for heart disease, stroke, various cancers, and type 2 diabetes [4].

As a result of obesity, the prevalence of type 2 diabetes has quickly risen during the twenty first century affecting over 150 million individuals in the U.S [5]. Evaluating obesity globally, the World Health Organization (WHO) estimates that the prevalence of diabetes has quadrupled since 1980 [6]. Studies from over 91 countries were used to estimate the global prevalence of diabetes for 2030 in which it's projected that diabetes will affect over 439 million adults worldwide [7].

A common feature of human obesity and type 2 diabetes is insulin resistance [8]. Insulin resistance can be clinically defined as the inability of an identified amount of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual compared with one in a normal population [9]. There are various mechanisms that may contribute to the development of insulin resistance which include genetic abnormalities in proteins of the insulin signal transduction cascade. In addition, other conditions such as fetal malnutri-

tion and increases in visceral adiposity may also be potential factors. Insulin resistance may occur as a component of other abnormalities referred to as the metabolic syndrome which can lead to the development of type 2 diabetes [9]. In addition to insulin resistance, patients with type 2 diabetes can also develop skeletal complications [10]. Studies have revealed that type 2 diabetic patients are at an increased risk for developing osteopenia and osteoporosis which is low or decreased bone density. In addition, they are at an increased risk for occurrence of fractures compared with those without diabetes [11].

Ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) are a family of enzymes hydrolyzing nucleotides and their derivatives. E-NPP1 is also known as plasma cell membrane glycoprotein 1 (PC-1) and was the first cloned one of five members in the family [12,13]. As a transmembrane glycoprotein, E-NPP1 was initially discovered in 1970 as a surface marker for antibody-secreting B-lymphocytes [14]. Since its discovery, E-NPP1 has become the best characterized E-NPP isoenzyme compared to the other four members. It is produced in a variety of tissues and is thought to have roles in interfering insulin signaling transduction and participating in bone mineralization.

To begin, key terms of E-NNP1 and type 2 diabetes were searched in PubMed. We read the relevant titles and abstracts first. The search results were further narrowed down with the key terms E-NPP1 and K121Q polymorphism, insulin resistance, and insulin signaling transduction. In addition, based on abstracts and articles read, key terms such as bone health, osteoporosis, and pyrophosphate were added in. We have presented our relevant findings in this review.

Overview of Insulin and Its Physiological Functions

Insulin is an anabolic hormone produced by the β-cells of the islet of Langerhans in the pancreas [15]. Insulin is first synthesized as preproinsulin, and removal of its signal peptide produces proinsulin [16,17]. The proinsulin is composed of three domains; an amino-terminal A chain, a carboxyl-terminal B chain, and a connecting peptide in the middle also known as the C peptide. The proinsulin is converted to insulin through the joint action of two proteases. These include proteases with trypsin-like endoprotease activity that cleave the C peptide and proteases with exopeptidase activity that remove any remaining residues after the tryptic cleavage [17]. The mature insulin is stored in secretory granules until be released in response to increased glucose levels or other stimuli [18]. The processed mature insulin is released in response to physiological secretagogues into the blood stream where it travels to the rest of the body. Glucose is the most important and physiologically relevant secretagogue.

Insulin is essential to regulate the use of glucose in many types of cells. It is also able to control lipid metabolism in various tissues such as the liver, muscle, and adipocytes. For muscle and adipocytes, one way for insulin to promote the use of glucose is to facilitate the transport of glucose into the cells through the translocation of glucose transporters type 4 (GLUT4) from the intracellular storage place to the cell membrane [19,20]. Increased levels of blood glucose stimulate the release of insulin from the pancreas, thus, allowing the insulin to act on the adipocytes and muscle cells to increase the uptake of glucose after the movement of GLUT4 onto the cell membrane [21]. In addition, insulin is responsible for signaling the liver and skeletal muscle to store glucose as glycogen. In the liver, this process starts from the increase of glucokinase activity, which results in the phosphorylation of glucose [22]. In the muscle, insulin stimulates synthesis of glycogen by promoting an overall decrease in the phosphorylation of glycogen synthase, a process that increases the activity of this rate-limiting enzyme in the glycogen synthesis pathway [23]. This allows glycogen synthase to synthesize glycogen from uridine diphosphate glucose for the incorporation of glucose into glycogen [24].

In addition to its role in the regulation of glucose metabolism, insulin has other physiological roles in the regulation of protein and lipid metabolism [20]. These roles include decreasing lipolysis in adipose tissues to lower the plasma fatty acid levels, stimulating triacylglycerol and fatty acid synthesis, increasing synthesis rates of very-low-density lipoproteins, increasing triglyceride uptake from blood into the adipose tissues, and decreasing fatty oxidation rates in both the muscle and liver [25]. Insulin stimulates the uptake of amino acids and the rate of protein synthesis in adipose and muscle tissues. Also, insulin may have a role in decreasing protein degradation and urea formation [25].

Insulin Signaling Transduction Pathways

Cellular responses to insulin are mediated through the insulin receptor. Currently, three signal transduction pathways known as Akt/protein kinase B, the casitas B-lineage lymphoma (Cbl) associated protein (CAP)/ (Cbl/CAP), and mitogen activated protein kinase (MAPK) pathways have been indicated to mediate the insulin signals in different tissues and cells [26,27]. These pathways can be observed in muscle and fat cells in which they transduce insulin signals for the control of glucose metabolism [28,29]. Since approximately seventy-five percent of the body mass is composed of skeletal muscles, the insulin signal transduction pathways in the muscle cells play an important role in the glucose usage [30]. Some of these signal transduction pathways can also be observed in other tissues and organs such as the liver [31].

The insulin receptor is glycosylated and located on the cell membrane [32]. As shown in Figure 1, the tetramer insulin receptor is composed of two α- and two β-subunits linked by disulfide bonds. The two extracellular α -subunits contain two insulin binding sites. The transmembrane β-subunit is a protein tyrosine kinase [32]. One insulin molecule binds to one binding site on one α-subunit, facilitating the binding of the second insulin molecule on the other α-subunit. This event results in a conformational change bringing the two β-subunits in close proximity, which allows the β-subunit tyrosine kinase to phosphorylate each other, a phenomenon known as cross phosphorylation or auto-phosphorylation [33]. The phosphorylation of the protein tyrosine kinase results in a phosphate group attaching to a tyrosine residue which will act as an attachment point for other proteins to interact with the insulin receptor. One family of proteins interacting with the insulin receptor is called insulin receptor substrates (IRS-1 to 4). For example, IRS-1 is attached to the phosphorylated tyrosine residue of the receptor kinase and is phosphorylated at four different sites by the β-subunit tyrosine kinase. Other IRS proteins, IRS-2, IRS-3, and IRS-4 all have roles in mediating insulins actions [34].

The phosphorylated IRS proteins will transduce the insulin signal further. For example, IRS-1 protein will function as an adaptor protein to interact with phosphoinositide 3 kinase (PI3K) which bind to the phosphorylated region of IRS-1, resulting in the phosphorylation of phosphatidylinositol, 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) in the membrane. For Akt activation, the PIP3 will travel along the membrane until binding to PIP3 dependent kinase-1 (PDK-1) to activate it. Upon activation, PDK-1 will phosphorylate Akt at residue threonine 308 (Thr308). In addition, a second phosphorylation will occur at serine 473 (Ser473) carried out by mammalian target of rapamycin (mTOR) complex [35,36]. The phosphorylation of both residues will result in the full activation of Akt. Once activated, Akt will mediate downstream signals such as the regulation of glucose uptake in fat and muscle cells. For example, the translocation of GLUT-4 from intracellular storage places to the cell membrane has been considered an important step for glucose uptake in those cells [32]. In addition, Akt can participate in the mTOR pathway by directly phosphorylating mammalian target of rapamycin complex 1 resulting in the promotion of cell growth, gene expression, and protein synthesis [37,38].

The Cbl/Cap pathway functions as an additional signaling pathway for insulin-stimulated glucose transport, specifically seen in adipocytes [29]. The Cbl protein is recruited to the insulin receptor through its association and interaction with an adaptor protein using adjacent SRC homology 3 domain (SH3) domains in the carboxyl terminus of CAP. The activation of the insulin receptor will result in phosphorylation of Cbl mediated by the adaptor protein CAP. After phosphorylation the Cbl-CAP complex will dissociate from the insulin receptor and translocate to the lipid rafts in the plasma membrane. Cbl can interact with proto-oncogene c-Crk, an adaptor protein which is associated with the Rho family guanine nucleotide exchange factor, guanine nucleotide-releasing factor 2, which can activate guanosine-5'-triphsophate (GTP) binding protein family members such as TC10 [29,39,40]. The activated and GTP bound TC10 mediates GLUT-4 containing vesicles to fuse to the plasma membrane of the adipocytes [41]. In addition, research suggests that TC10 functions in parallel with the PI3K pathway to stimulate GLUT-4 translocation in response to insulin [42].

As an important player of signal transduction pathways of many factors, the MAPK pathway promotes cell growth predominantly in eukaryotic cells [27]. In addition, the MAPK pathway is also present in cancer cells and neurodegenerative diseases such as Alzheimer's disease [43,44]. Insulin binds to the receptor resulting in the cross phosphorylation of tyrosine kinase domains. Growth factor receptor bound protein 2 (GRB2) binds to the phosphorylated insulin receptor β-subunit and then to the son of seven less homolog 1 (SOS). The

GRB2-SOS complex binds to RAS which binds to GTP. The binding to GTP results in the activation of RAS [45]. RAS is a member of the family of GTPases which hydrolyze GTP. It regulates cell growth, proliferation, and differentiation [46]. In its activated form, RAS can bind to B-Raf and phosphorylates and activates mitogen activated protein kinase (MEK) which phosphorylates extracellular signal-regulated kinase (ERK) resulting in their activation. The activated MAPKs will change the activities of transcription factors through various mechanisms such as transcriptional and post translational events [47]. For example, AP-1 is a transcription factor made of fos proto-oncogene and jun proto-oncogene [48]. ERK upon activation can activate AP-1 [49]. Once activated, AP-1 moves to the nucleus as a heterodimer, binds to the promoter DNA of their targeted genes, and in turn changes the expression levels of genes, which results in enhanced cell proliferation, increased expression of growth factors and cytokines [50,51].

Type 2 Diabetes

Diabetes is a group of metabolic diseases in which blood glucose level rises significantly higher than normal fasting blood glucose levels, above 130 mg/dL [52,53]. This hyperglycemia stems from either defects in insulin secretion, or insulin action or a combination of both [53]. Normal fasting blood glucose range recommendation is in between 80-130 mg/dl while postprandial blood glucose is under 130 mg/dl [54]. The majority of diabetes cases fall into two primary categories as either type 1 or type 2 diabetes [55]. Of the two primary categories of diabetes, 5%-10% of diabetes incidences are type 1, the more common, type 2 diabetes affects 90%-95% of diabetic individuals [53].

Type 1 diabetes is an auto-immune disease in which very little or absolutely no insulin is produced due to destruction of pancreatic β-cells, resulting in an inability to mediate the uptake and utilization of glucose [56]. Contributing factors to the development of type 1 diabetes includes genetic factors or even possibly exposure to viruses [57]. Despite extensive and active research, there is no current cure to type 1 diabetes, but with medical intervention and insulin treatment it can be managed in order to live a healthy life.

Type 2 diabetes is characterized by the body's inability to use insulin properly, resulting in insulin resistance. Despite not being completely understood, several risk factors have been associated with the development of type 2 diabetes. Weight, physical activity status, and body fat distribution are the key driving factors behind the development of type 2 diabetes. Other factors such as genetics, age, and race also play a role [58]. Due to weight, physical activity, and body fat distribution being the major risk factors, type 2 diabetes treatment often targets lifestyle interventions in order to reduce the risk factors associated with it and to manage the disease [59]. Without proper management, long-term complications of diabetes hyperglycemia can include, retinal problems, skin issues, neuropathy, organ damage such as nephropathy, and increased risk for stroke and heart attack [53]. Additional complications with type 2 diabetes include decreased bone quality and an increased risk for fractures [10]. Individuals with type 2 diabetes are at a greater risk for developing osteopenia and osteoporosis compared to those without type 2 diabetes [60]. Comorbidities associated with this disease often include hypertension, cardiovascular disease, cancer and depression [61,62] As a result, it is imperative that management and treatment of diabetes mellitus should be fully researched and explored.

Insulin resistance occurs when normal levels of insulin are unable to trigger the downstream metabolic actions in order for normal physiological processes to occur, which includes glucose utilization [63]. The pancreatic β-cells will attempt to produce sufficient insulin to meet the enhanced demand, but eventually will not be able to keep up with the demands to maintain normal blood glucose levels and will result in β-cell dysfunction [64]. As a result, there will be a decrease in glucose uptake of adipose and muscle tissues, and plasma glucose levels will be elevated leading to the development of overt type 2 diabetes [65].

Bone Development and Health Issues

The adult human body mass is supported by a skeletal system composed of two hundred and six different bones. Eighty percent of bones are composed of a dense and solid cortical bone while the remaining 20% is composed of a spongy-like network trabecular bone [66]. Location and type of bone determine the ratio of cortical to trabecular bone. Bone mass is composed of an extracellular matrix and cells which become mineralized by calcium hydroxyapatite in order to give the bone strength and rigidity for function. There are three distinct and essential bone cell types: the osteoblasts which are often referred to as the bone forming cells, the osteoclasts referred to

as the bone-resorbing cells, and finally the osteocytes which are osteoblasts within lacunae [67]. As bone forming cells, the osteoblasts are specialized mesenchymal cells that produce the matrix making up the bone. The matrix is composed of collagen fibers, calcium, and phosphate in order to provide both strength and rigidity. The osteoblasts will form and package the matrix molecules to release into the extracellular space, resulting in bone mineralization [68].

Bone mineralization is a complex physiological process requiring hydroxyapatite being deposited into a collagenous matrix. This matrix is highly regulated through calcium and phosphate in precise concentrations and configurations [69]. Bone mineralization occurs in two different stages; the first being within the matrix vesicles where hydroxyapatite is precipitated after being converted from calcium phosphate, and magnesium and the second stage resulting in the propagation of hydroxyapatite for formation on the extracellular matrix. Pyrophosphate has dual roles in regulating hydroxyapatite formation through inhibition and also acting as a precursor for phosphate [70]. The ratio of phosphate to pyrophosphate is controlled through enzyme reactions involving in both E-NPP1, and tissue-non-specific alkaline phosphatase (TNAP) [71]. Pyrophosphate can inhibit this process by inhibiting the growth and deposition of hydroxyapatite crystal during bone formation [72]. This process can occur through several mechanisms that include the pyrophosphate inhibiting TNAP activity and directly binding to growing crystals [73].

Bone has several primary functions which include mechanical support of soft tissues, muscle lever support, release of calcium for maintenance of an ionic environment in the extracellular fluid, nervous system protection, and hemopoietin support [74]. In order to accomplish these functions, bone homeostasis occurs through a dynamic process in which osteoblasts form bone and osteoclasts induce resorption while the osteocytes act as mechanosensors during the bone remodeling process [74]. Disruption of bone homeostasis in which bone is broken down and remodeled can result in damage to the bone from repeated stress due to being unable to repair the small cracks in the bone. In addition, accumulation of old bone can occur, which will result in lost resilience and possible brittle bones. Also, calcium and phosphorus stores in the bone can be affected, resulting in a compromised supply when there is a dietary deficiency of them [75].

Both osteopenia and osteoporosis can be defined as a skeletal disease characterized by low or decreased bone mass [76,77]. Osteopenia is considered the precursor for osteoporosis with bone mineral density levels considered abnormally low, but levels not severe enough to be classified as osteoporosis [78]. Low bone mass can be assessed by measuring bone mineral density using dual-energy x-ray absorptiometry in which a T-score is used to assess how an individual's bone density differs compared to a set standard from a healthy individual [77]. The WHO has determined that a T-score at or below -2.5 indicates osteoporosis, a T-score between -1.0 and -2.5 indicates osteopenia while a T-score above -1.0 indicates normal bone density [79]. Primary contributing factors to the development of osteoporosis include a variety of medical, behavioral, nutritional, clinical, and genetic variables [80]. In addition, age is a key risk factor, bone mass generally decreases with age [81]. General treatment strategies and recommendations for individuals with osteoporosis or who are at risk for osteoporosis include ensuring adequate intake of both calcium and vitamin D. Other recommendations include, partaking in muscle strengthening and weight bearing exercises along with ceasing tobacco use and reducing excessive alcohol consumption. Finally, the use of pharmaceutical drugs for osteoporosis can be recommended [82].

The Issue of Diabetes and Bone Health

In 1948, Albright and his colleagues first reported the concept that diabetic patients may experience bone mass loss leading to development of osteoporosis [83]. Since then, additional studies have established that type 2 diabetic individuals are at an increased risk for developing osteopenia and osteoporosis [11,83,84]. In addition, patients with type 2 diabetes are at an increased risk for experiencing fractures due to quality of the bone, rather than the bone mineral density as seen in patients with type 1 diabetes [84]. Bone quality is considered a combination of factors that determine bone resistance to fractures while bone mineral density is the amount of minerals such as calcium in the bone [85]. Additional studies reinforce this concept with one examining the association between type 2 diabetes, bone mineral density, and fractures in 6,655 individuals taken from the Rotterdam Study [86]. The population group was comprised of both men and women over 55 years old. Results showed that subjects with type 2 diabetes had a higher bone mineral density. However,

despite having higher bone mineral density it was observed that the subjects had an increased risk for fractures. In addition, subjects with impaired glucose tolerance had higher bone mineral density, but a decreased risk for fractures [86]. Thus, understanding the molecular influences on bone health is vital in order to prevent and treat bone diseases.

Ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs)

The E-NPPs were first known as nucleotide pyrophosphatases/phosphodiesterases (NPPs) are a family composed of 5 different membrane proteins that hydrolyze various nucleotides and their derivatives to release nucleoside-5-monophosphates. Of the members of these E-NPPs, only E-NPP1, E-NPP2, and E-NPP3 have been extensively characterized [12]. As membrane proteins, E-NPPs contain extracellular active sites and the ability to hydrolyze pyrophosphate and phosphodiester bonds in order to produce the nucleoside 5-monophsphates. E-NPP1 also serves as a surface marker with additional functions related to type 2 diabetes. As a glycoprotein E-NPP2 stimulates motility of melanoma cells and production of lysophosphatidic acid while E-NPP3 may potentially play a role during neurulation [12,13]. Tissue expression profile of the E-NPPs covers a wide range from muscle and adipose tissue to organs such as the kidney [13].

Due to what some considered illogical and perhaps confusing reasons, the nomenclature of the NPPs was readdressed. As the primary three well characterized E-NPPs appearing to be ecto-enzymes, it was proposed and successfully passed to refer to the NPPs as ecto-NPPs or E-NPPs [87]. In the current manuscript, we have used E-NPPs.

Ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP1)

E-NPP1 is the first member being cloned in the E-NPP family [12,13]. It is a 230-260 k Da homodimer. Human E-NPP1 has 873 amino acids. The protein structure consists of a short intracellular NH2 terminus, a single transmembrane domain, a carboxyl terminal nuclease domain, and two somatomedin-B-like domains [12,13,88]. As a member of the E-NPP family, E-NPP1 hydrolyzes the phosphodiester and pyrophosphate bonds of its substrates to release nucleoside-5-monophosphates. In addition, E-NPP1 can also hydrolyze the phosphodiester bonds of other substrates such as oligonucleotides and p-nitrophenyl ester of thymidine monophosphate in order to generate nucleoside 5- monophosphates [12].

Protein expression of human E-NPP1 can be observed in a large variety of tissues including adipose tissue, skeletal tissues, the liver and kidney [89,90]. E-NPP1 has been observed to be expressed weakly in the brain, placenta, heart, epididymis, lungs, pancreatic islets, lymphocytes, fibroblasts, and salivary glands [89]. Methods to detect protein expression level of E-NPP1 in various studies included immunoassays such as western blots [91].

Despite knowledge of expression of E-NPP1 in these tissues, the physiological functions of E-NPP1 in these locations have not been fully unearthed. However, evidence shows that over- expression of E-NPP1 in cells can inhibit tyrosine kinase activity of the insulin receptor, indicating its potential to cause insulin resistance [92,93]. Further studies have explored the possible negative roles that E-NPP1 may play in insulin resistance, glucose metabolism, and type 2 diabetes [32,88]. In addition to the possibility that E-NPP1 may be a negative influence related to insulin resistance and type 2 diabetes, E-NPP1 does have vital roles in both bone health and development [12,72,94]. E-NPP1's influence on insulin resistance and bone health related to type 2 diabetes can be complex, but in order to treat type 2 diabetes, a thorough understanding of E-NPP1's role is needed.

E-NPP1 Relation to Insulin Signaling and Glucose Homeostasis

Research done both *in vitro* and *in vivo* suggest that over-expression of E-NPP1 will inhibit insulin downstream signaling transduction cells, resulting in insulin resistance and hyperglycemia [63,70,92,95,96]. The current understanding is that E-NPP1 will not block the binding of insulin to the insulin receptor. It directly interacts with the insulin receptor itself. Maddux and Goldfine's research group had shown that the over-expression of E-NPP1 will inhibit the tyrosine kinase activity of the insulin receptor β-subunit by first interacting with the α-subunit (Figure 1) [26,32]. The increased levels of E-NPP1 result in decreased insulin-mediated activation of the tyrosine phosphorylation of the insulin receptor β-subunit and subsequently the downstream of insulin signaling transduction. E-NPP1 prevents the insulin-induced conformational changes that would result in the auto-phosphorylation of the insulin receptor β-subunits and thus the ac-

tivation of the tyrosine kinase domain [92] as evidenced by measuring the insulin receptor β-subunit and both the auto-phosphorylation and phosphorylation of the downstream proteins such as IRSs [90]. This could potentially lead to the development of insulin resistance and hyperglycemia [92]. However, the particular mechanisms in which E-NPP1 binds to the insulin receptor at the level of the α-subunit to inhibit the tyrosine kinase activity is still unclear at this time.

There are a few studies that do not support the theory that E-NPP1 interrupts insulin signal transduction. Specifically, in NIH-3T3 fibroblasts with a stable over-expression of insulin receptor, neither E-NPP1 nor its gene polymorphisms had the ability to interact with the insulin receptor [97]. In addition, an over-expression of E-NPP1 did not change the status of the insulin receptor auto-phosphorylation [97].

Another study showed that E-NPP1's ability to inhibit the insulin receptor is dependent on its enzyme activity [98]. Using HEK293 cells with stably expressed recombinant insulin receptor or insulin-like growth factor 1 (IGF1) receptor, a transient expression of a wild-type full length E-NPP1 inhibited insulin signaling transduction without affecting IGF1 signaling. In addition, an over-expression of wild-type full length E-NPP1 did not affect the insulin receptor at the mRNA level, cell surface levels, or total protein. Thus, suggesting that the inhibition of insulin signaling by E-NPP1 may be specific or dependent on the enzymatic activity of the phosphodiesterase/pyrophosphatase [98].

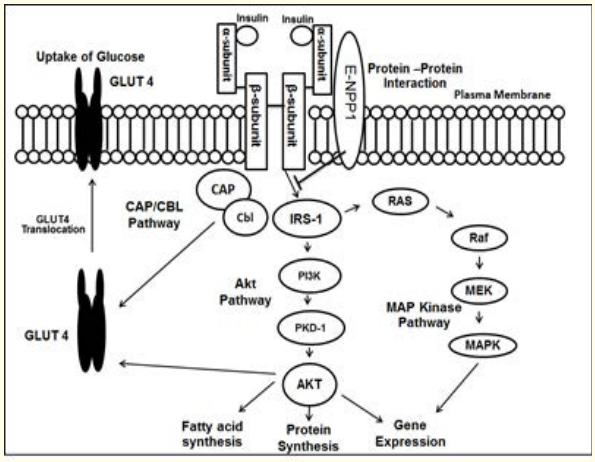

Figure 1: *In here, E-NPP1 interacts with insulin receptor α-subunit, an event that may interfere with insulin signal transduction, and in turn results in a decreased insulin-mediated signal transduction. The activation of the tyrosine phosphorylation of the insulin receptor β-subunit and subsequently the downstream of insulin signaling transduction including the translocation of GLUT4 have been described in the main text. For the Akt/protein kinase B pathway (Akt) the arrows denote the direction of the pathway in which phosphorylated insulin receptor substrate 1 (IRS-1) interacts with phosphoinositide 3 kinase (PI3K) in order to mediate the phosphorylation of phosphatidylinositol, 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) in the plasma membrane. PIP3 binds to PIP3 dependent kinase-1 (PDK-1) to activate it. The activated PDK-1 phosphorylates Akt allowing Akt to be involved in fatty acid synthesis, protein synthesis, and gene expression. For the mitogen activated protein kinase (MAPK) pathway in which the arrows show the general direction of the pathway, IRS-1 mediates activation of RAS which in turns binds to rapidly accelerated fibrosarcoma (Raf) resulting in the activation of mitogen activated protein/extracellular signal regulated kinase (MEK) which can be referred to as a MAPK which can mediate gene expression. The casitas B-lineage lymphoma (Cbl) associated protein (CAP)/ (Cbl/CAP) pathway results in GLUT4 translocation.*

E-NPP1's Relation to Type 2 Diabetes

Based on the evidence that expression of E-NPP1 can inhibit insulin downstream signaling which potentially can alter glucose homeostasis, additional human epidemiology studies have examined the relationship of E-NPP1 K121Q gene polymorphism with type 2 diabetes, specifically its role in the development type 2 diabetes [88,99-103]. Both the Q and K variants of this polymorphism interact directly with the insulin receptor. However, the Q allele interacts more strongly with the insulin receptor than the K allele does, which results in a reduction of auto-phosphorylation of the receptor. Although the K allele is more common, the Q allele has a stronger inhibitory effect on the insulin receptor, resulting in the reduction of insulin-induced phosphorylation of IRS-1 than the K allele [104]. As shown in table 1, multiple studies confirm the association between the E-NPP1 K121Q gene polymorphism and greater insulin resistance leading to development of type 2 diabetes [102,105-106]. Studies covering subjects in a variety of ethnicities suggest evidence that that individual's with the Q allele of the E-NPP1 K121Q gene polymorphism may be predisposed to development of type 2 diabetes [102,105-108].

Population/Ethnic Group	Results	Sample Size
Meta-Analysis of Chinese population with 2 diabetes mellitus [102]	Significant association between the E-NPP1 K121Q gene polymorphism and type2 diabetes in this population.	11,855 subjects
South Asians and Causations (US) with type 2 diabetes mellitus [105]	E-NPP1 121Q predicts genetic susceptibility for type 2 diabetes development.	2,620 subjects
Hubei Han Chinese with type 2 diabetes mellitus [106]	Association found between the E-NPP1 K121Q polymorphism and type 2 diabetes in this population.	943 subjects
Korean Men with type 2 diabetes and impaired fasting glucose [100].	Individuals with K121 variant reduced more over all BMI responsive to intervention.	448 subjects
Obese Polish Caucasians with type 2 diabetes mellitus [107].	E-NPP1 SNP, rs997509 polymorphism associated with risk of type 2 diabetes in obese individuals of this population.	796 subjects
Meta-Analysis of Caucasian European Population with type 2 diabetes [108].	The E-NPP1 Q121 variant increases risk of developing type 2 diabetes.	15,801 subjects

Table 1: *Studies indicating a positive association between the K121Q polymorphism of E-NPP1 and type 2 diabetes or related quantitative metabolic traits.*

However, there are also multiple studies reporting no association between the E-NPP1 K121Q gene polymorphism and type 2 diabetes. As shown in table 2, multiple studies conducted on Danish, North Indian, and Chinese Han populations did not find associations between the E-NPP1 K121Q gene polymorphism and insulin resistance leading to type 2 diabetes [109-111]. The primary goal of these studies was to investigate if there was any association between the E-NPP1 gene or its K121Q polymorphism and the development or presence of type 2 diabetes and obesity in their respective populations. The study with the Danish population involved genotyping over 400 individuals with type 2 diabetes or who were considered glucose intolerant [109]. Results indicated that for the diabetic or glucose intolerance individuals there was no significant difference in the frequency of the variant compared to a matched control population. The same results were also shown in studies conducted on different population groups that included North Indians and Chinese Han population [110,111]. Again for both studies, associations between type 2 diabetes and E-NPP1 and its variants were explored by genotyping the individuals with type 2 diabetes and comparing them to matched control populations. Results for both of these populations indicated there was no significant association between E-NPP1 or its K121Q variant and the development or presence of type 2 diabetes.

Due to the conflicting information between E-NPP1 K121Q gene polymorphism and type 2 diabetes, a meta-analysis was conducted on a Chinese population as shown table 2. With 11,855 subjects the meta-analysis indicated that individuals carrying the Q allele may be pre-disposed to type 2 diabetes development [102]. This reinforces the hypothesis that the Q allele of the E-NPP1 K121Q gene polymorphism may increase individual's risk of developing type 2 diabetes. In addition, another study examined three different populations that differ in susceptibility to diabetes in order to evaluate E-NPP1 K121Q polymorphism influence on the development of type 2 diabetes.

The cohorts included, 858 Caucasians living in Dallas, Texas with 141 individuals having type 2 diabetes 679 non-migrant South Asians living in Chennai, India with 223 having type 2 diabetes; and 1,083 migrant South Asians located in Dallas, Texas with 121 having type 2 diabetes. The study results showed that prevalence of subjects with the Q allele in the South Asians located in Chennai was 25% in the non-diabetic group and 34% in the diabetic group. For the South Asians living in Texas it was 33% for non-diabetic and 45% for diabetic groups and 26% and 39% respectively for the Caucasians. These results seemed to support the hypothesis that the E-NPP1 121Q allele is associated with genetic susceptibility for developing type 2 diabetes in these two population groups [105]. Additional studies support the hypothesis that E-NPP1 and its genetic polymorphisms contribute to the development of type 2 diabetes in a wide range of individuals [100,107,108].

Population/Ethnic Group	Results	Sample Size
Danish Caucasians with type 2 diabetes mellitus [109].	E-NPP1 K121Q variant was not associated with either insulin resistance or type 2 diabetes mellitus in this population.	582 subjects
North Indian Punjabi with type 2 diabetes mellitus [110].	E-NPP1 K121Q polymorphism is not associated with type 2 diabetes or related traits in this population	654 subjects
Chinese Han with type 2 diabetes mellitus [111].	E-NPP1 K121Q Polymorphism is not associated with type 2 diabetes or obesity in this population	1,912 subjects

Table 2: *Studies that did not find an association between the K121Q polymorphism of E-NPP1 and type 2 diabetes or related quantitative metabolic traits.*

E-NPP1's Relation to Bone Structure

Studies have been conducted to investigate the role of E-NPP1 in bone formation and mineralization [12,72,94,112]. It has been established that E-NPP1 is highly expressed on the surface of osteoblasts and chondrocytes, mineralizing cells [12,89,94]. There are two predominant mechanisms in which E-NPP1 can influence bone formation and development; pyrophosphate levels and osteoblast differentiation.

Osteoblast differentiation is characterized by three predominant stages that include; cell proliferation, matrix maturation, and matrix mineralization. Various transcription and growth factors promote the different stages of osteoblast differentiation such as type 1 collagen, alkaline phosphatase, and osteopontin [113]. The first stage of cell proliferation includes extracellular matrix proteins being excreted in which to produce osteoprogenitor cells. The second stage of matrix maturation is characterized by over-expression of alkaline phosphatase and the production of pre-osteoblasts. Finally, the last stage of matrix mineralization occurs when genes for various proteins are expressed, resulting in calcium depositions and mature osteoblasts [114,115].

Through the hydrolysis of nucleotides and nucleotide sugars, E-NPP1 is capable of generating a supply of pyrophosphate which can serve as a source of phosphate through hydrolysis by TNAP which is expressed in conjunction with E-NPP1 in the plasma membranes and matrix vesicles of mineralizing cells, such as the chondrocytes [71,116]. In addition, pyrophosphate acts as an inhibitor of hydroxyapatite crystal in order to regulate bone formation [117]. The link between over-expression of E-NPP1 and altered mineralization has been demonstrated in the mutant "tiptoe walking" mouse models which were compared to E-NPP$^{-/-}$ mice [112]. These "tiptoe walking" mice exhibited the same behaviors as the E-NP$^{-/-}$ and had decreased levels of extracellular inorganic pyrophosphate which had resulted in alterations of mineralization of the long bones [112,118]. Thus, the pyrophosphate generated from E-NPP1 has several roles in tissue mineralization and varying levels of E-NPP1 can greatly influence the process of mineralization in bone.

In addition, reduced levels of E-NPP1 may cause inhibition of osteoblast differentiation, resulting in reduced mineralization of bones [70,116]. Nam and colleagues have shown that E-NPP1 expression is vital for osteoblastic differentiation independent of extracellular levels of phosphate and pyrophosphate or catalytic activities. Using MC3T3E1 (C4) pre-osteoblastic cells, suppression of E-NPP1 resulted in defective osteoblastic differentiation as indicated by inability to mineralize matrix, no evidence of osteoblast marker gene expression,

and no sign of morphological change in the cells. In addition, using the same cells, over-expression of E-NPP1 resulted in increased differentiation as evidenced by increased mineralization and increased expression of osteoblast marker genes [116]. Thus, the expression level of E-NPP1 can have a profound influence on bone development through osteoblast differentiation.

Therefore, E-NPP1 has direct roles in bone formation, by first providing a source of phosphate from pyrophosphate in which osteoblasts can use to facilitate bone mineralization [71]. Second, E-NPP1 levels have a direct influence on osteoblast differentiation during the matrix mineralization stage as evidenced by mineralization markers during osteoblast gene expression [72].

Conclusion and Future Perspectives

Type 2 diabetes is a widespread public health issue with an array of factors affecting the development and treatment of this disease. E-NPP1 probably plays a role in interference of insulin signaling transduction and bone health, two serious issues for diabetic patients. The research results summarized here indicate E-NPP1 may have an inhibitory effect towards insulin signaling transduction, resulting in the reduction of insulin sensitivity and altered glucose homeostasis. In addition, evidence suggests that E-NPP1 K121Q gene polymorphism may be a marker for development of type 2 diabetes. However, information is still conflicting at this time with multiple studies indicating no association between E-NPP1 and type 2 diabetes for a variety of population groups. It will also be worth analyzing the relationship of polymorphism with the bone health in normal and diabetic populations.

E-NPP1 seems to play a vital role in bone mineralization through production of pyrophosphate and osteoblast differentiation. Thus, it suggests that suppressing E-NPP1 levels in order to reduce insulin resistance may have negative effects on bone health which could result in decreased bone mineralization and osteoblast differentiation. In addition, increasing E-NPP1 levels to improve bone mineralization and osteoblast differentiation may result in increased insulin resistance. Therefore, targeting E-NPP1 through the manipulation of its expression level or activity for the treatment of a specific aspect of type 2 diabetes may have consequences on other issues of the disease. Any decision based on this mechanism should be evaluated carefully before moving forward. As a result, if studies on E-NPP1 indicate that either a suppression or over-expression would be beneficial to a specific aspect of type 2 diabetes, then tissues specific drugs should be developed which could prevent undesired consequences in other tissues. Due to the desire to prevent and treat type 2 diabetes and its complications, further exploration and studies of E-NPP1 and its roles related to insulin signaling transduction and bone health should be conducted. Even though it appears that suppression of E-NPP1 may reduce insulin resistance, there are multiple studies on a variety of populations that do not support this hypothesis. Further studies may be needed to elucidate E-NPP1's potential effect on particular populations that may be influenced by this protein. This allows to develop specific treatments to target unique populations.

Bone mineralization and osteoblast differentiation appear to be directly influenced by the expression level of E-NPP1. Additional studies are needed to reinforce this concept. These studies could explore E-NPP1s effects on osteoblast differentiation in bone specifically at an increased risk for fractures such as seen in type 2 diabetes. Finally, studies in which either suppression or over-expression of E-NPP1 in type 2 diabetic conditions should be conducted in order to evaluate its potential effect on both insulin resistance and bone health. Bone growth and health are critical issues for human nutrition. On the other hand, insulin is an anabolic hormone for human metabolism and growth. It will be interesting to see whether E-NPP1 serves as a link between insulin and bone health in growth and development. For example, whether insulin regulates E-NPP1 expression is still an open question that remains to be determined.

In summary, the body of an organism can be considered as a system integrating multiple signals from the environment and internal organs and cells at any moment. The sum of these signals reflects the balance of organs and cells to a certain physiological state. The beneficial or harmful role of an enzyme or its products in certain physiological state should always be considered at the same time. How to utilize the findings of E-NPP1 in insulin resistance and bone health to treat diabetes and osteoporosis is certainly a question deserved to be explored further.

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Volume 4 Issue 6 September 2016

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